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Bioinformatics analysis of *CUL2/4A/9* and its function in head and neck squamous cell carcinoma

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Abstract

Introduction: Several previous studies have shown that differential expression of cullin (*CUL*) family proteins may be involved in mediation of the signal transduction pathways associated with cancer. However, the function of CULs is still unclear in head and neck squamous cell carcinoma (HNSCC).

Material and methods: We used The Cancer Genome Atlas (TCGA) database, cBioPortal, Metascape, STRING, Cytoscape, Tumor Immune Estimation Resource (TIMER), Kaplan-Meier plotter, and Tumor Immune System Interaction Database (TISIDB) to access the expression of CULs and the possible correlation with the tumourigenesis, development, prognosis, immunity, and transcriptional level of CULs in HNSCC. Furthermore, real-time quantitative polymerase chain reaction (RT-qPCR) was used to detect messenger ribonucleid acid (mRNA) levels in HNSCC tissues and cell samples. We also explored the cell proliferation and migration separately by CCK8 assay and wound healing assay.

Results: The results showed that the expressions of *CUL2/4A* were upregulated and *CUL9* was downregulated in HNSCC patients as compared with normal patients. *CUL2/4A/9* were also linked to the clinicopathological features and overall survival of HNSCC in bioinformatics analysis. Moreover, we noticed that *CUL2/4A/9* may take part in tumour-specific immune response by modulating the tumour-infiltrating lymphocytes (TILs) and immunomodulators. Lastly, we found that *CUL2/4A/9* could promote cellular proliferation and migration.

Conclusion: These results suggest that the transcriptional levels of CUL2/4A/9 were upregulated and these genes could affect proliferation and migration of HNSCC cells. Therefore, *CUL2/4A/9* could potentially function as novel independent biomarkers in HNSCC patients.
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Key words: *head and neck squamous cell carcinoma; cullin; prognosis; proliferation; migration*

Introduction

Head and neck squamous cell carcinoma (HNSCC), the sixth most common malignancy worldwide, arises from the oral cavity, pharynx, and larynx mucosal epithelium [1]. Conventional treatments for HNSCC include surgery, radiotherapy, and chemotherapy, but they are incompletely effective, with only 50% of patients being cured [2]. Therefore, the exploration of molecular targeted therapy for HNSCC is becoming a current treatment trend [3]. New prognostic biomarkers are urgently needed to accurately forecast the progression of precancerous lesions in HNSCC, thereby predicting overall survival and optimizing treatment regimens.

In addition, cullin ring ubiquitin ligases (CRLs), which consist of 4 distinct parts: CULs, ring-finger proteins (RINGs), adaptor proteins, and substrate recognition receptors/proteins, which can catalyse the movement of ubiquitin to the substrate, play a fundamental role in regulating cell cycle, gene expression, apoptosis, etc. [4] Several recent studies have reported the potential involvement of CRLs in the progression of several types of cancer [5–7]. The *CUL* gene family has been reported to be evolutionarily conserved, and at present it consists of 8 distinct members in the human genome: *CUL1*, *CUL2*, *CUL3*, *CUL4A*, *CUL4B*, *CUL5*, *CUL7*, and *CUL9* [8].

Several previous studies have suggested that the differential transcriptional levels of CULs may also play a vital role in mediating the signal transduction pathways related to cancer. For instance, *CUL2* E3 ligase complexes may participate in the von Hippel-Lindau (VHL) signalling transduction mechanism in clear cell renal cell carcinoma [9]; thus, *CUL3* can serve as major regulator of human malignancies and emphasize the importance of developing novel agents targeting this protein to prevent or treat the tumourigenesis. However, the potential functions of distinct CULs are still unknown in HNSCC. Thus, an in-depth study about the possible involvement of different CULs in HNSCC is needed to reveal the molecular pathways related to the occurrence and development of HNSCC and might reveal novel prognostic as well as clinical treatment biomarkers for

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this high morbidity tumour. The purpose of this study is to investigate and identify the predictive role of different CULs in HNSCC occurrence and progression.

Material and methods

Expression level analysis of different CULs

In the present research, we explored the transcriptional levels of CULs in multiple cancers and their potential relationship with individual stages, tumour grade, and prognosis in HNSCC by using the University of ALabama at Birmingham CANcer (UALCAN) (http://ualcan.path.uab.edu/analysis.html) [10] and The Cancer Genome Atlas (TCGA) databases (http://cancergenome.nih.gov) [11]. A Kaplan-Meier plotter (http://kmplot.com/analysis/) was used to explore the prognosis of transcriptional levels of different *CULs* in HNSCC, and thereafter the information about the potential interactions among gene expression and the overall survival (OS) of cancer patients was processed easily.

Functional enrichment analysis of different CULs

The possible relationship between CULs and their neighbouring genes was built by STRING (https://string-db.org/) [12] and visualized by Cytoscape [13]. Furthermore, the "stat" R package was used to identify the top 20 similar genes that have been linked with *CUL* expression in HNSCC from the TCGA database. We used Metascape (http://metascape.org) to perform the function enrichment analysis of CULs and their similar genes by using Gene Oncology (GO) and the Kyoto Encyclopaedia of Genes and Genomes (KEGG) [14].

Immune infiltration analysis of different CULs

In the present study, we used TIMER (https://cistrome.shinyapps.io/timer/) [15] and TISIDB (http://cis.hku.hk/TISIDB/index.php) [16] to study the possible interactions among *CUL* expression levels and immune cell infiltration, immune-related molecular markers, and multiple types of immunomodulators. Finally, we used a heatmap to demonstrate the coaction display details in scatterplots and provide the partial Spearman correlation and p-values, as shown in Supplementary File — Tables S1 and S2.

Patient tissue samples

Head and neck squamous cell carcinoma tissue samples (n = 10) and matched para-carcinoma tissue (n = 10) were obtained from the Department of Otolaryngology-Head and Neck Surgery of the Affiliated Hospital of Qingdao University. The Hospital Accreditation Committee of the Affiliated Hospital of Qingdao University (approval #QYFY WZLL 27078) and patients approved this study, and the confidentiality of patient information was maintained.

Cell culture

Hypopharyngeal cancer cells (FaDu), human oral epidermoid carcinoma cells (KB), and human normal epithelial cells (HNEpC) cells were cultured in Dulbecco's Modified Eagle Medium (Meilunbio, China), while nasopharyngeal carcinoma (CNE-2) cells were cultured in RPMI1640 Medium (Meilunbio, China), containing 10% foetal bovine serum (FBS) (Procell, China), 100 μg/mL streptomycin, and 100 U/mL penicillin (Procell, China), maintained at 37°C in an atmosphere of 5% CO_2, and grown to 70–90% confluence.

Plasmid construction and transient transfection

CUL2 and CUL4A were subcloned into PB-3FLAG-IRES2-EGFP-CMV-IRES2-puro-M13 vector, while CUL9 shRNA was inserted into in the pLKO.1 vector. Both vector and control plasmids were purchased from GeneChem Company. Transient transfection was performed with Lipofectamine 3000 following the instructions of the manufacturer.

Quantitative real-time polymerase chain reaction (RT-qPCR)

RNA from HNSCC tissue samples and cells was extracted using TRIzol reagent following the manufacturer's instructions. After the RNA concentration was quantified using NanoDrop One (Thermo Fisher Scientific, United States), we reversed RNA into cDNA by using Evo M-MLV RT Premix for quantitative polymerase chain reaction (qPCR) (Accurate Biotechnology [Hunan] Co., Ltd). Real-time qPCR (RT-qPCR) was performed to quantify mRNA expression of *CUL2*, *CUL4A*, and *CUL9* via SYBR^R Green Premix *Pro Taq* HS qPCR Kit [Accurate Biotechnology (Hunan) Co., Ltd]. For normalization of expression levels, *18sRNA* and *GAPDH* were,

Table1. Databases ***used for bioinformatics analysis***

Name	Link	This study	Keywords
UALCAN	http://ualcan.path.uab.edu/analysis.html	To explore potential relationship between the transcriptional level of different *CULs* in HNSCC samples and their individual stages and tumour grade	Clinicopathological features
TCGA	http://cancergenome.nih.gov	To explore the similar genes that have been linked with *CULs* expression in HNSCC	Gene expression Similar gene detection
String	https://string-db.org/	To the possible relationship between CULs and their neighbouring genes	Protein-protein interaction network
Metascape	http://metascape.org	To perform the function enrichment analysis of *CULs* and their similar genes by GO and KEGG	Functional enrichment analysis
TIMER	http://timer.cistrome.org/	To evaluate the interaction among *CULs* expression levels and immune cell infiltration	Gene expression Immune infiltration
Kaplan-Meier plotter	http://kmplot.com/analysis/	To explore the prognosis of transcriptional levels of different *CULs* in HNSCC	Gene expression Survival curve
TISIDB	http://cis.hku.hk/TISIDB/index.php	To study the possible interactions among immunomodulators and transcriptional level of *CULs*	Gene expression Immune modulation

UALCAN — University of ALabama at Birmingham CANcer; TCGA — The Cancer Genome Atlas; TIMER — Tumor Immune Estimation Resource; TISIDB — Tumor Immune System Interaction Database; CUL — cullin; GO — Gene Ontology; KEGG — Kyoto Encyclopaedia of Genes and Genomes; HNSCC — head and neck squamous cell carcinoma

Table 2. *The clinical materials of head and neck squamous cell carcinoma patients*

Patients	Gender	Age (years)	Tumour types
1	Male	63	Skull base squamous cell carcinomas
2	Female	45	Nasopharynx cancer (squamous cell carcinoma)
3	Female	40	Nasopharynx cancer (squamous cell carcinoma)
4	Male	55	Nasopharynx cancer (squamous cell carcinoma)
5	Male	35	Oropharynx cancer (squamous cell carcinoma)
6	Male	67	Larynx cancer (squamous cell carcinoma)
7	Male	59	Larynx cancer (squamous cell carcinoma)
8	Female	39	Hypopharynx cancer (squamous cell carcinoma)
9	Male	44	Hypopharynx cancer (squamous cell carcinoma)
10	Male	59	Hypopharynx cancer (squamous cell carcinoma)

Table 3. *Primer sequences of different genes designed for real-time quantitative polymerase chain reaction (RT-qPCR)*

Gene	Primer sequence
CUL2	Forward: 5′-GTCTTACTCCGTGCTGTGTCCA-3′
	Reverse: 5′-CTGACTCCACAAATAGTGTTGGC-3′
CUL4A	Forward: 5′-GAATGAGCGGTTCGTCAACCTG-3′
	Reverse: 5′-CTGTGGCTTCTTTGTTGCCTGC-3′
CUL9	Forward: 5′-GTGAGGACTCAAGCTACATGCC-3′
	Reverse: 5′-CAGGTTCTCCAAGAGGATCACC-3′
GAPDH	Forward: 5′-GTCTCCTCTGACTTCAACAGCG-3′
	Reverse: 5′-ACCACC CTGTTGCTGTAGCCAA-3′
18sRNA	Forward: 5′-GGGAGGTAGTGACGAAAAATAACAAT-3′
	Reverse: 5′-TTGCCCTCCAATGGATCCT-3′

CUL — cullin; GAPDH— glyceraldehyde 3-phosphate dehydrogenase; 18sRNA — 18S ribosomal RNA

respectively, used per tissue and cell sample. *CUL2, CUL4A, CUL9, 18sRNA,* and *GAPDH* primer sequences are described in Table 3. After normalization, the ΔΔCt method was used to compare the relative expression for different CULs.

Cell viability

Above cells, seeded at density of 5×10^3 per well in a 96-well plate, were transfected with control or OE-*CUL2*, OE-*CUL4A*, and *CUL9* shRNA. After 48 h, we followed the instructions of the Cell Counting Kit-8 (Yeason) so that the attached cells were incubated with 100 μL of medium containing 10 μL of cell counting kit 8 (CCK-8) solution for between 30 minutes and 2 hours. The absorbance was measured at 450 nm. Cell confluence was calculated to reflect cell proliferation.

Wound healing assay

The cells were seeded in 6-well plates and transfected until they were fused to 60–70%. After 48 h, we used 200-μL pipet tips to create similar scratches. The plate was washed well once with phosphate-buffered saline (PB). Then, 2 mL of fresh media supplement was added without Fetal Bovine Serum (FBS). The images of the scratches were recorded in the same position at different time points. The scratch areas were quantitatively analysed by ImageJ software.

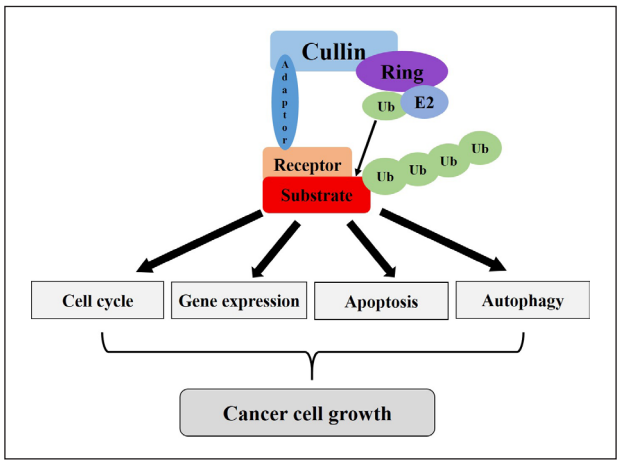


Figure 1. *A model for regulating cancer cell growth by cullin ring ubiquitin (Ub) ligases (CRLs). E2 — ubiquitin-conjugating enzyme 2*

Statistical analysis

SPSS version 23 (IBM, Ehningen, Germany) or GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, United States) were used to analyse the data. We analysed the CULs mRNA expression between paired clinical tumours and normal tissues by using Student's t-test because these experimental data were accorded with the normal distribution and homogeneous variance. It was also applied to explore the differences in *CUL* expression levels, proliferation, and migration in multiple HNSCC cell lines. Meanwhile, the Wilcoxon rank sum test was used to analyse the data that did not conform to the normal distribution. In all graphs, the mean value ± 1 standard deviation (SD) was performed. $p < 0.05$ was considered as statistical significance.

Results

Up-regulation of different CULs in patients with HNSCC

To investigate the transcriptional levels of different *CULs* in HNSCC, we used the TCGA database to analyse the mRNA levels of CULs in different types of tumour. The results displayed in Figure 2 indicate that the mRNA

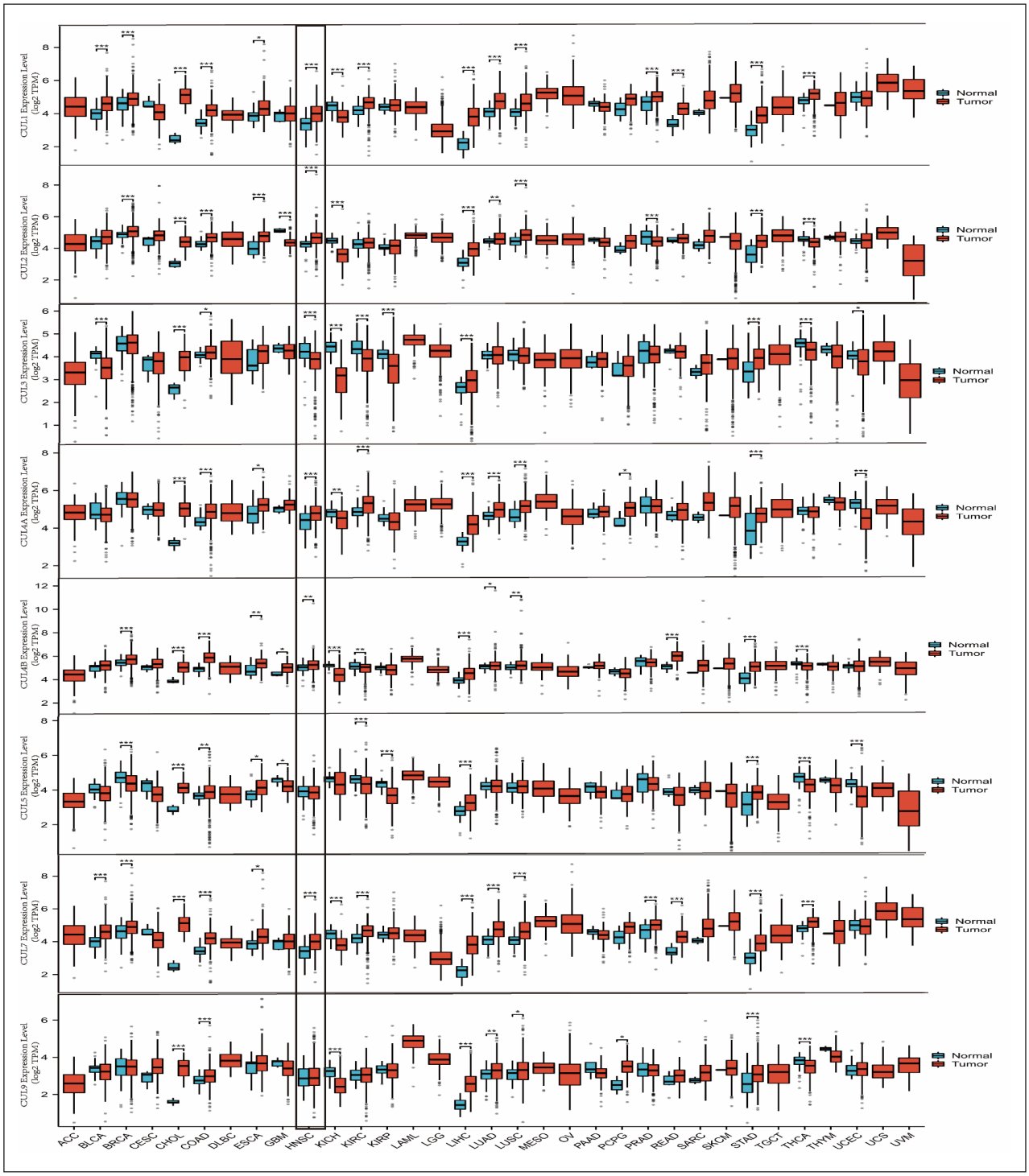


Figure 2. *Transcriptional expression of cullins (CULs) in different types of cancer diseases [The Cancer Genome Atlas (TCGA) database]. Difference of transcriptional expression was compared by the Wilcoxon rank sum test. Data type: mRNA. *p < 0.05, **p < 0.01, ***p < 0.001. ACC — adrenocortical carcinoma; BLCA — bladder urothelial carcinoma; BRCA — breast invasive carcinoma; CESC — cervical endocervical adenocarcinoma and squamous cell carcinoma; CHOL — cholangiocarcinoma; COAD — colon adenocarcinoma; ESCA — oesophageal carcinoma; GBM — glioblastoma; HNSC — head and neck squamous cell carcinoma; KICH — kidney chromophobe; KIRC — kidney renal clear cell carcinoma; KIRP — kidney renal papillary cell carcinoma; LAML — acute myeloid leukaemia; LGG — brain lower-grade glioma; LIHC — liver hepatocellular carcinoma; LUAD — lung adenocarcinoma; LUSC — lung squamous cell carcinoma; MESO — mesothelioma; OV — ovarian serous cystadenocarcinoma; PRAD — prostate adenocarcinoma; READ — rectum adenocarcinoma; SKCM — skin cutaneous melanoma; STAD — stomach adenocarcinoma; TGCT — testicular germ cell tumours; THCA — thyroid carcinoma; THYM — thymoma; UCEC — uterine corpus endometrial carcinoma; UVM — uveal melanoma*

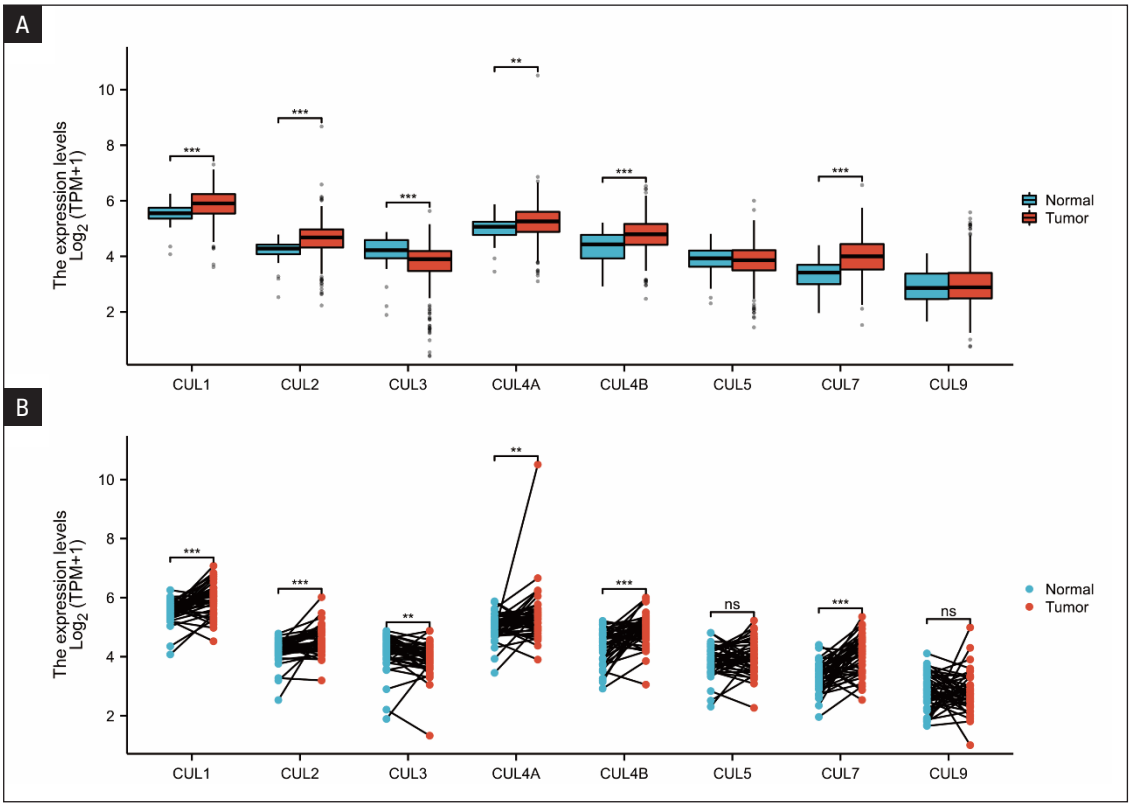


Figure 3AB. *messenger ribonucleid acid (mRNA) expression of distinct cullin (CULs) family members in normal tissues and head and neck squamous cell carcinoma (HNSCC) tissues [The Cancer Genome Atlas (TCGA)]. mRNA expressions of 8 CUL family members were found to be over-expressed or low-expressed in primary unpaired or paired HNSCC tissues compared to normal samples. The transcriptional expression of CULs in unpaired HNSCC patients was compared by the Wilcoxon rank sum test, while using paired t test in paired HNSCC patients. *p < 0.05, **p < 0.01, ***p < 0.001, ns: no significance*

levels of *CUL1/2/4A/4B/7* were significantly higher in HNSCC patients than in normal patients, while the mRNA level of *CUL3* was markedly lower in HNSCC samples than in normal samples. The expression of *CUL5* and CUL9 was statistically insignificant. Next, we also analysed the transcriptional level of *CULs* between HNSCC samples and normal samples in the TCGA database; the results of CULs mRNA expression in HNSCC tissues are consistent with Figure 1 (Fig. 3).

Relationship between mRNA expression of CULs and the clinicopathological features in HNSCC patients

After the expression levels of *CULs* were found to be upregulated in HNSCC, we used UALCAN to explore the potential relationship between the mRNA levels of *CULs* and various clinicopathological features of HNSCC, including patients' individual cancer stages and tumour grades. As shown in Figure 4, the transcriptional levels of 3 *CULs* were found to be significantly related to cancer stage in individual patients, and those in more severe tumour stages expressed significantly higher mRNA levels of *CULs*. The highest transcriptional level of *CUL2/4A/4B* was observed in stage 4 (Fig. 4B, D, and E), the highest transcriptional expression of *CUL3* as well as *CUL9* was found in the normal stage (Fig. 4C and H), and the highest mRNA expressions of *CUL1* and *CUL7* were noted in stage 2 (Fig. 4A and G). The higher transcriptional expression of *CUL2/4A/4B* in stage 4 is possibly related to poor prognosis. Similarly, as shown in Figure 5, the transcriptional levels of 7 *CULs* were primarily related to the tumour grade, and with increasing tumour grade the transcriptional level of *CULs* was observed to augment proportionately. The highest transcriptional level of *CUL1/2/4A/4B/9* was found in grade 4 (Fig. 5A, B, D, E, H), but the highest mRNA level of *CUL7* was noticed in tumour grade 2 (Fig. 5G). Nevertheless, the highest transcriptional level of CUL3 was observed in normal tissues, and with the increase in the tumour grade the mRNA level of *CUL3* was detected to be downregulated (Fig. 5C). In brief, the above results indicated that the transcriptional level of 7 *CULs* was noteworthy and correlated with clinicopathological features in HNSCC.

Prognosis of transcriptional level of CULs in HNSCC

Next, we applied a Kaplan-Meier plotter (http://kmplot.com/analysis/) to assess the impact of variation

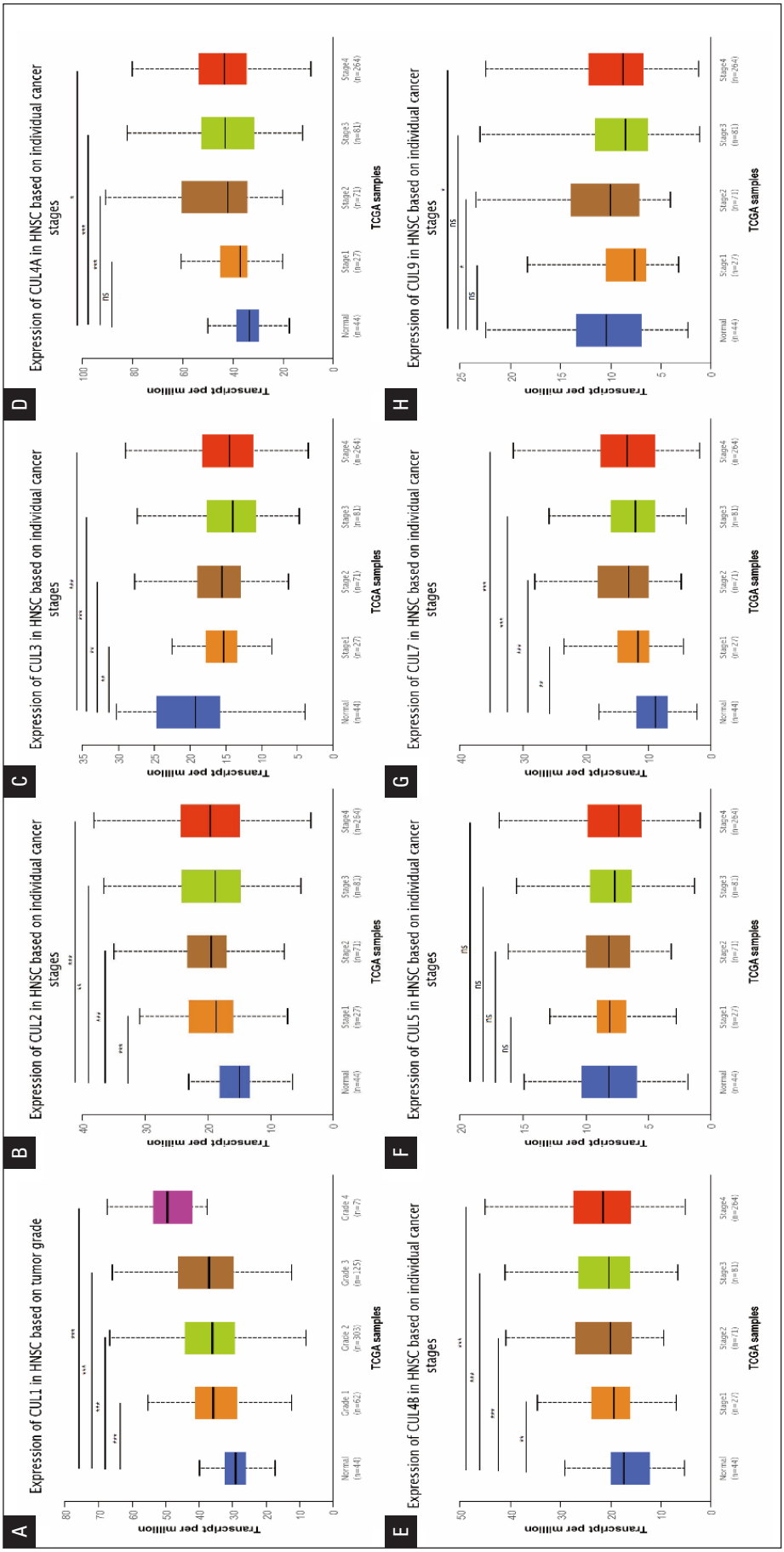


Figure 4. *Relationship between messenger ribonucleid acid (mRNA) expression of distinct cullin (CULs) family members and individual cancer stages of neck squamous cell carcinoma (HNSCC) patients. The highest mRNA expressions of CUL1/4A were found in stage 3* (**A**, **D**)*, while the highest mRNA expressions of CUL2/4B/7 were found in stage 4* (**B**, **E**, **G**)*. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$*

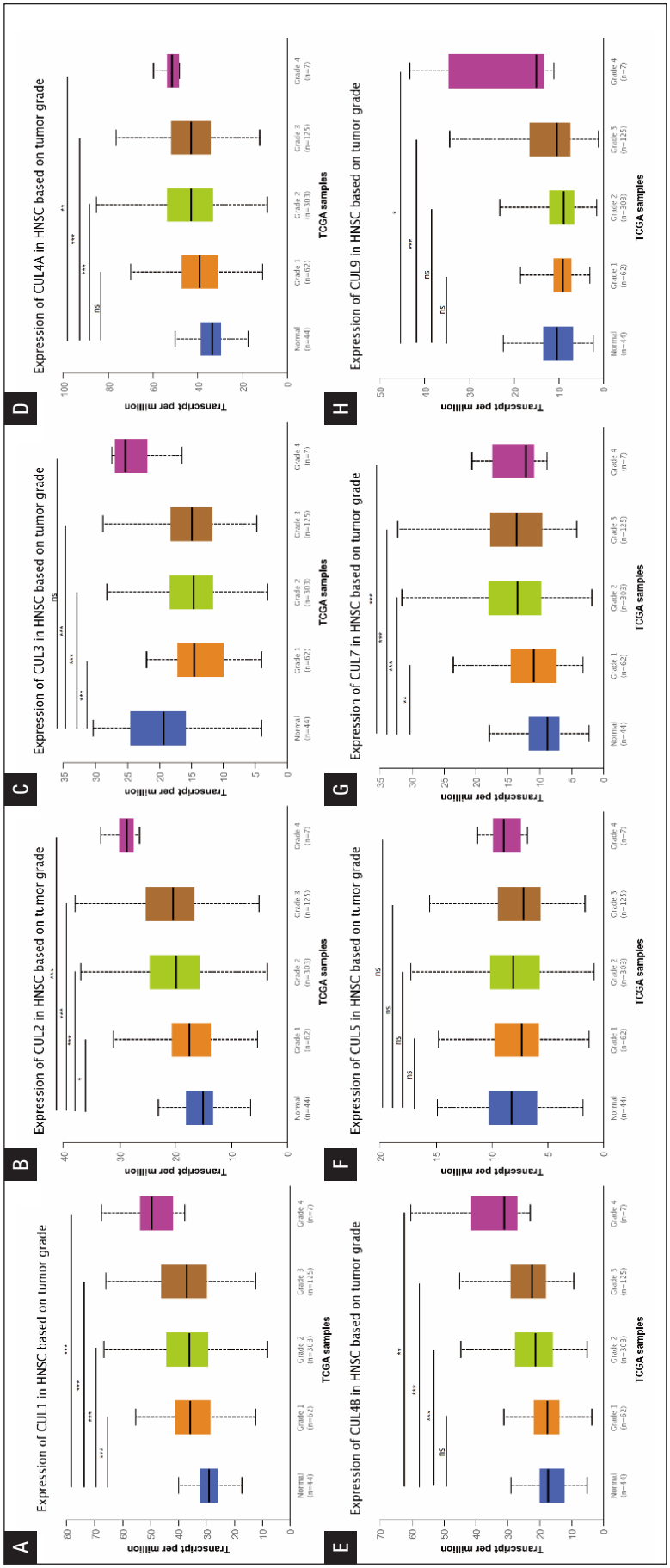


Figure 5. *Association of messenger ribonucleid acid (mRNA) expression of distinct cullin (CUL) family members with tumour grade of head and neck squamous cell carcinoma (HNSCC) patients. The mRNA expressions of 5 CUL family members were significantly related to tumour grade, and as tumour grade increased, the mRNA expressions of CULs tended to be higher. The highest mRNA expressions of CUL1/4A/7/9 were found in tumour grade 4* (**A**, **D**, **G**, **H**)*, while the highest mRNA expression of CUL2/4B was found in grade 3* (**B**, **E**)*. However, the highest mRNA expression of CUL3 was found in grade 1* (**C**)*. *p < 0.05; **p < 0.01; ***p < 0.001. TCGA — The Cancer Genome Atlas*

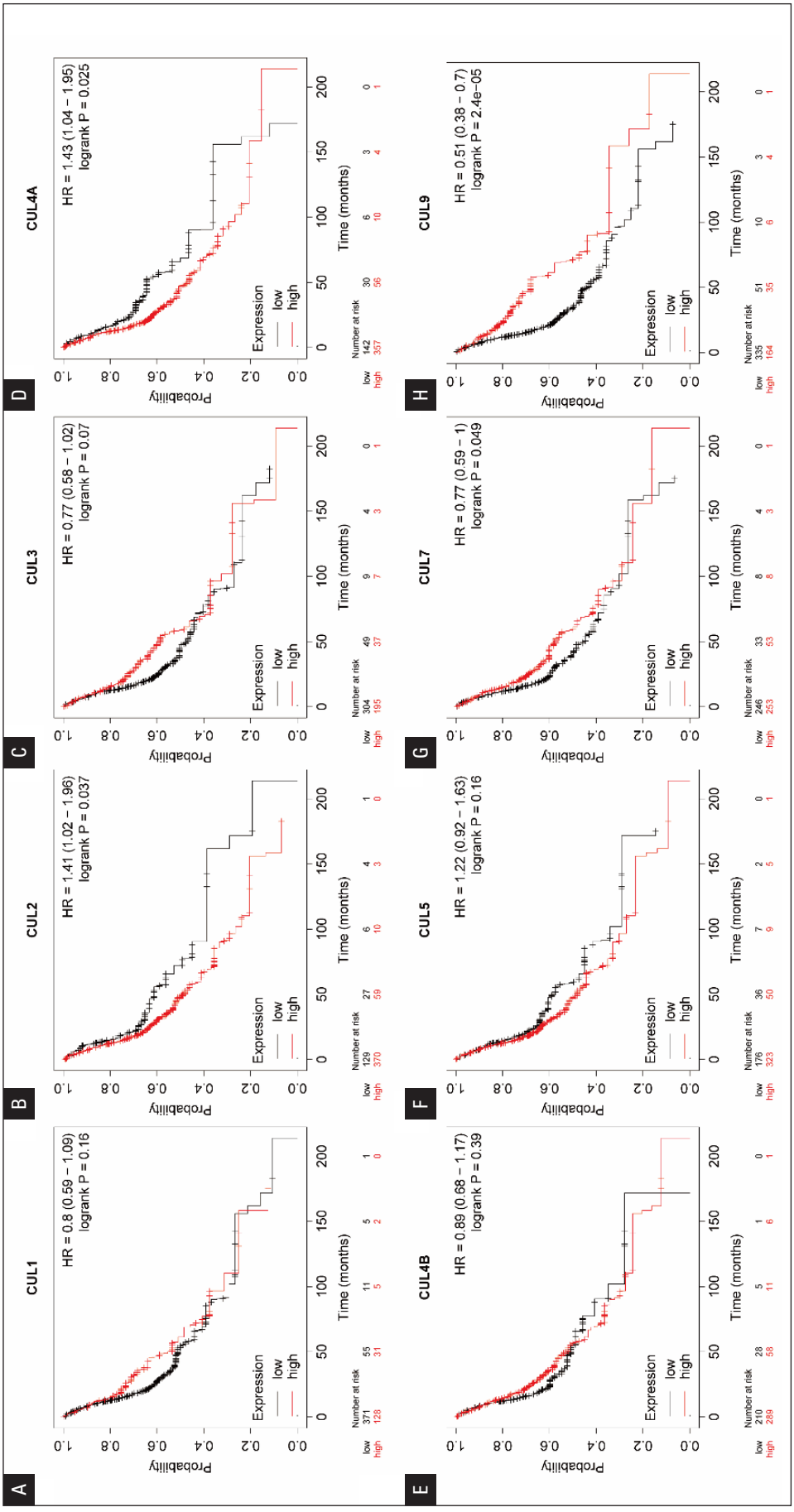


Figure 6. *Prognostic value of messenger ribonucleid acid (mRNA) expression of distinct cullin (CUL) family members in head and neck squamous cell carcinoma (HNSCC) patients (Kaplan-Meier Plotter). Higher mRNA expressions of CUL7/9 were significantly associated with favourable overall survival (OS) of HNSCC patients* (**B**, **G**, **H**)*, while higher mRNA expression of CUL2/4A was significantly related to shorter OS of HNSCC patients* (**D**)*. However, CUL1/3/4B/5 mRNA expression showed no correlation with prognosis in HNSCC patients* (**A**, **C**, **E**, **F**)*. HR — hazard ratio*

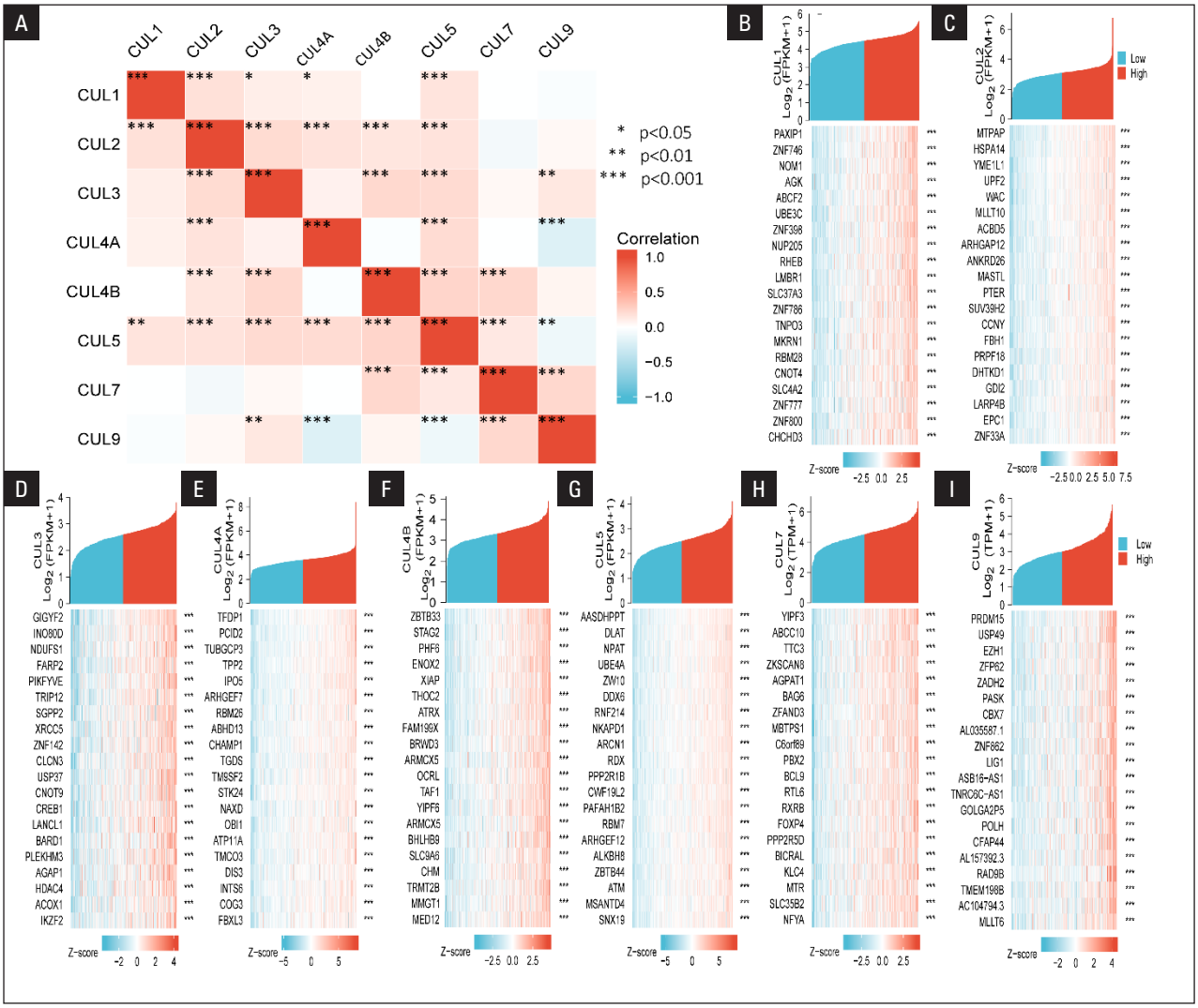


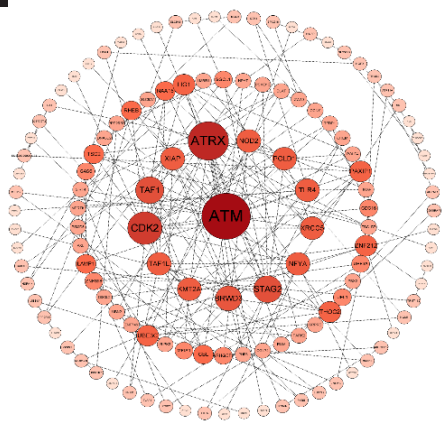
Figure 7. *Correlation heatmaps of cullins (CULs) and their most correlated genes in head and neck squamous cell carcinoma (HNSCC).* **A.** *Correlation heatmap of different expressed CULs in HNSCC. Red and blue cells indicate co-occurrence and mutual exclusivity, respectively;* **B–I.** *Correlation heatmap of CUL1-9 expression and its top 20 correlated genes. *p < 0.05; **p < 0.01; ***p < 0.001*

in the transcriptional level of *CULs* on the prognosis in HNSCC (Fig. 6). The results showed that upregulation of *CUL2* [hazard ratio (HR) = 1.41, p = 0.037] and *CUL4A* (HR = 1.43, p = 0.025) mRNA were significantly linked to shorter overall survival (OS) in HNSCC, while overexpression of *CUL7* (HR = 0.77, p = 0.049) and *CUL9* (HR = 0.51, p < 0.001) resulted in favourable prognosis in HNSCC patients.

Co-expression, similar gene network, and interaction analyses of CULs in HNSCC

After studying the interaction between the transcriptional level of CULs and clinicopathological features as well as with the prognosis in HNSCC, we further investigated the latent co-expression of different CULs in HNSCC. We noticed that there were mild to moderate positive interactions between *CUL1* and *CUL3*, *CUL1* and *CUL4A*, *CUL3* and *CUL9* (Fig. 7A, p < 0.05). We found medium to strong positive correlations between *CUL1* and *CUL2*, *CUL1* and *CUL5*, *CUL2* and *CUL3*, *CUL2* and *CUL4A*, *CUL2* and *CUL5*, *CUL3* and *CUL4B*, *CUL3* and *CUL5*, *CUL4A* and *CUL5*, *CUL4B* and *CUL5*, *CUL4B* and *CUL7*, *CUL5* and *CUL7*, *CUL7* and *CUL9* (Fig. 7A, p < 0.01). Interestingly, we also noticed the negative correlations between *CUL5* and *CUL9*, *CUL4A*, and *CUL9* (Fig. 7A, p < 0.001). The relationship between *CUL1-9* and their top 20 similar genes is shown in Figure 7B–I. We next designed a protein-protein interaction network analysis by STRING (www.string-db.org) to analyse the latent coactions among CULs and their associated genes (Fig. 7A). We further used the plug-in MCODE of Cytoscape to detect the top 10 hub genes with higher

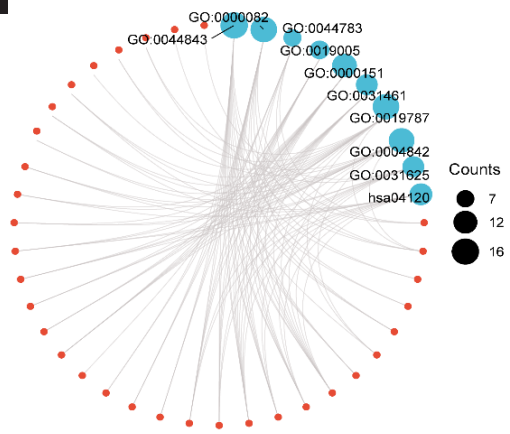
A



B

Top 10 hub genes with a high degree of connectivity ranked by MCC method				
Gene Symbol	Type of Gene	Description	Gene Location	Degree
ATM	Protein-coding	ATM serine/threonine kinase	Chr11:108222484-108369102	159
CDK1	Protein-coding	cyclin dependent kinase 1	Chr10:60778331-60794852	132
ATRX	Protein-coding	ATRX chromatin remodeler	ChrX:77504880-77786235	75
XRCC5	Protein-coding	X-ray repair cross complementing 5	Chr2:216109348-216206293	75
BARD1	Protein-coding	BRCA1 associated RING domain 1	Chr2:214725645-214809683	74
CDK2	Protein-coding	cyclin dependent kinase 2	Chr12:55966830-55972789	73
EZH2	Protein-coding	enhancer of zeste 2 polycomb repressive complex 2 subunit	Chr7:148807374-148884344	73
PAXIP1	Protein-coding	PAX interacting protein 1	Chr7:154943690-155003419	30
DDX6	Protein-coding	DEAD-box helicase 6	Chr11:118747763-118791696	28
TAF1	Protein-coding	TATA-box binding protein associated factor 1	ChrX：71366220-71530525	26

C



Ontology	ID	Description
BP	GO:0044843	cell cycle G1/S phase transition
BP	GO:0000082	G1/S transition of mitotic cell cycle
BP	GO:0044783	G1 DNA damage checkpoint
CC	GO:0019005	SCF ubiquitin ligase complex
CC	GO:0000151	ubiquitin ligase complex
CC	GO:0031461	cullin−RING ubiquitin ligase complex
MF	GO:0019787	ubiquitin−like protein transferase activity
MF	GO:0004842	ubiquitin−protein transferase activity
MF	GO:0031625	ubiquitin protein ligase binding
KEGG	hsa04120	Ubiquitin mediated proteolysis

Figure 8. *Enrichment analysis of cullins (CULs) and their most correlated genes in head and neck squamous cell carcinoma (HNSCC).* **A.** *Gene-gene interaction network for CULs and their most correlated genes (Cytoscape);* **B.** *Top ten hub genes with a higher degree of connectivity;* **C.** *The functions of CULs and their most correlated genes were predicted by the analysis of gene ontology [Gene Oncology, and Kyoto Encyclopedia of Gene and Genomes (KEGG) by Metascape tolls]. Go and KEGG enrichment analysis predicted the functional roles of target host genes based on 4 aspects: biological process, cellular components, molecular functions, and KEGG pathway analysis*

correlation: *ATM, CDK1, ATRX, XRCC5, BARD1, CDK2, EZH2, PAXIP1, DDX6*, and *TAF1* (Fig. 8A, B).

Predicted functions and pathways of similar genes with CULs

Thereafter, we used GO and KEGG in Metascape to explore the potential functions of *CULs* and their co-expressed genes. As shown in Figure 8C, various biological processes, for example GO:0044843 (cell cycle G1/S phase transition), GO:0000082 (G1/S transition of mitotic cell cycle), and GO:0044783 (G1 DNA damage checkpoint), were found to be significantly associated with the *CULs'* co-expressed genes in HNSCC. Moreover, different cellular components, including skp1-cullin-F-box (SCF) ubiquitin ligase complex (GO:0019005), ubiquitin ligase complex (GO:0000151), and cullin-RING ubiquitin ligase complex (GO:0031461), were significantly connected with the *CULs'* co-expressed genes. In addition, *CULs'* co-expressed genes also notably influenced diverse molecular functions, such as ubiquitin-like protein transferase activity (GO:0019787), ubiquitin-protein transferase activity (GO:0004842), and ubiquitin protein ligase binding (GO:0031625). The results shown in Figure 8C suggest that *CULs* and their associated genes were primarily enriched in ubiquitin-mediated proteolysis (hsa04120), which was markedly associated with both the occurrence and progression of HNSCC.

Relationship between the transcriptional level of CULs and immune cell infiltration in HNSCC

We next analysed the correlation between the expression of *CULs* and immune infiltration degree in HNSCC using TIMER database. As Figure 9A and Figure S1 show, we noticed positive interactions between *CUL1* and infiltration of CD4+ T cells, dendritic cells, neutrophil, and macrophages, while the transcriptional levels of *CUL2, CUL3, CUL4B*, and *CUL9* were found to be positive in connection with the degree of B cells,

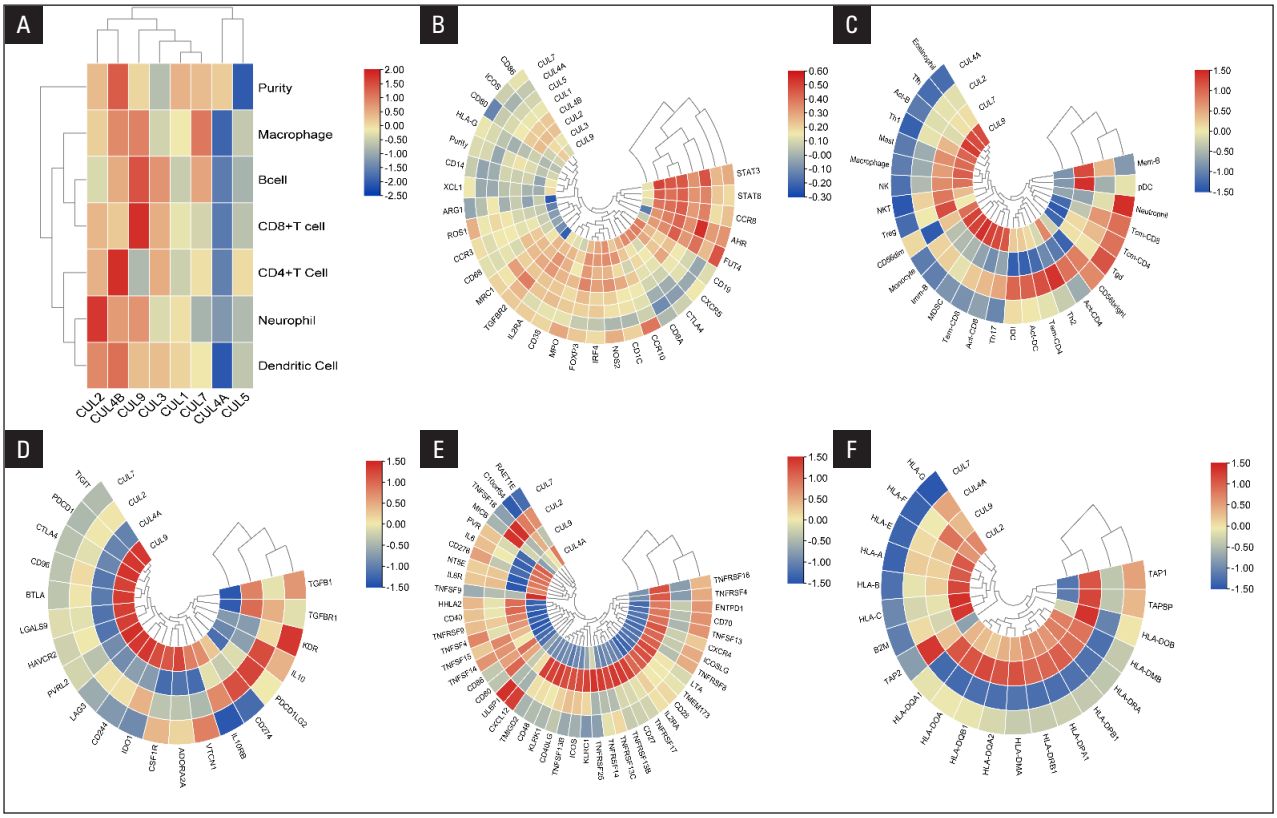


Figure 9. *Interactions of cullins (CULs) expression and immune system.* **A.** *Correlation of CULs expression and immune cell infiltration in head and neck squamous cell carcinoma (HNSCC);* **B.** *Correlation heatmap of CULs expression and immune cell-related gene markers;* **C.** *Correlation heatmap of CUL expression and 28 tumour-infiltrating lymphocytes (TILs) types;* **D.** *Correlation heatmap of CUL2/4A/7/9 expression and 21 immunoinhibitor types;* **E.** *Correlation heatmap of CUL2/4A/7/9 expression and 45 immunostimulator types;* **F.** *Correlation heatmap of CUL2/4A/7/9 expression and 21 major histocompatibility complex (MHC) molecule types.* **C–F.** *The correlation values were normalized by row scale*

CD8+ T cells, CD4+ T cells, dendritic cells, neutrophils, and macrophage infiltration (Supplementary File — Fig. S1, C, E, and H). The transcriptional level of *CUL4A* was found to be negatively correlated with the degree of CD8+ T cell infiltration, but it was positively connected with the degree of CD4+ T cell infiltration (Supplementary File — Fig. S1D). The *CUL5* expression was significantly related to the degree of CD4+ T cell, macrophage, and dendritic cell infiltration (Supplementary File — Fig. S1F). In the meantime, the transcriptional level of *CUL7* was significantly connected with the degree of B cell, CD4+ T cell, macrophage, and dendritic cell infiltration. The above results suggest that *CULs* may participate in the pathogenesis of HNSCC by regulating immune cells infiltration, especially T cells.

Correlation between the expression of CULs and typical immune markers in HNSCC

Despite the above results indicating that *CULs* were not significantly connected with HNSCC prognosis, as observed through their potential interactions with immune infiltration, we next analysed the possible correlation between the transcriptional level of *CULs* and representative immune biomarkers. As shown in Figure 9B and in Supplementary File — Table S1, prominent correlations were found primarily for *CULs* and T cell markers such as STAT3, STAT8, CCR8, AHR, and FUT4.

Correlation between the expression of CULs, tumour-infiltrating lymphocytes (TILs), and immunomodulators in HNSCC

Several previous studies have shown that TILs may act as distinct prognostic indicators in multiple tumours [17, 18], and the above results suggest that *CUL2/4A/7/9* may play a pivotal role in the survival of HNSCC. We next applied the TISIDB database to analyse the possible relationship between the immune-related characters of 28 TILs, 3 types of immunomodulators (21 major histocompatibility complex [MHC] molecules, 45 immunostimulators and 21 immunoinhibitors) and the expression of *CUL2/4A/7/9*. As displayed in Figure 9C–F and in Supplementary File — Table S2, *CUL2* was found to be negatively connected to 8 TILs, 4 immunoinhibitors, 13 immunostimulators, and 6 of the 21 MHC molecules, but just 3 TILs and 5 immunostimulators were

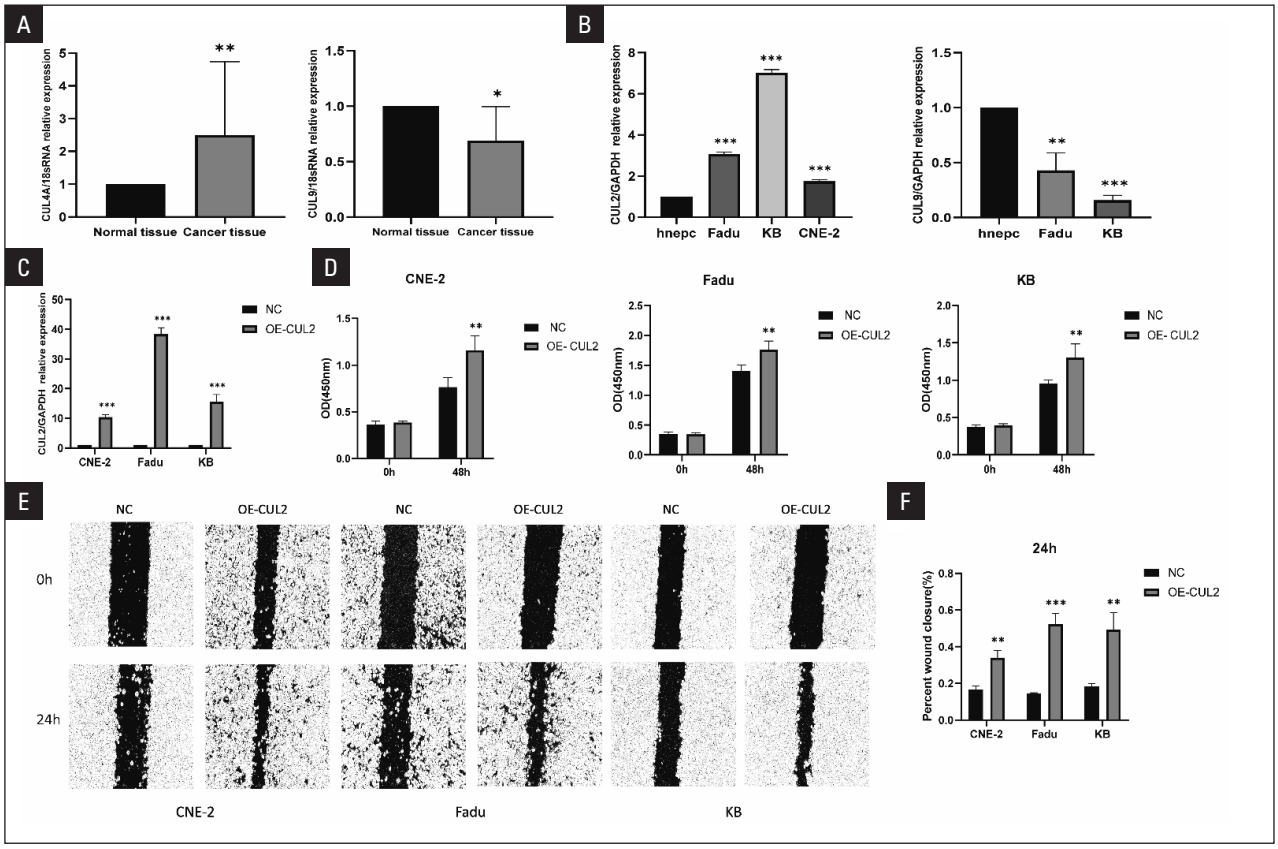


Figure 10. *The effects of cullin 2 (CUL2) on proliferation and migration of head and neck squamous cell carcinoma (HNSCC) cells.* **A.** *Different CULs expression in HNSCC tissues and cell lines;* **B.** *The relative expression of CUL2 messenger ribonucleid acid (mRNA) in HNSCC cells transfected with normal control (NC) or overexpressed CUL2 (OE-CUL2);* **C.** *The cell counting kit 8 (CCK-8) assay between 0 h and 48 h group in transfection with NC and OE-CUL2;* **D.** *The wound healing assay between 0 h and 24 h group in transfection with NC and OE-CUL2;* **E.** *The quantitative result of (D). *p < 0.05, **p < 0.01, ***p < 0.001*

observed to be positively related to *CUL2*. However, for *CUL4A*, we noticed that just one immunoinhibitor, 4 immunostimulators, and one MHC molecule were associated with the gene transcriptional level, but 18 TILs, 14 immunoinhibitors, 31 immunostimulators, and 12 MHC molecules were negatively correlated with *CUL4A*. Lastly, only 6 TILs, 4 immunoinhibitors, 7 immunostimulators, and one MHC molecule were noted to be negatively associated with *CUL9*, but *CUL9* was correlated to 9 TILs, 13 immunoinhibitors, 28 immunostimulators, and 12 MHC molecules. To sum up, it can be predicted that *CUL2* perhaps takes part in the tumour-specific immune response by modulating immunomodulators, *CUL4A* can participate by regulating TILs, immunoinhibitors, immunostimulators, and MHC molecules, while *CUL9* perhaps aids by affecting the various immunoinhibitors, immunostimulators, and MHC molecules.

Expression of different CULs in HNSCC tissues and cell lines

Because the tendency of *CUL7* overexpression in tumours contradicts its prognostic trend, we obtained HNSCC tissue samples (n = 10) and matched para-carcinoma tissue samples (n = 10) to explore the transcriptional expression of *CUL2/4A/9*. The clinical materials of these HNSCC patients are shown in Table 2. The results suggest that the relative mRNA expression of *CUL4A* was significantly higher in tumour compared with in normal tissue, while *CUL9* was lower in caner tissues ($p_{CUL4A} = 0.0019$, $p_{CUL9} = 0.0363$; Fig. 10A). Moreover, we used multiple HNSCC cell lines to study the gene different expressions: HNEpC, CNE-2, FaDu, and KB. We found that *CUL2* expression was higher but *CUL9* was lower in tumour cell lines than HNEpC, which was regarded as a control cell line ($p_{CUL2} < 0.001$, $p_{CUL9\ in\ \mathrm{Fadu}} = 0.0363$, $p_{CUL9\ in\ \mathrm{KB}} < 0.001$; Fig. 10B).

Effect of CULs on proliferation and migration of HNSCC cells

Subsequently, we built overexpressed *CUL2* (*OE-CUL2*) or *CUL4A* (*OE-CUL4A*) plasmid and short hairpin RNAs of *CUL9* (*CUL9* shRNA), respectively, to identify the effects on the proliferation and migration of HNSCC cells. The 3 plasmids were transfected separately into CNE-2, Fadu, and KB. After 48 hours, we detected

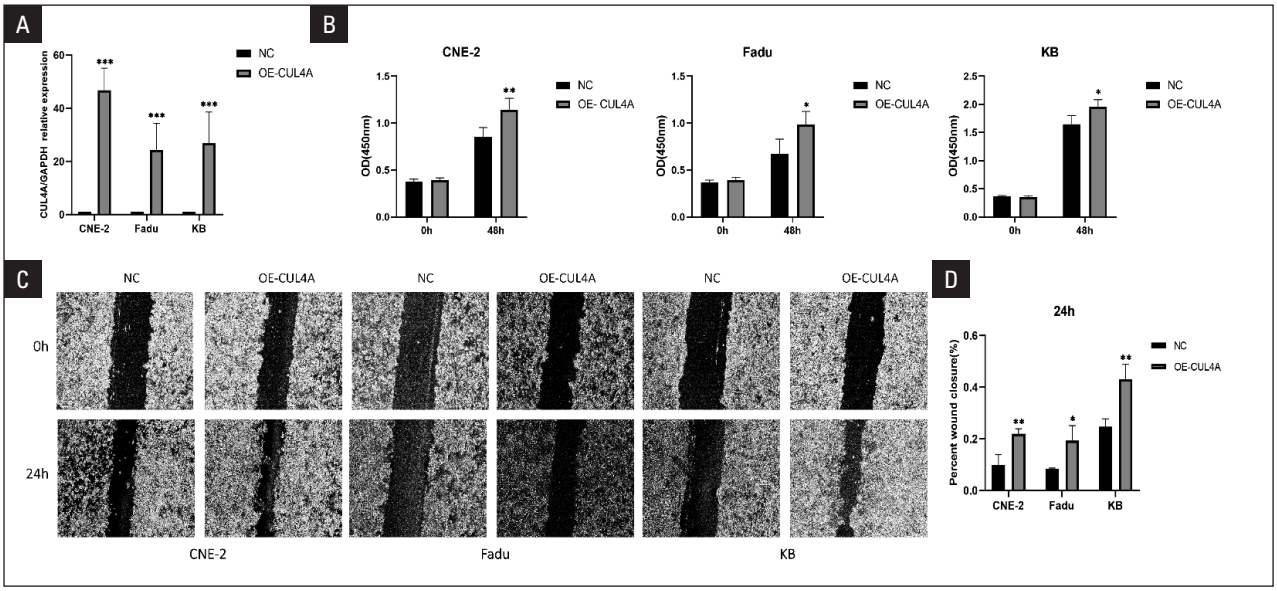


Figure 11. *The effects of cullin 4A (CUL4A) on proliferation and migration of head and neck squamous cell carcinoma (HNSCC) cells.* **A.** *The relative expression of CUL4A messenger ribonucleid acid (mRNA) in HNSCC cells transfected with normal control (NC) or overexpressed CUL2 (OE-CUL4A);* **B.** *The cell counting kit 8 (CCK-8) assay between 0 h and 48 h group in transfection with NC and OE-CUL4A;* **C.** *The wound healing assay between 0 h and 24 h group in transfection with NC and OE-CUL4A.* **E.** *The quantitative result of (***C***). *p < 0.05, **p < 0.01, ***p < 0.001*

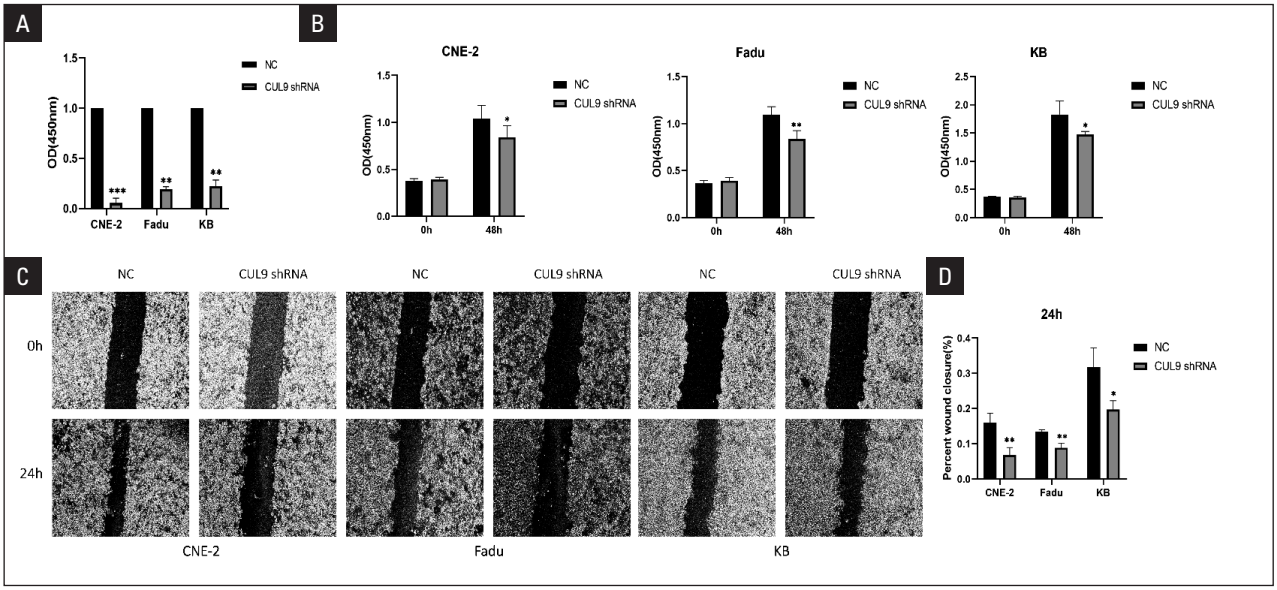


Figure 12. *The effects of cullin 9 (CUL9) on proliferation and migration of head and neck squamous cell carcinoma (HNSCC) cells.* **A.** *The relative expression of CUL9 messenger ribonucleid acid (mRNA) in HNSCC cells transfected with normal control (NC) or short hairpin RNAs of CUL9 (CUL9 shRNA);* **B.** *The cell counting kit 8 (CCK-8) assay between 0 h and 48 h group in transfection with NC and CUL9 shRNA;* **C.** *The wound healing assay between 0 h and 24 h group in transfection with NC and CUL9 shRNA;* **D.** *The quantitative result of (***C***). *p < 0.05, **p < 0.01, ***p < 0.001*

the relative expression of *CULs* mRNA to validate whether the transfection was successful ($p_{CNE-2} < 0.001$, $p_{Fadu} < 0.001$, $p_{KB} < 0.001$; Fig. 10C, 11A, and 12A). Next, CCK-8 assay and wound healing assay were used to explore the function of different CULs in HNSCC cell lines. The results indicate that *CUL2* can promote cellular proliferation ($p_{CNE-2} = 0.0019$, $p_{Fadu} = 0.0015$, $p_{KB} = 0.0051$, Fig. 10D) and migration ($p_{CNE-2} = 0.0021$, $p_{Fadu} < 0.001$, $p_{KB} = 0.0046$, Fig. 10EF), and *CUL4A* can enhance the migration ($p_{CNE-2} = 0.0081$, $p_{Fadu} = 0.0317$, $p_{KB} = 0.0090$, Fig. 11C, D) and proliferation ($p_{CNE-2} = 0.0058$, $p_{Fadu} = 0.0237$, $p_{KB} = 0.0199$, Fig. 10B) of

HNSCC cells. However, *CUL9* might inhibit the ability of cell proliferation (p_{CNE-2} = 0.0447, p_{Fadu} = 0.0051, p_{KB} = 0.0449, Fig. 12B) and migration (p_{CNE-2} = 0.0095, p_{Fadu} = 0.0055, p_{KB} = 0.0243, Fig. 12C, D).

Discussion

Due to genetic factors that can contribute to an increased risk of HNSCC, it is urgent to find more novel molecular indicators to accurately predict the tumourigenesis, progression, prognosis, and potential therapeutic targets of HNSCC. It has been established that different members of the CUL family play a pivotal role in both the occurrence and development of HNSCC [19–21]. Although certain members of the *CUL* family have been shown to be referred to the pathogenesis of HNSCC, the distinct role of the *CUL* family in HNSCC remains unknown. The purpose of the present research study was to explore the expression, prognostic values, co-expressed genes, and immune cell infiltration of different *CULs* in HNSCC.

Our findings detected an upregulation of *CUL1/2/4A/4B/7* mRNAs, while *CUL3* mRNA was low-expressed, and the expression of *CUL5/9* was not significant. Next, the transcriptional level of *CULs* was observed to be correlated with clinicopathological materials in HNSCC. In addition, higher mRNA expressions of *CUL7/9* were significantly correlated to favourable OS of HNSCC, while lower mRNA expression of *CUL2/4A* was predominantly associated with that of HNSCC. Moreover, the functions and pathways of the *CULs* and 20 co-expressed genes in HNSCC were explored, and we found that the top 10 hub genes, including *ATM, CDK1, ATRX, XRCC5, BARD1, CDK2, EZH2, PAXIP1, DDX6*, and *TAF1*, were in correlation with CUL expression. Various biological processes such as cell cycle G1/S phase transition (GO:0044843), cellular components, including GO:0000151 and molecular functions, such as GO:0019787, were markedly regulated by *CUL* similar genes in HNSCC. These results suggest that *CULs* were more enriched in pathways related to cell proliferation and ubiquitin. Finally, we analysed the correlation between *CUL* expression and immune cell infiltration, especially T cells in HNSCC. The above results clearly indicat that since the tendency of *CUL7* overexpression in HNSCC contradicts its prognostic trend, and *CUL9* was the most significant gene related to overall survival in HNSCC, *CUL2/4A//9* may serve as a potential marker of prognosis in HNSCC patients.

Several previous studies have indicated that cullin 2 (*CUL2*) could be overexpressed in some types of tumours, and that it is significantly associated with prognosis. For instance, in glioblastoma multiforme (GBM), increased *CUL2* expression, which functions in encoding the scaffold protein *CUL2* in the CRL2 E3 ligase, was found to predict GBM progression and prognosis [22]. Recently, it has been suggested that the transcriptional activity of *CUL2* might vary according to the size of oral squamous cell carcinoma (OSCC) [23]. In the present research, we found that the mRNA expression of *CUL2* was markedly elevated, and it was significantly connected with clinicopathological features, while it also was directly associated with the degree of infiltration of B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells. Also, a lower transcriptional level of *CUL2* was observed in connection with the favourable OS of HNSCC. Therefore, we verified the overexpression of CUL2 in HNSCC cell lines and identified that CUL2 could accelerate the proliferation and migration of HNSCC cells, thereby clearly indicating an independent biomarker role of *CUL2* in HNSCC.

Cullin 4A (*CUL4A*) is one of the most extensively studied *CULs* in cancer. A variety of previous studies have reported that increased expression of *CUL4A* could serve as a latent prognostic indicator in intrahepatic cholangiocarcinoma [24]. Mechanistically, overexpression of *CUL4* could mediate the proliferation and apoptosis of colon cancer cells by modulating the Hippo pathway [25]. *CUL4A* has similar oncogenic effects in nasopharyngeal carcinoma. The expression of *CUL4A* has been directly related to cancer stage and prognosis in nasopharyngeal carcinoma [26]. Meanwhile, we found that an increased transcriptional level of *CUL4A* was shown in HNSCC samples, and the level of *CUL4A* mRNA could be correlated to clinic-pathological features. The transcriptional level of *CUL4A* was positively connected with CD4+ T cell infiltration. In addition, it was observed that an increased transcriptional level of *CUL4A* promoted cellular proliferation and migration, which was memorably linked to poor OS of HNSCC, indicating that *CUL4A* may actively control the occurrence of HNSCC by regulating T-cell immune response.

The conflicting roles of cullin 7 (*CUL7*) have been found in multiple types of human cancers. First, overexpression of *CUL7* can play a major role in the tumourigenesis and progression of hepatocellular carcinoma (HCC) and potentially serves as an important biomarker for HCC [27]. Moreover, *CUL7* promoted proliferation and invasion of breast cancer (BC) cells by causing down-regulation of protein p53 (p53) activity. In addition, studies have also proven that *CUL7* can function as a new breast cancer oncogene and act as a potential treatment biomarker for BC [28]. Furthermore, the transcriptional level of *CUL7* was found to be increased in primary lung cancer samples [29]. In the present research, conflicting findings about the function of

CUL7 in HNSCC were obtained. First, increased mRNA expression of *CUL7* was observed in HNSCC samples, and the transcriptional level of *CUL7* was dramatically linked to clinicopathological features. However, an increased mRNA level of *CUL7* was correlated with better OS in HNSCC, and the tendency of *CUL7* overexpression in tumours might contradict its prognostic trend. As a result, more in-depth studies are needed to evaluate the exact function of *CUL7* in HNSCC.

The role of cullin 9 (*CUL9*) in cancer remains unclear. A few recent studies have reported that the *CUL9* (formerly *Parc*) gene can function in encoding the E3 ubiquitin ligase, which can directly bind to p53 and translocate in the cytoplasm. Moreover, *CUL9* deletion led to spontaneous cancer progression and accelerated transgenic mice bearing a c-myc oncogene (EC-MYC)-induced lymphomas, and predisposed mice to cancer [30]. Likewise, the expression of *CUL9* had no significance in the TCGA database, but in this study we found that the lower mRNA expression of *CUL9* was noted in HNSCC samples and cells. Furthermore, higher transcriptional level of *CUL9* was observed to favour the OS of HNSCC, which was in keeping with our experimental result. The bioinformatics analysis also suggested that *CUL9* perhaps takes part in the regulation of the immune response primarily through controlling the potential relationship between immunoinhibitors, immunostimulators, and MHC molecules. Lastly, we found that knockdown of *CUL9* could inhibit the capacity for proliferation and migration in HNSCC cell lines. Overall, our results suggest that *CUL9* also played an important tumour-promoting role in HNSCC.

There are some limitations associated with our study. First, more in-depth research, which also include a greater number of samples, are needed to verify our conclusions and to decipher the clinical application of *CULs* in both the diagnosis and treatment of HNSCC. Second, we did not analyse the latent diagnostic and clinical treatment functions of *CULs* in HNSCC, and lastly, other limitations such as the lack of investigation of the downstream molecular mechanisms contributing to the tumour-promoting function of *CULs* in HNSCC and lack of preclinical validation should also be discussed. Thus, whether *CULs* could be used as symptomatic biomarkers or therapeutic indicators remains to be further studied.

Conclusion

On the one hand, multivariate bioinformatics analysis suggests that the transcriptional levels of *CUL2/4A/9* serve as latent prognostic indicators for overall survival of HNSCC. On the other hand, the results also suggest that *CUL2/4A/9* could affect the proliferation and migration of HNSCC cells in vitro. Overall, these results indicate that *CUL2/4A/9* potentially function as novel independent indicators for predicting survival in HNSCC patients. Nevertheless, the detailed downstream mechanism regarding the role of *CUL2/4A/9* in the prognosis of HNSCC needs more in-depth research.

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Data availability
The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of interest
The authors declare no conflict of interest.

Author's contributions
Substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data: B.X., R.W., F.C., X.D. Drafting the article or revising it critically for important intellectual content: B.X., R.W., F.C., X.D. Final approval of the version to be published: J.Z., L.W., X.C., Y.J. All authors have reviewed and approved the submitted manuscript and agree to be accountable for all aspects of the work submitted.

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