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Original Article

Comprehensive analysis of the Cullin family of genes reveals that CUL7 and CUL9 are the significant prognostic biomarkers in colorectal cancer

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Abstract: Objectives: The purpose of this study is to decipher the role of Cullin family genes in colorectal cancer (CRC), drawing insights from comprehensive analyses encompassing multiple databases and experimental validations. Methods: UALCAN, GEPIA2, Human Protein Atlas (HPA), KM plotter, cBioPortal, TISIDB, DAVID, colon cancer cell lines culturing, gene knockdown, CCK8 assay, colony formation, and Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) assays. Results: Initial scrutiny of The Cancer Genome Atlas (TCGA) CRC datasets through the UALCAN and GEPIA databases unveiled significant alterations in Cullin family gene expressions. Elevations in CUL1, CUL2, CUL4A, CUL4B, CUL5, CUL7, and CUL9 were observed in CRC tissues compared to normal counterparts, while CUL3 demonstrated down-regulation consistently across datasets. Further exploration revealed notable correlations between Cullin gene expressions and various clinical parameters of CRC patients, substantiating the potential diagnostic and prognostic utility of these genes. Protein expression analyses conducted via the HPA corroborated the transcriptomic findings, indicating high levels of Cullin proteins in CRC tissues. Prognostic assessments identified CUL7 and CUL9 as significant predictors of poor survival outcomes in CRC patients, emphasizing their clinical relevance. Genetic alterations within the Cullin family genes were elucidated through the cBioPortal database, shedding light on the mutation landscape and prevalence of missense mutations in CRC. Immune subtype and tumor immune microenvironment analyses underscored the intricate interplay between Cullin family genes and immune processes in CRC. Experimental validation in CRC cell lines demonstrated the functional significance of CUL7 and CUL9 in promoting CRC growth, further solidifying their roles as potential therapeutic targets. Conclusion: Overall, these multifaceted analyses elucidated the intricate involvement of Cullin family genes in CRC pathogenesis and provided valuable insights for future diagnostic and therapeutic endeavors in CRC management.

Keywords: Colorectal cancer, Cullin family genes, biomarkers, therapeutic targets

Introduction

Colorectal cancer (CRC) accounts for around 10% of all cancer cases and is globally recognized as the third most frequently diagnosed and second most lethal cancer [1]. In 2020, CRC contributed to approximately 9.4% of cancer-related deaths [1, 2]. Typically asymptomatic until advanced stages, CRC underscores the urgency to discover novel biomarkers for early diagnosis and treatment, aiming to improve prognosis, especially considering the prevalence of small undetectable metastases in over half of colon cancer patients before surgery [3].

The ubiquitin-proteasome system is a primary pathway governing protein degradation [4]. Protein ubiquitination-mediated degradation involves two distinct steps: initially, ubiquitins are catalyzed and transferred to specific substrates through the sequential actions of activating (E1), conjugating (E2), and ligase (E3) enzymes. Subsequently, the poly-ubiquitinated substrates are recognized and degraded by the 26S proteasome complex [5]. The Cullin-Ring

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ubiquitin ligase (CRL) stands as the largest family of E3 ubiquitin ligases, responsible for ubiquitinating approximately 20% of intracellular proteins [6]. Structurally, the Cullin protein functions as a scaffold by binding to an adaptor protein at the N-terminus, and it interacts with a RING protein (RBX1 and RBX2) at the C-terminus, forming a CRL [7]. The activity of CRL is intricately regulated by various proteins, including neural-precursor-cell-expressed developmentally down-regulated 8 (NEDD8), Cullin-associated NEDD8-dissociated protein 1 (CAND1), and the COP9 signalosome complex (CSN). These regulatory proteins modulate the association/dissociation cycles of CRL subunits, thereby influencing the activity of CRLs [8].

In mammals, eight Cullin proteins have been identified, namely CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, CUL7, and CUL9, with the latter closely related to the p53-associated parkin-like cytoplasmic protein (PARC) [9-14]. The CRL degradation pathway plays a crucial role in mediating several proto-oncoproteins and tumor suppressors [12]. Dysregulation of Cullins' expression can lead to aberrant levels of various cancer-related proteins, potentially contributing to tumorigenesis. Prior investigations have noted the overexpression of certain Cullin members in breast cancers, correlating with unfavorable histology grades and poorer survival outcomes [15-18]. However, specific Cullin members have received limited attention in CRC studies, and their prognostic significance remains unclear. Consequently, there is considerable interest in conducting a systematic examination of the prognostic roles of each individual Cullin for breast cancer patients.

In this study, we conducted a comprehensive analysis of the mRNA expression levels and clinical stage characteristics of Cullin members in CRC patients using the TCGA database. Our findings revealed elevated expression levels of CUL1, CUL2, CUL4A, CUL4B, CUL7, and CUL9 in CRC patients compared to normal samples. Subsequently, utilizing the Kaplan-Meier Plotter database, we assessed the prognostic significance of Cullins, highlighting that CUL5 and CUL7 expressions were associated with the prognosis of CRC. Furthermore, our investigation delved into the relationship between Cullin expression and immune infiltration components, uncovering notable differences in immune subtype and cells among CUL members. In conclusion, our study establishes CUL5 and CUL7 as promising diagnostic and prognostic markers and reveals their involvement in the immune processes of CRC patients.

Methodology

Expression profiling of Cullin family genes in The Cancer Genome Atlas (TCGA) database

UALCAN (https://ualcan.path.uab.edu/) is a user-friendly web portal for cancer data analysis, providing easy access to TCGA expression data [19]. It offers in-depth analyses of gene expression and patient survival across cancer types. GEPIA2 (Gene Expression Profiling Interactive Analysis 2, http://gepia2.cancer-pku.cn/) is a versatile tool offering customizable gene expression analyses using TCGA and GTEx data, facilitating comprehensive investigations into gene expression patterns and their implications in various diseases [20]. In the present study, both UALCAN and GEPIA2 databases were used for the expression profiling of Cullin family genes in TCGA datasets of CRC.

Expression profile of Cullin family genes across different clinical variables of CRC patients

In this study, UALCAN [19, 21] was utilized to assess the expression patterns of Cullin family genes among CRC patients, considering diverse clinical variables such as cancer stages, races, genders, and age groups.

Proteomic expression of Cullin family genes in CRC

The Human Protein Atlas (HPA, https://www.proteinatlas.org/) is a comprehensive resource offering detailed information on the expression and localization of proteins in human tissues and cells [22, 23]. Combining immunohistochemistry and antibody-based profiling, HPA provides a valuable platform for researchers to explore protein expression patterns and their relevance in health and disease. In the current work, HPA was used to analyze proteomic expression of Cullin family genes in CRC.

Survival analysis of Cullin family genes in CRC

KM Plotter (https://kmplot.com/analysis/index.php?p=service) is an online survival analysis

tool that leverages gene expression data from multiple cancer studies [24, 25]. Focused on breast, colorectal, ovarian, lung, and gastric cancers, it enables users to explore the impact of specific gene expression on patient survival. KM plotter was used in this study to perform the survival analysis of Cullin family genes in CRC.

Mutational analysis of Cullin family genes in CRC

cBioPortal (https://www.cbioportal.org/) is a versatile platform for cancer genomics, offering a user-friendly interface to explore complex molecular data [26, 27]. Integrating diverse datasets, it empowers researchers to visualize and analyze genetic alterations, mRNA expression, and protein levels across various cancers. cBioPortal is invaluable for elucidating cancer biology and identifying potential therapeutic avenues. In the present study, cBioPortal database was used to perform the mutational analysis of Cullin family genes in CRC.

Correlation between Cullin family gene expression and immune components

TISIDB (http://cis.hku.hk/TISIDB/) represents an online resource designed to analyze interactions between tumors and the immune system [28]. In this study, we conducted a detailed correlation analysis using the Kruskal-Wallis test to examine the relationship between Cullins and both immune and molecular subtypes. Additionally, we investigated the link between members of the Cullin family and immune infiltrating cells by utilizing the GSCA database [29], a web server that integrates multiomics data from the TCGA database.

Correlation of Cullin family genes with immune cells

The Gene Set Cancer Analysis (GSCA, https://guolab.wchscu.cn/GSCA//) database is a comprehensive platform for exploring cancer-associated gene sets [30, 31]. Integrating diverse data sources, GSCA enables researchers to investigate the molecular mechanisms underlying cancer development. In the present work, we explored the association between Cullin family members and immune infiltrating cells via GSCA database.

Gene enrichment analysis

DAVID (Database for Annotation, Visualization, and Integrated Discovery, https://david.ncifcrf.gov/) is a robust bioinformatics tool for functional annotation and enrichment analysis of gene sets [32, 33]. Widely used in genomics research, DAVID facilitates the interpretation of high-throughput data, providing valuable insights into the biological relevance and functional characteristics of gene lists. In the present study, DAVID was utilized to perform Gene enrichment analysis of Cullin family genes.

Cell culture and Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) analysis

Normal colonic epithelial cells (HCoEpiC and HCEC) and five colon cancer cell lines (SW480, SW620, SW1116, RKO, and HCT116) were acquired from the American Type Culture Collection (ATCC) and cultured per the manufacturer's guidelines. Regular mycoplasma contamination checks were conducted. Total RNA extraction from both normal and colon cells followed Sigma Trizol instructions (Invitrogen). cDNA synthesis was performed through RNA reverse transcription, and subsequent RT-qPCR utilized AceQ qPCR SYBR Green Master Mix as per Hiscript QRT supermix instructions for RT-qPCR (Vazyme). GAPDH served as a reference control. Primer sequences were as follows: GAPDH-F Primer, 5'-TGACTTCAACAGCGACACCCA-3', GAPDH-R Primer, 5'-CACCCTGTTGCTGTAGCCAAA-3'; CUL1-F Primer, 5'-CAATGACGCTGGCTTTGTGGCT-3', CUL1-R Primer, 5'-CAAGGAGTCACAGTATCGAGCC-3'; CUL2-F Primer, 5'-GTCTTACTCCGTGCTGTGTCCA-3', CUL2-R Primer, 5'-CTGACTCCACAAATAGTGTTGGC-3'; CUL3-F Primer, 5'-TCGACAGCTCACACTCCAGCAT-3', CUL3-R Primer, 5'-GTGCTTCCGTGTATTAGAGCCAG-3'; CUL4A-F Primer, 5'-GTGCTTCCGTGTATTAGAGCCAG-3', CUL4A-R Primer, 5'-CTGTGGCTTCTTTGTTGCCTGC-3'; CUL4B-F Primer, 5'-GAAGCTACAGATGAAGAACTTGAG-3', CUL4B-R Primer, 5'-GCACTCTTTCCGACTAACAGGC-3'; CUL5-F Primer, 5'-CCTGATGCTGAACTTAGGAGGAC-3', CUL5-R Primer, 5'-GGTTCACTGAGAAGAGGGTACC-3'; CUL7-F Primer, 5'-CCGCAAACTCATCACCAACATCC-3', CUL7-R Primer, 5'-GGCACAGGTATCTGAGGAACAC-3'; CUL9-F Primer, 5'-GTGAGGACTCAAGCTACATGCC-3', CUL9-R Primer, 5'-CAGGTTCTCCAAGAGGATCACC-3'.

RNA interference

HCT116 cells were seeded at a density of 50,000 cells/well in a six-well plate with antibiotic-free fresh media 24 hours before transfection, aiming for 30-50% confluency during transfection. Sequences of CUL7-siRNA (Forward: 5'-UGAGAUCCUAGCUGAACUGTT-3'; and Reverse: 5'-AGAACUCCGCUACAGGGAAUU-3') and CUL9-siRNA (Forward: 5'-CAAGUGUAUATUAATAUUTT-3'; and Reverse: 5'-AGUAUTACUGTTAUAGTUGTT-3') were procured from Thermo Fisher Scientific (Waltham, MA, USA). Transfection was carried out using 100 nmol/L of siRNA and Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Following siRNA transfection, plates were incubated for 48 hours at 37°C before subsequent analysis. Later on, gene knockdown efficiency was checked with the help of RT-qPCR assay using the above mentioned protocol.

Cell proliferation assay

HCT116 cells seeded at a density of 1×10^3 cells per well in 96-well plates. Cell proliferation was assessed using Cell Counting Kit-8 (CCK-8; Dojindo, Rockville, USA) following the manufacturer's guidelines. In brief, 10 μL of CCK-8 solution was introduced into the culture medium and incubated for 2 hours. The absorbance at a wavelength of 450 nm was measured, with a reference wavelength set at 570 nm.

Colony-formation assay

After transfection with CUL7-siRNA and CUL9-siRNA, HCT116 cells were seeded into three 6-cm cell culture dishes. Following a 2-week incubation in complete growth media, cell colonies were fixed using cold methanol and stained with 0.1% crystal violet for 30 minutes. Colony counting was performed manually using a microscope.

Statistical analysis

Statistical analyses were conducted using the SPSS statistical software package (version 16.0; SPSS, Inc., Chicago, IL, USA). Comparisons were assessed using the Student's t-test. All *p* values were two-sided, and statistical significance was defined as $P < 0.05$.

Results

Gene expression of Cullin family in The Cancer Genome Atlas (TCGA) CRC datasets

We investigated the expression patterns of Cullin family genes in TCGA CRC patients utilizing the UALCAN and GEPIA databases. Analysis from the UALCAN database revealed significant up-regulation (*p*-value < 0.05) of CUL1, CUL2, CUL4A, CUL4B, CUL5, CUL7, and CUL9 in CRC patients compared to normal controls, while CUL3 exhibited significant down-regulation (*p*-value < 0.05) (**Figure 1A**). Similarly, analysis conducted using the GEPIA2 database yielded consistent results, confirming the up-regulation of the CUL1, CUL2, CUL4A, CUL4B, CUL5, CUL7, and CUL9 Cullin family genes and down-regulation of CUL3 in CRC tissues compared to normal tissues (**Figure 1B**).

Association between Cullin family gene expression and clinical parameters of CRC patients

Subsequently, we examined the correlation between Cullin family expression and clinical parameters of CRC patients using the UALCAN database. In comparison to normal tissues, the expression levels of CUL1, CUL2, CUL4A, CUL4B, CUL5, CUL7, and CUL9 showed significant (*p*-value < 0.05) elevation across different cancer stages, age groups, and genders in CRC patients (**Figure 2A**, **2B**, **2D-H**). Conversely, CUL3 exhibited significantly lower expression (*p*-value < 0.05) in CRC patients across different cancer stages, age groups, and genders compared to normal controls (**Figure 2C**).

Protein expression of Cullin family genes in CRC

Next, we utilized the HPA database to examine the protein expression profiles of Cullin family genes in CRC. Immunohistochemistry-based results showed that the protein expression of CUL1, CUL2, CUL4A, CUL4B, CUL5, CUL7, and CUL9 was higher (Staining: High) in CRC tissue samples relative to the normal samples (Staining: Low, **Figure 3**). Regarding CUL3, the expression of this protein was lower (Staining: Low) in CRC samples relative to the control sample (Staining: High, **Figure 3**).

Prognostic roles of Cullin family genes

To assess the prognostic significance of the differentially expressed Cullin family genes in

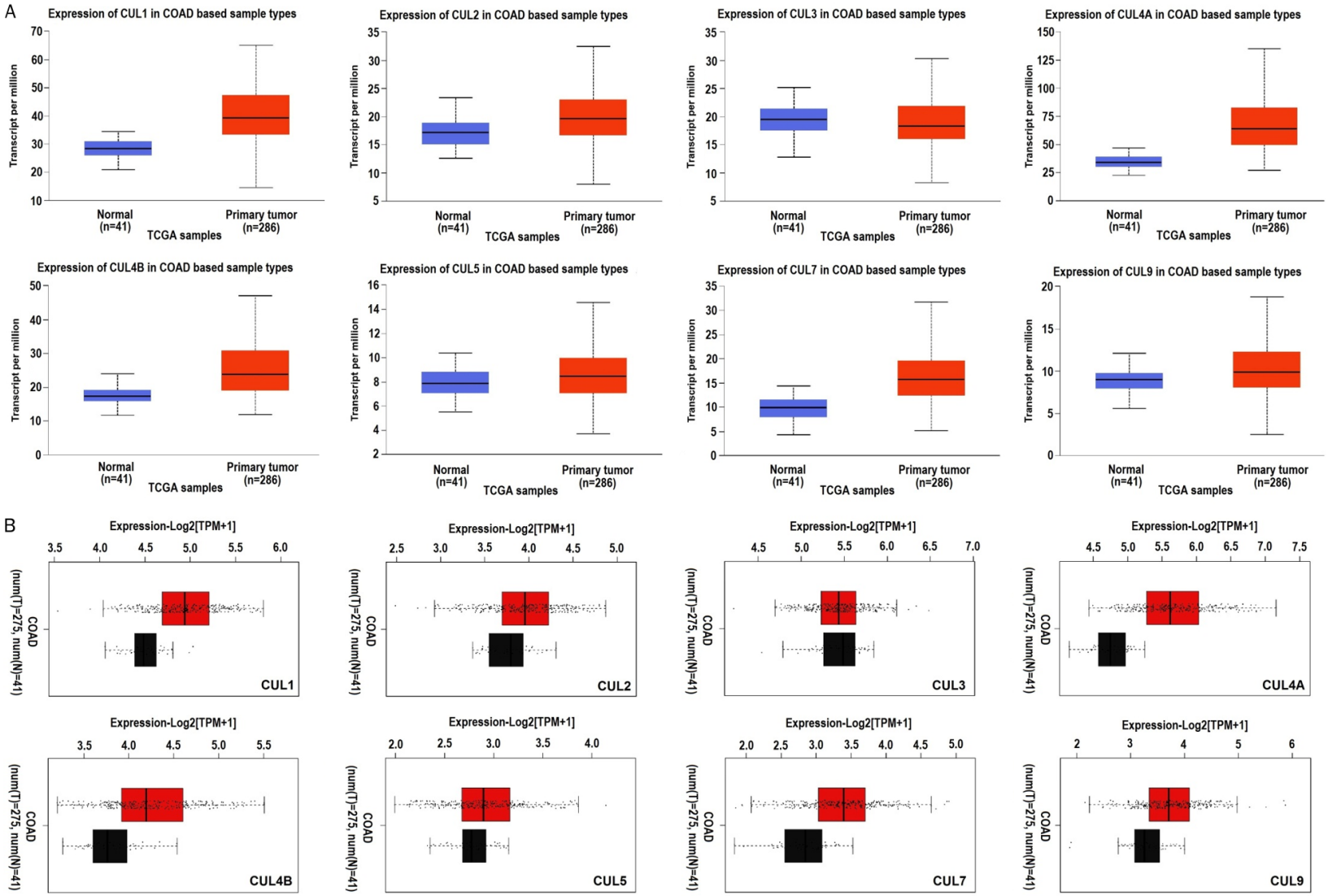
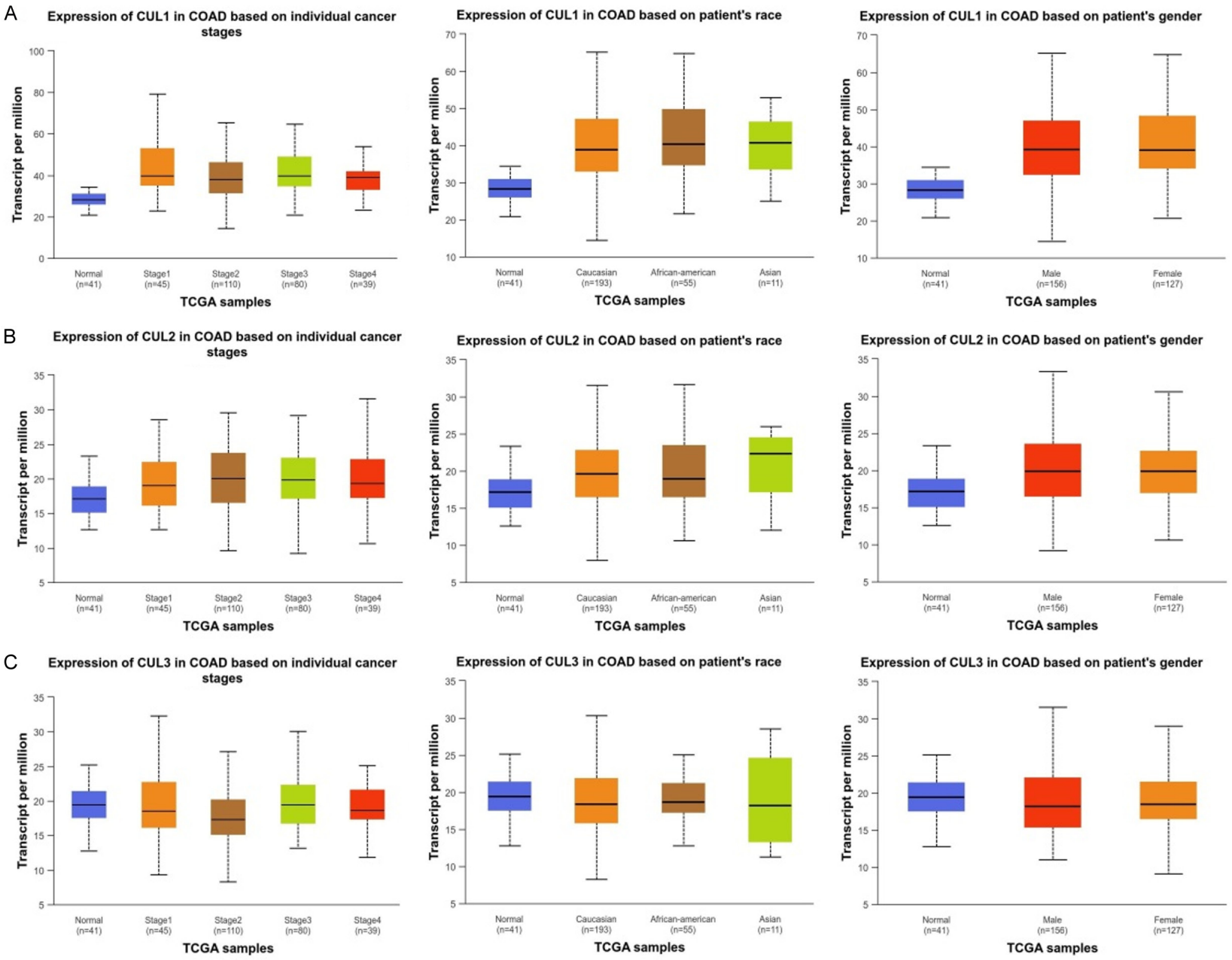
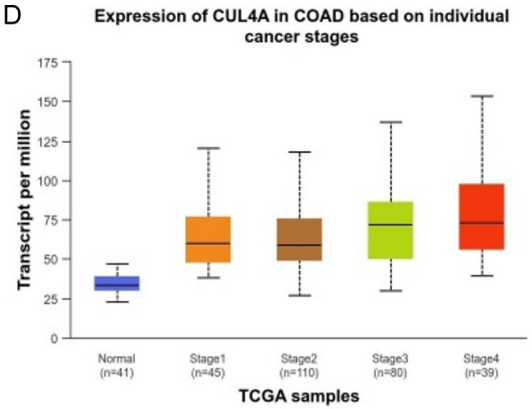
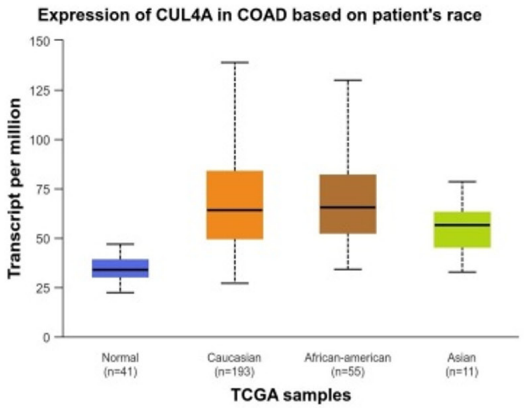


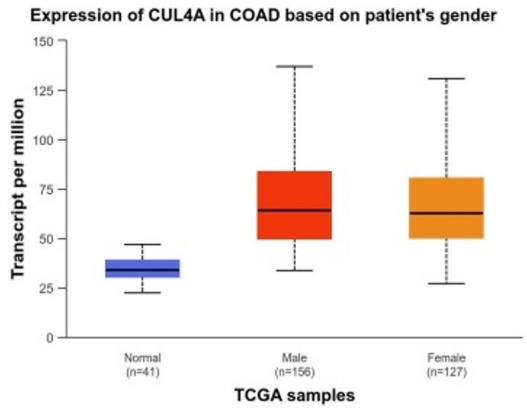
Figure 1. The expression levels of the Cullin family genes in The Cancer Genome Atlas Colon Adenocarcinoma (COAD) samples and adjacent control tissue samples. A. mRNA expression of Cullin family genes as depicted in the UALCAN database. B. mRNA expression of Cullin family genes as presented in the UALCAN database. *P*-value < 0.05.


A
Expression of CUL1 in COAD based on individual cancer stages
Transcript per million
Normal (n=41)
Stage1 (n=45)
Stage2 (n=110)
Stage3 (n=80)
Stage4 (n=39)
TCGA samples
Expression of CUL1 in COAD based on patient's race
Normal (n=41)
Caucasian (n=193)
African-american (n=55)
Asian (n=11)
TCGA samples
Expression of CUL1 in COAD based on patient's gender
Normal (n=41)
Male (n=156)
Female (n=127)
TCGA samples
B
Expression of CUL2 in COAD based on individual cancer stages
Expression of CUL2 in COAD based on patient's race
Expression of CUL2 in COAD based on patient's gender
C
Expression of CUL3 in COAD based on individual cancer stages
Expression of CUL3 in COAD based on patient's race
Expression of CUL3 in COAD based on patient's gender

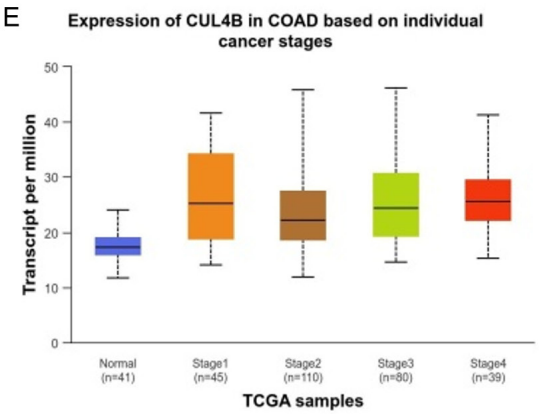
D

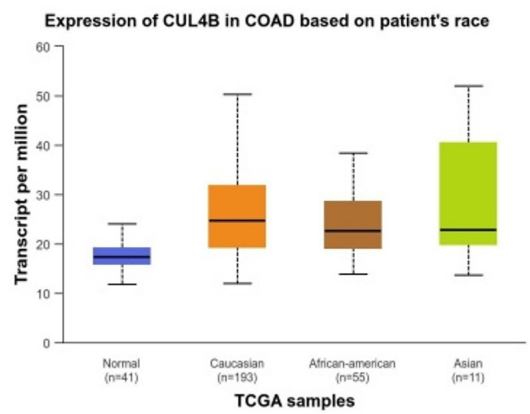

Expression of CUL4A in COAD based on individual cancer stages
Transcript per million
Normal (n=41)
Stage1 (n=45)
Stage2 (n=110)
Stage3 (n=80)
Stage4 (n=39)
TCGA samples

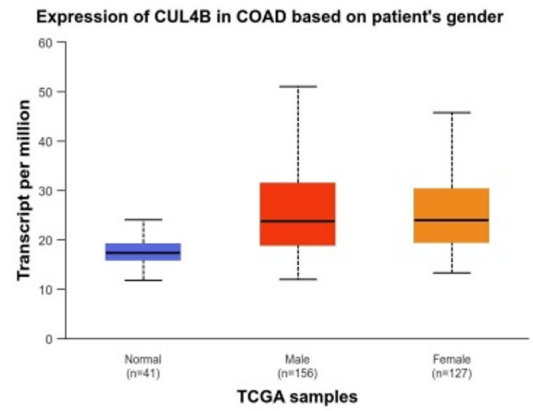

Expression of CUL4A in COAD based on patient's race
Transcript per million
Normal (n=41)
Caucasian (n=193)
African-american (n=55)
Asian (n=11)
TCGA samples


Expression of CUL4A in COAD based on patient's gender
Transcript per million
Normal (n=41)
Male (n=156)
Female (n=127)
TCGA samples

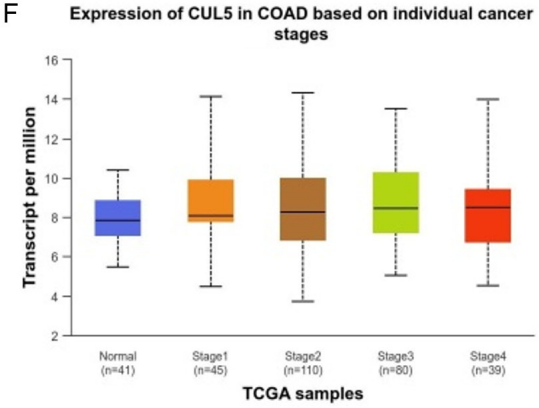
E

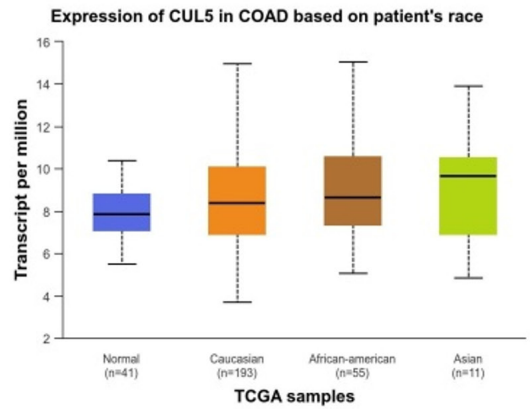

Expression of CUL4B in COAD based on individual cancer stages
Transcript per million
Normal (n=41)
Stage1 (n=45)
Stage2 (n=110)
Stage3 (n=80)
Stage4 (n=39)
TCGA samples

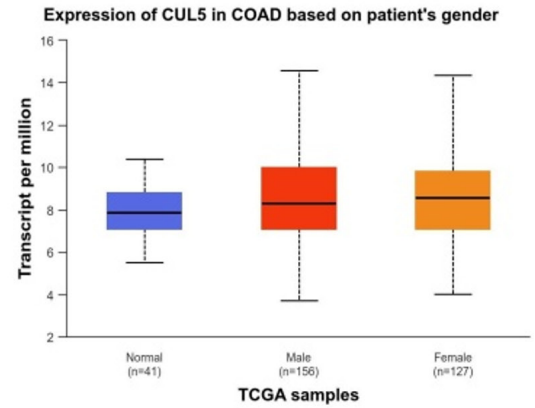

Expression of CUL4B in COAD based on patient's race
Transcript per million
Normal (n=41)
Caucasian (n=193)
African-american (n=55)
Asian (n=11)
TCGA samples


Expression of CUL4B in COAD based on patient's gender
Transcript per million
Normal (n=41)
Male (n=156)
Female (n=127)
TCGA samples

F


Expression of CUL5 in COAD based on individual cancer stages
Transcript per million
Normal (n=41)
Stage1 (n=45)
Stage2 (n=110)
Stage3 (n=80)
Stage4 (n=39)
TCGA samples


Expression of CUL5 in COAD based on patient's race
Transcript per million
Normal (n=41)
Caucasian (n=193)
African-american (n=55)
Asian (n=11)
TCGA samples


Expression of CUL5 in COAD based on patient's gender
Transcript per million
Normal (n=41)
Male (n=156)
Female (n=127)
TCGA samples

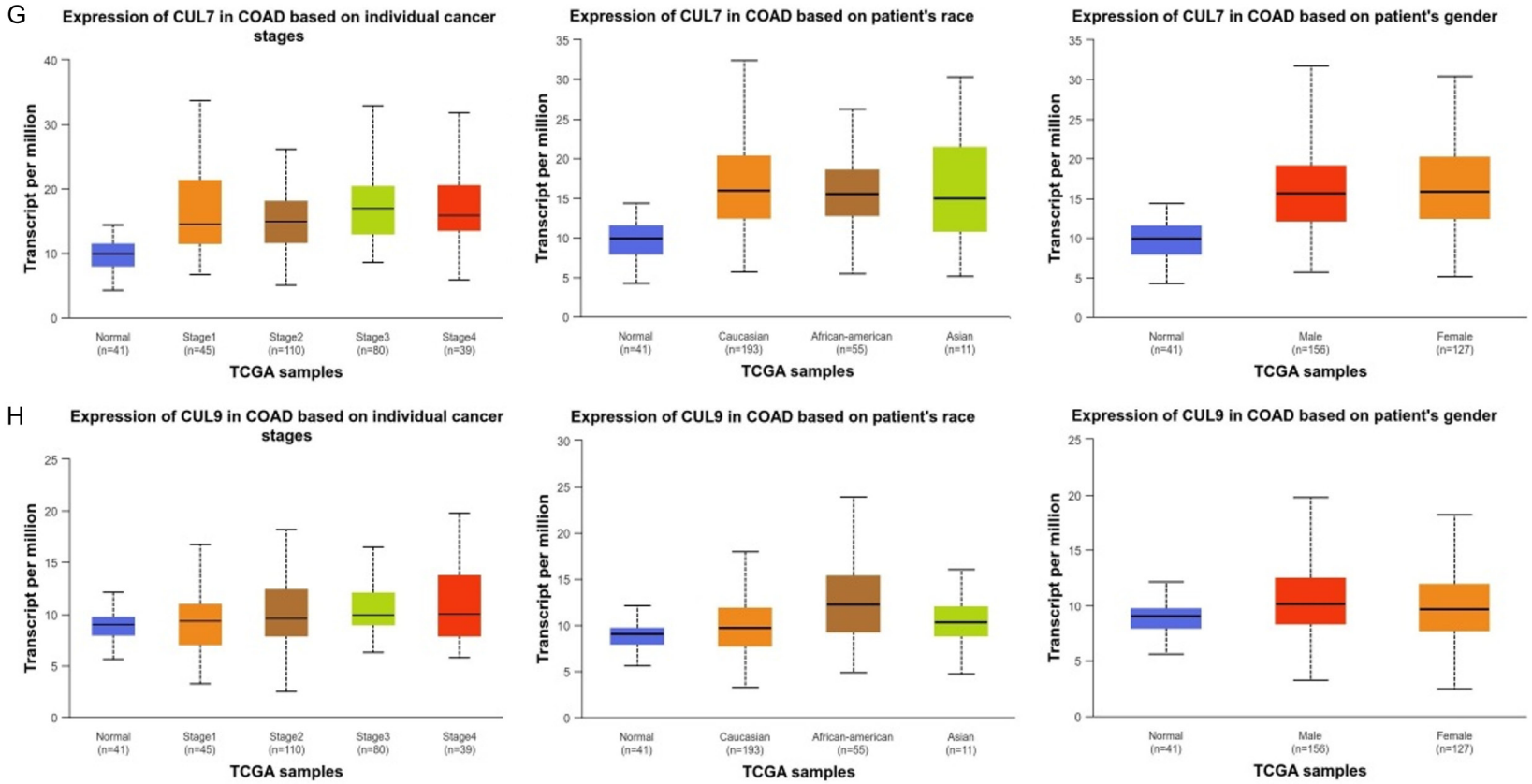


Figure 2. The association between Cullin family gene expression and various clinical variables of Colon Adenocarcinoma (COAD) patients. Boxplots illustrate Cullin transcript levels based on stage, race, and gender as extracted from the UALCAN database. (A) CUL1 expression in CRC patients of different stages, races, and genders, (B) CUL2 expression in CRC patients of different stages, races, and genders, (C) CUL3 expression in CRC patients of different stages, races, and genders, (D) CUL4A expression in CRC patients of different stages, races, and genders, (E) CUL4B expression in CRC patients of different stages, races, and genders, (F) CUL5 expression in CRC patients of different stages, races, and genders, (G) CUL7 expression in CRC patients of different stages, races, and genders, and (H) CUL9 expression in CRC patients of different stages, races, and genders. *P*-value < 0.05.

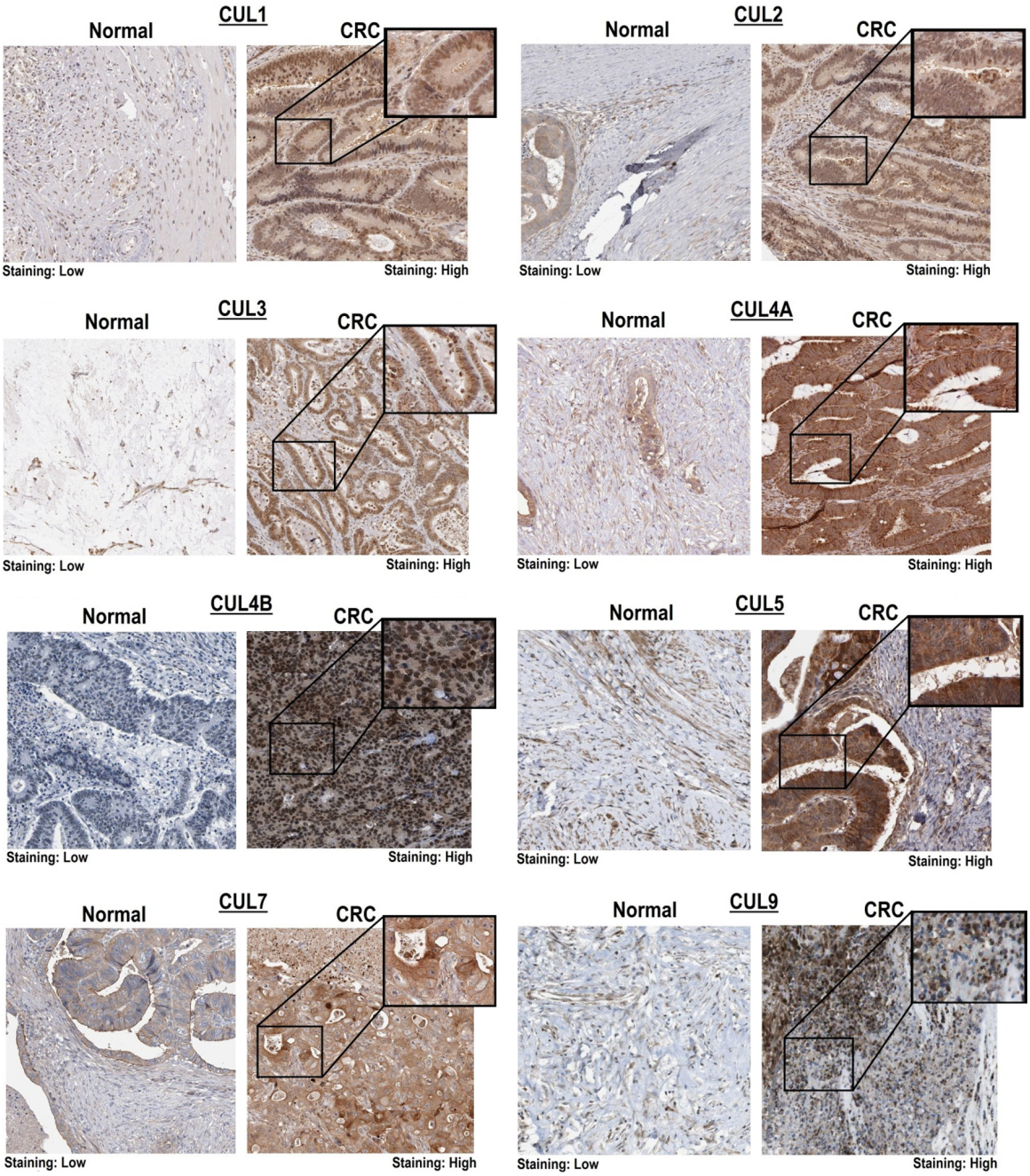


Figure 3. The proteomic expression patterns of Cullin family members in colorectal cancer (CRC) tissues compared to adjacent normal control tissues, as sourced from the Human Protein Atlas database.

CRC, we employed gene chip data from Kaplan-Meier Plotter for survival analysis. The results indicated that among all Cullin family members, only CUL7 and CUL9 exhibited notable predictive value (**Figure 4**). Elevated expressions of CUL7 and CUL9 were significantly (p-value < 0.05) correlated with shorter overall survival (OS) in CRC patients (**Figure 4**). Conversely, other members of the Cullin family, such as CUL1, CUL2, CUL3, CUL4A, CUL4B, and CUL5, did not demonstrate significant associations with OS of the CRC patients (**Figure 4**).

Genetic alterations among Cullin family genes in CRC

Utilizing the cBioPortal database, we investigated the genetic alterations within the Cullin

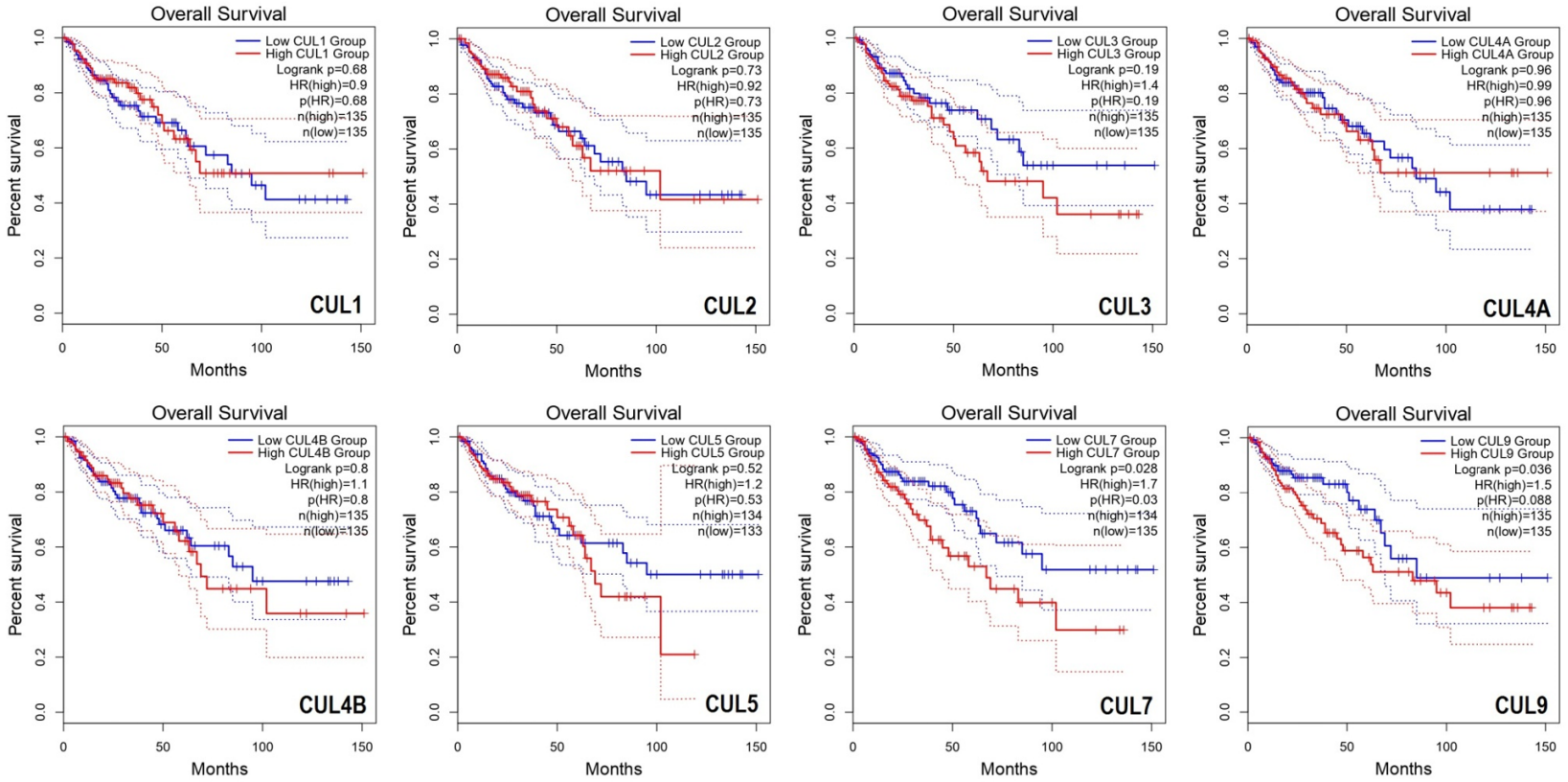


Figure 4. The prognostic analysis of Cullin family genes in Colon Adenocarcinoma (COAD) patients via the Kaplan-Meier Plotter. The survival plots of CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, CUL7, and CUL9 are shown. *P*-value < 0.05.

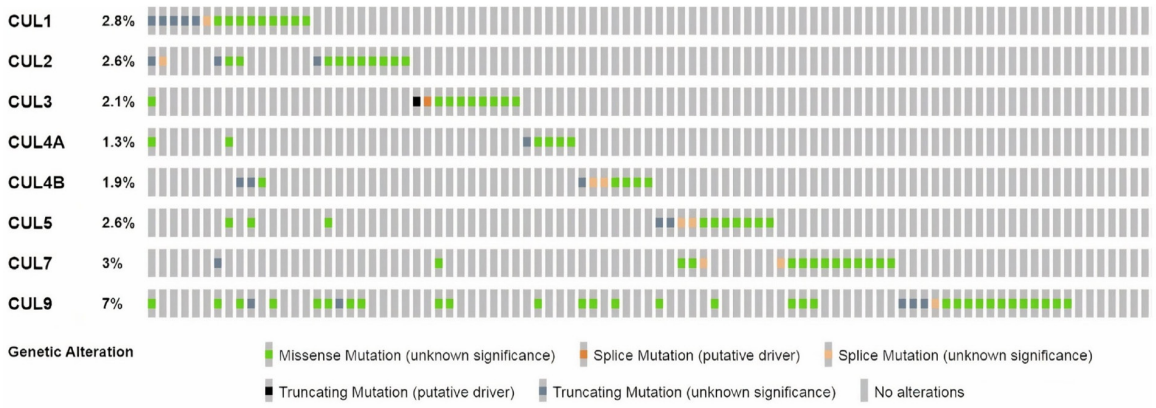


Figure 5. Mutational analysis of Cullin family genes among Colon Adenocarcinoma (COAD) patients via the cBioPortal database. The oncoplots show the mutational frequencies of CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, CUL7, and CUL9 in the COAD samples.

family among CRC patients. The mutation rates for Cullin family genes, including CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, CUL7, and CUL9, were determined as 2.8%, 2.6%, 2.1%, 1.3%, 1.9%, 2.6%, 3.0%, and 7%, respectively (**Figure 5**). Notably, missense mutations predominated among the observed mutations within the Cullin family genes in CRC patients (**Figure 5**).

Immune subtype and tumor immune microenvironment analysis of Cullin family genes

Prior research has highlighted the potential involvement of the Cullin family in immune processes across various cancers [34]. Motivated by these findings, we proceeded to investigate the immune subtype associations of Cullin family genes. The immune subtypes encompassed C1 (wound healing), C2 (IFN-gamma dominant), C3 (inflammatory), C4 (lymphocyte depleted), C5 (immunologically quiet), and C6 (TGF-β dominant) categories. Our analysis revealed significant correlations between the expression levels of CUL2, CUL3, CUL4A, CUL5, CUL7, and CUL9 and immune subtypes in COAD (**Figure 6A**). Furthermore, we examined the relationship between Cullin family gene expression and molecular subtypes. The findings demonstrated significant associations between most Cullin family members, with the exception of CUL9, and different molecular subtypes in COAD (**Figure 6B**).

Validation of Cullin family gene expression in cell lines

In this phase of our study, two normal colonic epithelial cell lines (HCoEpiC and HCEC) and five colon cancer cell lines (SW480, SW620, SW1116, RKO, and HCT116) were utilized to validate the expression of Cullin family genes through RT-qPCR. Results of the RT-qPCR analysis validated that the expression of CUL1, CUL2, CUL4A, CUL4B, CUL5, CUL7, and CUL9 genes was significantly (p-value < 0.5) up-regulated, while the expression of CUL3 was significantly (p-value < 0.5) down-regulated in CRC cell lines as compare to the control cell lines (**Figure 7A**). Overall, these findings shed light on the involvement of expression variances within the Cullin gene family in the development of CRC.

Cullin family gene expression and immune cell infiltration

Gaining insight into the connection between cancers and the immune system is beneficial for devising therapeutic approaches. Hence, this study delved into the associations between Cullin family genes and immune cells. Our findings revealed significant correlations (p-value < 0.05) between Cullin family genes and various immune cell types in COAD. For instance, the expression of CUL7 exhibited a negative correlation with immune-active cells such as cytotoxic, CD8-T, NK, Th1 cells, among others (**Figure 7B**). Conversely, CUL5 expression showed a positive correlation with immune regulatory cells including iTreg, macrophage, and DC immune cells (**Figure 7B**).

Gene enrichment analysis of Cullin family

Gene Ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment

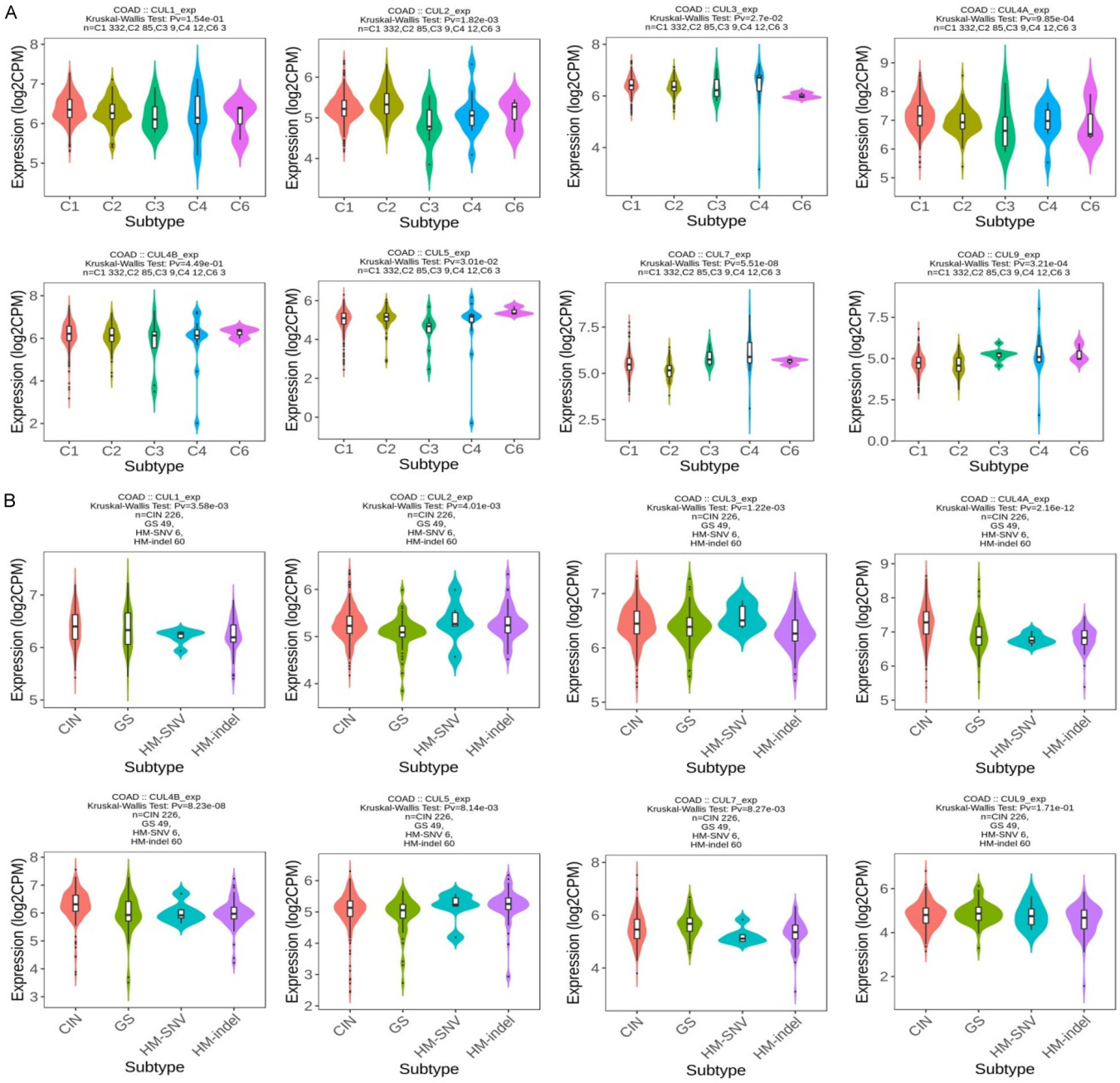

A
COAD :: CUL1_exp
Kruskal-Wallis Test: Pv=1.54e-01
n=C1 332,C2 85,C3 9,C4 12,C6 3
COAD :: CUL2_exp
Kruskal-Wallis Test: Pv=1.82e-03
n=C1 332,C2 85,C3 9,C4 12,C6 3
COAD :: CUL3_exp
Kruskal-Wallis Test: Pv=2.7e-02
n=C1 332,C2 85,C3 9,C4 12,C6 3
COAD :: CUL4A_exp
Kruskal-Wallis Test: Pv=9.85e-04
n=C1 332,C2 85,C3 9,C4 12,C6 3
COAD :: CUL4B_exp
Kruskal-Wallis Test: Pv=4.49e-01
n=C1 332,C2 85,C3 9,C4 12,C6 3
COAD :: CUL5_exp
Kruskal-Wallis Test: Pv=3.01e-02
n=C1 332,C2 85,C3 9,C4 12,C6 3
COAD :: CUL7_exp
Kruskal-Wallis Test: Pv=5.51e-08
n=C1 332,C2 85,C3 9,C4 12,C6 3
COAD :: CUL9_exp
Kruskal-Wallis Test: Pv=3.21e-04
n=C1 332,C2 85,C3 9,C4 12,C6 3
Expression (log2CPM)
C1 C2 C3 C4 C6
Subtype
B
COAD :: CUL1_exp
Kruskal-Wallis Test: Pv=3.58e-03
n=CIN 226,
GS 49,
HM-SNV 6,
HM-indel 60
COAD :: CUL2_exp
Kruskal-Wallis Test: Pv=4.01e-03
n=CIN 226,
GS 49,
HM-SNV 6,
HM-indel 60
COAD :: CUL3_exp
Kruskal-Wallis Test: Pv=1.22e-03
n=CIN 226,
GS 49,
HM-SNV 6,
HM-indel 60
COAD :: CUL4A_exp
Kruskal-Wallis Test: Pv=2.16e-12
n=CIN 226,
GS 49,
HM-SNV 6,
HM-indel 60
COAD :: CUL4B_exp
Kruskal-Wallis Test: Pv=8.23e-08
n=CIN 226,
GS 49,
HM-SNV 6,
HM-indel 60
COAD :: CUL5_exp
Kruskal-Wallis Test: Pv=8.14e-03
n=CIN 226,
GS 49,
HM-SNV 6,
HM-indel 60
COAD :: CUL7_exp
Kruskal-Wallis Test: Pv=8.27e-03
n=CIN 226,
GS 49,
HM-SNV 6,
HM-indel 60
COAD :: CUL9_exp
Kruskal-Wallis Test: Pv=1.71e-01
n=CIN 226,
GS 49,
HM-SNV 6,
HM-indel 60
Expression (log2CPM)
CIN GS HM-SNV HM-indel
Subtype

Figure 6. Correlation between the Cullin family gene expression and immune and molecular subtypes of Colon Adenocarcinoma (COAD). A. Correlation of Cullin family genes with immune subtypes. B. Correlation of Cullin family genes with molecular subtypes. *P*-value < 0.05.

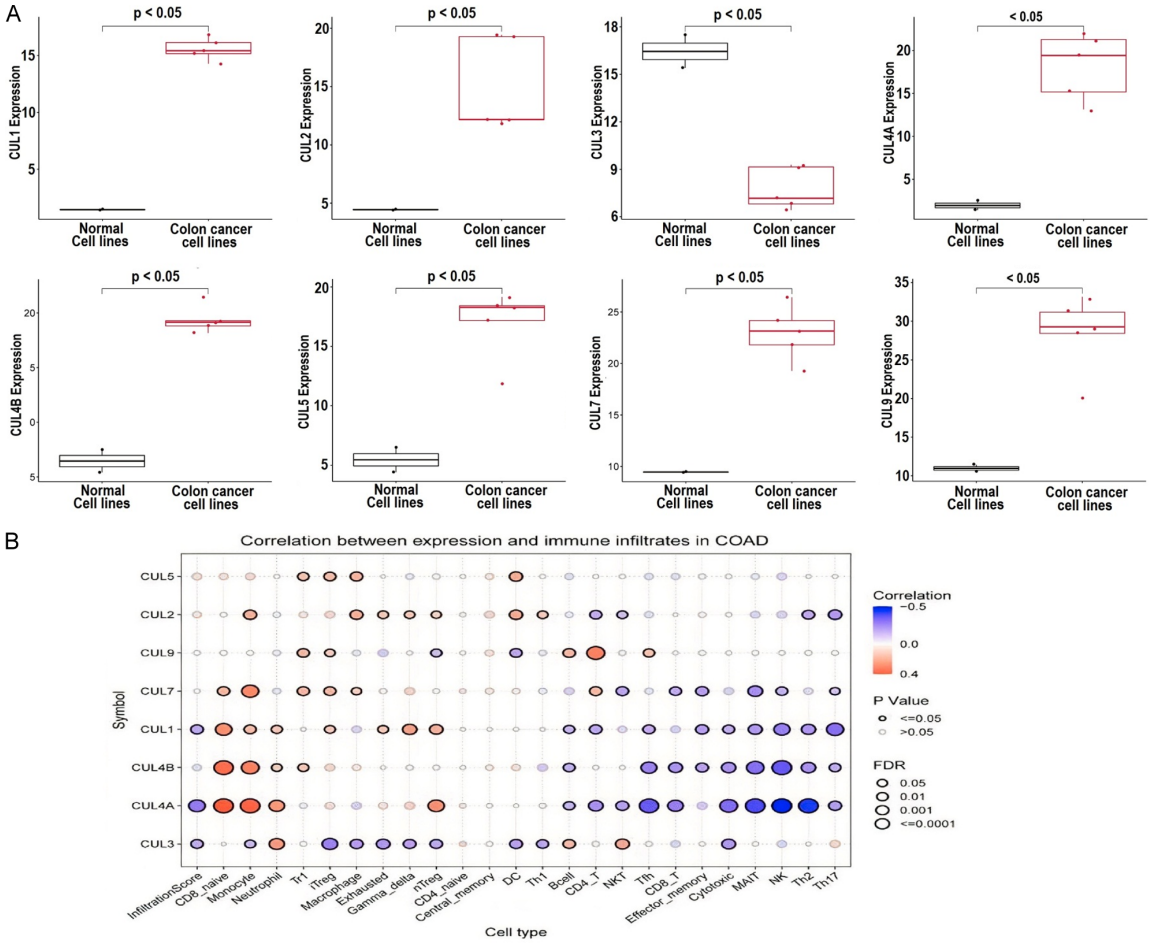


Figure 7. Expression of Cullin family genes in cell lines and association analysis of Cullin family gene expression with the immune-infiltration cells in Colon Adenocarcinoma (COAD). A. Expression of Cullin family genes in normal and colon cancer cell lines. B. Association of Cullin family gene expression with immune-infiltration cells in COAD. *P*-value < 0.05.

analysis of Cullin family genes were performed via the DAVID. In the CC (cellular components) category, Cullin family genes were enriched in "VCB complex, parkin-FBX-W7-Cul7 ubiquitin ligase complex, and Cul7-RING ubiquitin ligase complex, etc., terms (**Figure 8A**)". In the MF (molecular function) category, Cullin family genes were mainly involved in "POZ domain binding, Cul7 ubiquitin protein ligase binding, and ubiquitin-like protein ligase binding, etc., terms (**Figure 8B**)". In the BP (biological process) category, Cullin family genes were enriched in "SCF-dependent proteaosomal ubiquitin-dependent protein catabolic proc., G1/S transition of mitotic cell cycle, and cell cyle G1/S phase transition, etc., terms (**Figure 8C**)". Finally, KEGG analysis outcomes revealed that Cullin family genes were involved in diverse signaling pathways, including "Ubiquitin mediated proteolysis, Base excision repair, Hedgehog signaling pathway, and circadian rhythm, etc. (**Figure 8D**)".

Reduction in the expression of CUL7 and CUL9 inhibited CRC growth

We designed two si-RNAs, including si-RNA1 for CUL7 and si-RNA2 for CUL9 gene. Later on,

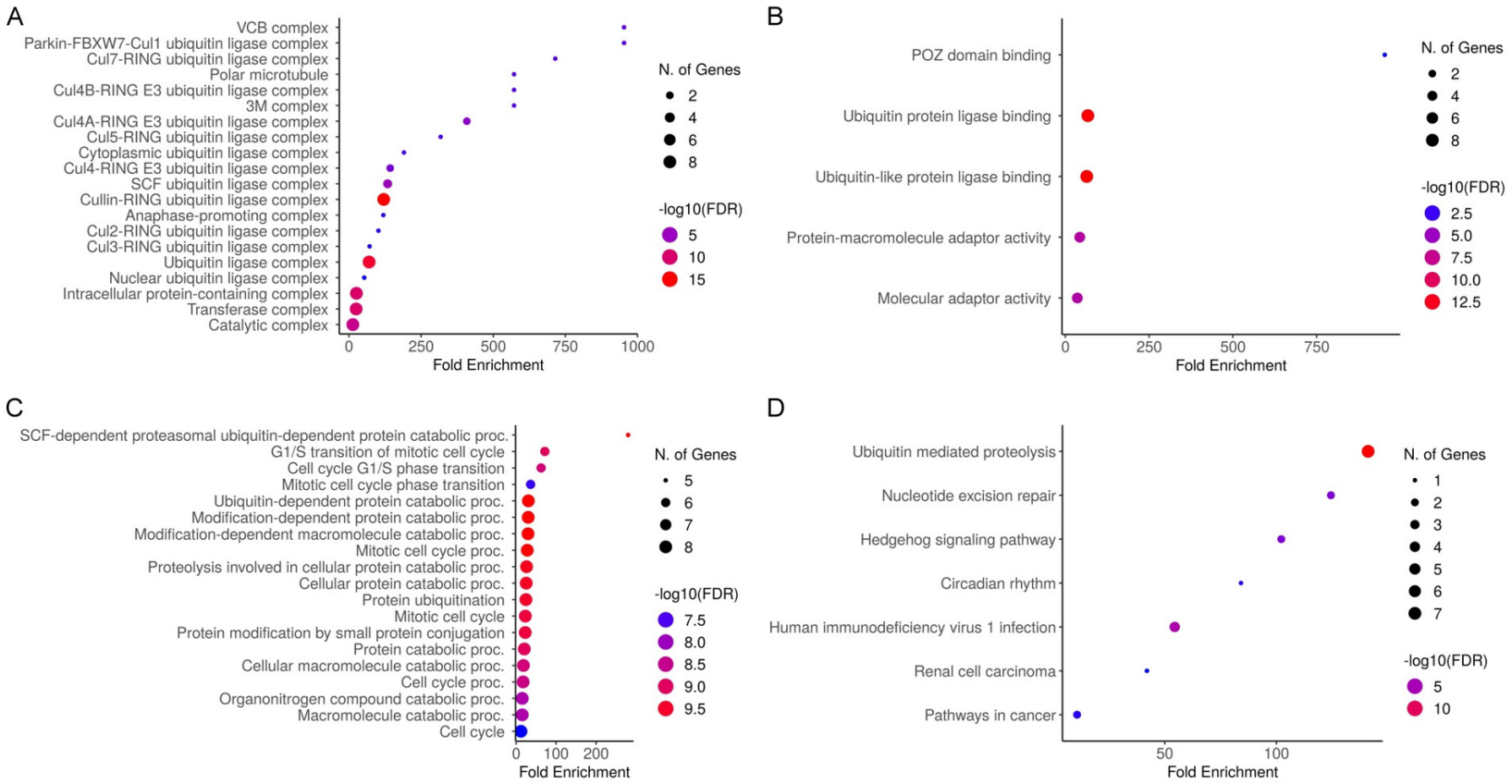


Figure 8. Functional and pathway enrichment analyses conducted on Cullin family genes. (A) Cellular Component (CC) terms, (B) Molecular Function (MF) terms, (C) Biological Process (BP) terms, and (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) terms associated with the Cullin family genes. *P*-value < 0.05.

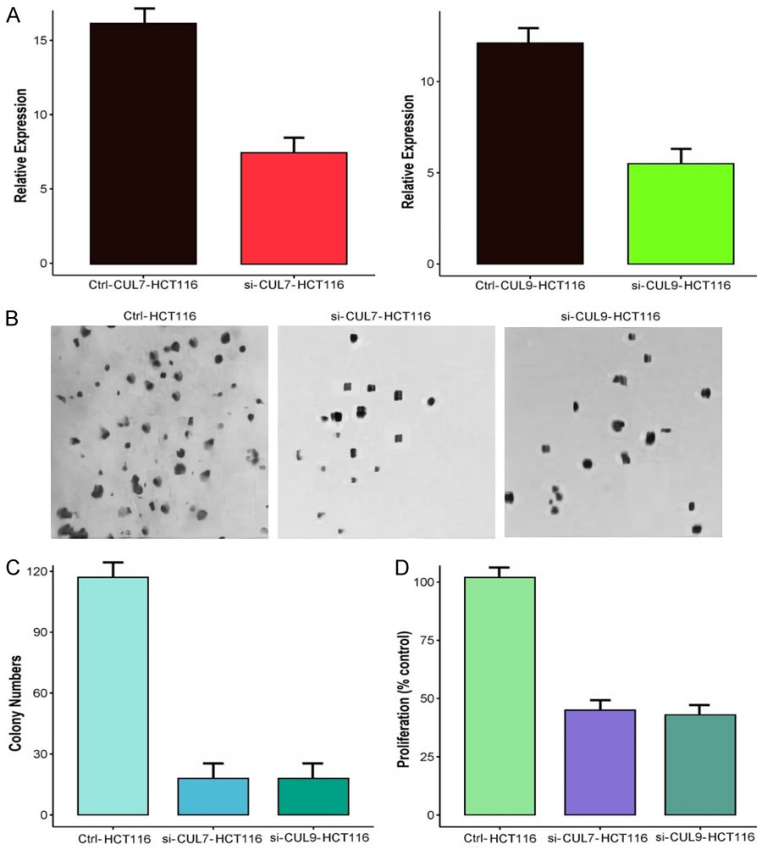


Figure 9. Biological functions of CUL7 and CUL9 in colorectal cancer (CRC). A. Evaluation of gene knockdown efficiency in colon cancer cell lines transfected with siRNAs. B. The assessment of colony formation ability in colon cell lines transfected with siRNAs targeting CUL7 and CUL9, respectively, via colony formation assay. C. Colony numbers graph. D. The assessment of cell proliferation in colon cancer cells transfected with siRNAs targeting CUL7 and CUL9 using CCK8 assay.

HCT116 cells were transfected with the constructed siRNAs. The efficiency of si-RNAs was verified by qRT-PCR (**Figure 9A**). Colony formation assays revealed a significant reduction in colony numbers in transfected colon cancer cells (si-CUL7-HCT116 and si-CUL9-HCT116) compared to control colon cells (Ctrl-HCT116) (**Figure 9B**, **9C**), indicating that silencing CUL7 and CUL9 led to notable suppression of colony formation. Additionally, CCK8 assays demonstrated a considerable decrease in the proliferation rate of colon cancer cells (si-CUL7-HCT116 and si-CUL9-HCT116) relative to control colon cells (Ctrl-HCT116) (**Figure 9D**). Collectively, results from colony formation and CCK8 assays underscored the pivotal roles of CUL7 and CUL9 genes in promoting the growth of colon cancer cells.

Discussion

Numerous studies have found that the abnormal expression of Cullin family members is closely related to the occurrence, development, metastasis, and recurrence of various malignant tumors [6]. For example, Min et al. demonstrated that the expression of Cullin 1 in breast cancer cells is positively correlated with the expression of p53 and regulates cell apoptosis [35]. Cullin 3 accelerates the progression of breast cancer by regulating the effect of speckle-type POZ protein on the expression of breast cancer metastasis suppressor 1 [36, 37]. The overexpression of Cullin 4A promotes the growth and metastasis of basal-like breast tumors [38]. Cullin 7 serves as a vital structural component of E3 ubiquitin ligases and functions as an oncogene, exerting a crucial impact on the proliferation and differentiation of pancreatic cancer cells [39]. It has been demonstrated to inhibit Myc-induced apoptosis and facilitate Myc-mediated malignant transformation of cells [40]. Moreover, Cullin 7 has been found to impede p53-dependent DNA repair function [41] and trigger epithelial-mesenchymal transition (EMT) in choriocarcinoma [42]. Despite these insights, the precise roles of Cullin family members in CRC remain elusive. Hence, the present study aims to comprehensively elucidate the roles of Cullin family members through a combination of bioinformatics analysis and molecular experiments.

Analysis of Cullin family gene expression across multiple TCGA datasets, the HPA database, and various cell lines revealed a significant up-regulation of CUL1, CUL2, CUL4A, CUL4B, CUL5, CUL7, and CUL9 in CRC patients compared to normal controls, while CUL3 exhibited significant down-regulation. Additionally, prognostic

analysis indicated that elevated expressions of CUL7 and CUL9 were significantly correlated with shorter overall survival (OS) in CRC patients. In contrast, other members of the Cullin family, including CUL1, CUL2, CUL3, CUL4A, CUL4B, and CUL5, did not show significant associations with OS in CRC patients. These findings collectively suggest that CUL7 and CUL9 serve as effective prognostic biomarkers for CRC. In a recent investigation, heightened levels of Cullin 7 were observed in hepatocellular carcinoma (HCC) tumor tissues, especially in metastatic HCC, correlating with reduced survival rates among HCC patients [43]. Furthermore, increased expression of Cullin 7 was noted in primary lung cancer tissues, with overexpressed Cullin 7 mRNA significantly linked to unfavorable prognosis in individuals with non-small cell lung carcinoma [44, 45]. Moreover, in breast cancer tissues, significant associations were found between overexpression of Cullin 9 and overall survival [15, 35].

The investigation of genetic alterations within the Cullin family among CRC patients sheds the light on the landscape of mutations in this gene family. The mutation rates vary among different Cullin family members, with CUL9 showing the highest mutation rate at 7%, while others range from 1.3% to 3.0%. Interestingly, missense mutations appear to be the predominant type of mutation observed within the Cullin family genes in CRC patients. These findings underscore the importance of genetic alterations in Cullin genes and suggest potential avenues for further research into their roles in CRC tumorigenesis and progression.

Furthermore, we observed a close relationship between several Cullin family genes and immune subtypes in colorectal adenocarcinoma (COAD). Likewise, most Cullins showed significant associations with immune cell infiltrations. Specifically, CUL7 expression exhibited a negative correlation with immune-active cells such as cytotoxic, CD8-T, NK, and Th1 cells in COAD. Previous studies have suggested that tumor-infiltrating regulatory T cells (Tregs) contribute to adverse clinical outcomes by suppressing anti-tumor immunity and promoting angiogenesis [46]. Therefore, CUL7 might contribute to the formation of an immunosuppressive microenvironment, enabling evasion of the tumor immune response, and potentially contributing to poor prognosis.

Conclusion

In conclusion, our study highlights the significance of CUL7 and CUL9 overexpression in the malignant phenotype of CRC, serving as prognostic indicators of poor patient outcomes. The identification of CUL7 and CUL9 as potential biomarkers offers promising avenues for precise diagnosis and treatment strategies in CRC.

Disclosure of conflict of interest

None.

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