Comprehensive analysis of cyclin-dependent kinase 6 and its correlation with immune infiltration in uterine corpus endometrial carcinoma

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Abstract

Background: CDK6, a member of the cyclin-dependent kinases (CDKs) family, is known as a classic cell cycle regulating kinases and plays an important role in promoting cancer initiation as well as progression. However, how CDK6 affects the immune system and the prognosis of uterine corpus endometrial carcinoma(UCEC) remains to be determined.

Methods: TIMER, UALCAN, TCGA, HPA, LinkedOmics, STRING, cBio-Portal and COSMIC were used to analyze the expression and mutation of CDK6 in patients with UCEC and its clinical value, and to explore the possible mechanism of CDK6 in the occurrence and development of UCEC through the biological functional analysis and immune infiltration.

Results: Our results suggested that the overall expression level of CDK6 in UCEC was significantly lower than that in adjacent normal tissues. Inversely, the CDK6 level in the intermediate-/high-risk endometrial carcinoma group was significantly higher than patients in the low-risk group. Subgroup analysis showed that the serous histological type, grade 3, and stage III had significantly higher CDK6 levels. The result of promoter methylation analysis served as a good explanation of the above conclusion. What's more, patients with high CDK6 expression had a poorer prognosis compared with those patients with low CDK6 expression using the ULCAN and Kaplan–Meier plotter data analysis. **GO and KEGG enrichment analysis** revealed that the Gene Co-Expression Network of CDK6 was mainly involved in extracellular structure organization and focal adhesion. PPI analysis validated that CDK6 was controlled by the Cip/Kip family and the INK4 family. Importantly, we found that CDK6 was significantly correlated with most of the immune markers of different T cells in UCEC, which suggested that CDK6 plays an important role in regulating UCEC tumor immunity and is probably involved in the T cell immune response. In addition, M1, M2 macrophage, TAM, Monocyte, Neutrophil, and Dendritic cell were also verified to correlate with CDK6 significantly.

Conclusions: CDK6 plays an important role in endometrial carcinoma progression and may serve as a potential prognostic biomarker and novel risk stratification target for endometrial carcinoma.

Background

Uterine corpus endometrial carcinoma (UCEC) is the second most commonly diagnosed cancer in the female genital system worldwide, with nearly 414,000 new cases reported in 2020, and this may be further exacerbated by increasing risk factors associated with globalization and a growing economy[1]. It takes place frequently in perimenopausal and postmenopausal women and the important prognostic factors include the age of onset, pathological stage, degree of tumor differentiation, and histological subtype[2]. Total abdominal hysterectomy and bilateral salpingo-oophorectomy with or without lymph node dissection remain the primary treatment for UCEC, followed by chemotherapy and/or radiotherapy according to risk stratification[3]. Although most UCEC is detected at an early stage and surgical intervention is curative, a subset of patients termed 'high-intermediate risk'(H-IR) experience an increased

rate of recurrence[4]. As genetic testing technologies rapidly evolve, there has already been some progress in the molecular features of UCEC: polymerase epsilon mutated(POLEmut), p53 abnormal(p53abn), mismatch repair deficient(MMRd), and no specific molecular profile (NSMP)[5]. The four groups have prognostic value and represent an encouraging appliance for clinical decision-making concerning adjuvant treatment[6]. However, evidence on the clinical efficacies of targeted therapies for these recurrence patients based on specific molecular features is limited. More molecular-based analyses are needed for an enhanced understanding of patients' true risk of recurrence, particularly patients of high-intermediate risk, and to simplify the rise of personalized medicine and improve patients' survival.

The family of cyclin-dependent kinases (CDKs), in complex with cyclins, regulate various critical cellular processes including cell cycle progression as well as transcription[7, 8]. CDK6 and the highly homologous enzyme CDK4 are known as classic cell cycle regulating kinases and play an important role in promoting cancer initiation as well as progression, which have attracted extraordinary attention in cancer research over the last years. Altered expression and dysregulated function have made them attractive targets for pharmacological inhibition[9, 10]. For example, the CDK4/6 inhibitors palbociclib (PD0332991), ribociclib (LEE011) and abemaciclib (LY835219) have been approved by the US Food and Drug Administration(FDA) for the treatment of hormone-receptor-positive breast cancer[11] and lymphomas[12]. However, it is not clear whether UCEC could benefit from these agents. Even if CDK6 and CDK4 are stated to confer important functions as critical regulators in cell cycle progression, the two kinases differ exclusively in cell-cycle independent tissue-specific functions presented in findings over the last years. CDK4 was highly expressed on different human skin tumors[13] while CDK6's expression was found to be upregulated in resistant esophageal cancer tissues and cell lines[14]. What's more, the critical role of CDK6 in Notch-Akt-dependent T-cell development and tumorigenesis cannot be compensated by CDK4[11]. Moreover, kinase-independent functions, as described for the transcriptional regulator CDK6, are not targeted by dual CDK4/6 inhibitors[15]. To address this issue, different selective CDK6 degraders have been designed and showed promising in-vitro results. For example, CDK6-selective proteolysis-targeting chimeras(PROTACS) remarkably reduced leukemia burden in mice injected with patient-derived Philadelphia-positive (Ph+) ALL[16]. Several studies have found that increased expression of nuclear CDK4 is involved in the carcinogenesis of endometrial carcinomas[17–19]. Although CDK6 interacts with CDK4, limited evidence was found about the role of CDK6 activity in UCEC.

Cancer immunology is the most rapidly expanding field in cancer research, with the importance of immunity in cancer pathogenesis now well accepted including in the endocrine-related cancers[20]. Nevertheless, how CDK6 affects the immune system and the prognosis of UCEC remains to be determined.

In summary, CDK6 was associated with cell cycle, cell proliferation and apoptosis, and poor prognosis in cancer patients. However, the expression pattern and role of CDK6 in UCEC were unclear. In this study, we investigated the expression of cdk6 and its correlations with clinicopathological features, prognostic value, and the influence of CDK6 on the tumor immune microenvironment in UCEC using comprehensive bioinformatic analysis.

First, we compared the mRNA expression pattern of CDK6 between carcinoma tissues and normal tissues and estimated the prognostic role of CDK6 mRNA expression in UCEC patients. Consequently, we investigated the relationship between CDK6 mRNA expression and clinicopathological parameters in patients with UCEC, and determine the biological pathways related to CDK6 using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. Finally, the association of CDK6 expression and immune infiltration in UCEC was assessed. Our results proposed that CDK6 had a robust effect on the immune microenvironment and may be a promising diagnostic and prognostic biomarker for UCEC.

Methods

Expression analysis and transcriptional analysis

We used the Tumor Immune Estimation Resource(TIMER)(https://cistrome.shinyapps.io/timer/) database to evaluate the expression of CDK6 in various human cancers[21]. Then, the UCEC dataset from The Cancer Genome Atlas (TCGA) (www.tcga-data.nci.nih.gov/tcga/), which contains more than 10,000 samples of 39 tumor types, was selected for analysis of the mRNA expression of CDK6 in UCEC and different subgroups, using the UALCAN database(http://ualcan.path.uab.edu)[22]. Subgroups covered BMI, age, race and diabetes, histological type, histologic grade, clinical stage, surgical approach, overall survival event, disease-specific survival event, and progression-free interval event. Patients were divided into high and low expression groups based on the median mRNA expression values.

The promoter methylation levels of CDK6 in UCEC and the subgroups of UCEC were also assessed compared with those in normal controls. Moreover, we explored the protein expression of CDK6 in the Human Protein Atlas(HPA) database(www.proteinatlas.org)[23]. What's more, we validated the prognostic value of CDK6 in UCEC using ULCAN and Kaplan-Meier plotter databases(https://kmplot.com/analy sis/)[24].

Biological functional analysis

we used the LinkedOmics database(http://www.linkedomics.org/login.php) to analyze the CDK6 co-expression in UCEC[25]. Gene expression data of CDK6 in HTSeq-FPKM were downloaded from TCGA's official website for analysis. The Pearson correlation coefficient was used for statistical analysis of CDK6 co-expression and exhibited in the form of a volcano map and heat map. The rank criterion was an FDR<0.05.

Gene ontology(GO) and Kyoto Encyclopedia of Genes and Genome(KEGG) analysis were performed on co-expressed genes with the package R 'clusterProfiler' to explore possible biological functions and signaling pathways affected by CDK6[26]. GO analysis included biological process(BP), cell composition(CC) and molecular function(MF). Genes were determined to be differentially expressed based on an absolute fold change>1.5 and Padj<0.05. The PPI network was constructed using both the STRING database(http://string-db.org)(interaction score >0.4) and Cytoscape software (version 3.9.0)[27].

Mutation and immune infiltration analysis

We also evaluated the mutation frequency of CDK6 in UCEC in the cBio-Portal database(http://www.cbioportal.org/)[28]. Five datasets (MSK, CPTAC, TCGA-Firehose Legacy, TCGA-Nature 2013, and TCGA-PanCancer Atlas) were included. The mutation types of CDK6 in UCEC were further calculated using the Catalogue of Somatic Mutations in Cancer(COSMIC) database(http://cancer.sanger.ac.uk)[29]. The correlations between CDK6 transcription level and immune cell infiltration were evaluated using the TIMER database, including B cells, neutrophils, CD4+T cells, macrophages, CD8+T cells, and dendritic cells, as well as the tumor purity. We also analyze the correlation between CDK6 and immune cell markers to evaluate the role of CDK6 in tumor immunity. Immune cell gene markers are selected from the website of R&D Systems(www.rndsystems.com/cn/resources/cell-markers/immune-cells). These gene markers include markers of B cells, CD8+T cells, follicular helper T cells (Tfh), T-helper 1 (Th1) cells, T-helper 2(Th2) cells, T-helper 17(Th17) cells, Treg, T cells exhausted, macrophages, M1 macrophages, M2 macrophages, tumor-associated macrophages(TAM), monocytes, natural killer (NK) cells, neutrophils, and dendritic cells (DC). In addition, we used the somatic copy number alteration (SCNA) module of the TIMER tool to link the genetic copy number variations (CNV) of CDK6 with the relative abundance of tumor-infiltrating cells.

Statistical analysis

The Pearson χ2 test was employed to analyze the relationship between CDK6 expression and clinicopathological variables; Fisher's exact test was utilized when needed. OS is defined as the time from random assignment until death due to any cause. DSS is defined as the percentage of people in a study or treatment group who have not died from a specific disease in a described period. Kaplan-Meier analysis was applied to evaluate the survival of patients, and the log-rank test was availed to test the significance. $P<0.05$ indicates statistical significance, and $P<0.01$ indicates highly statistical significance. All reported P-values were two-sided.

Results

The expression and transcriptional levels of CDK6 in uterine corpus endometrial carcinoma (UCEC)

TIMER database was used to evaluate the expression of CDK6 in human cancers, as shown in Figure 1A. Compared with adjacent normal tissues, UCEC(Uterine Corpus Endometrial Carcinoma) has significantly lower CDK6 expression, which was similar in BLCA(Bladder Urothelial Carcinoma), BRCA(Breast invasive carcinoma), LUAD(Lung adenocarcinoma), TGCT(Testicular Germ Cell Tumors), THCA(Thyroid carcinoma) and UCS(Uterine Carcinosarcoma).

In contrast, expression of CDK6 was significantly higher in COAD(colon adenocarcinoma), DLBC(Lymphoid Neoplasm Diffuse Large B-cell Lymphoma), EC(Esophageal carcinoma), GBM(Glioblastoma multiforme), HNSC(Head and Neck squamous cell carcinoma), KIRP(Kidney renal papillary cell carcinoma), LAML(Acute Myeloid Leukemia), LGG(Brain Lower Grade Glioma), LIHC(Liver

hepatocellular carcinoma), LUSC(Lung squamous cell carcinoma), PAAD(Pancreatic adenocarcinoma), PRAD(Prostate adenocarcinoma), READ(Rectum adenocarcinoma), SKCM(Skin Cutaneous Melanoma), STAD(Stomach adenocarcinoma) and THYM(Thymoma).

Furthermore, we validated the above result using unpaired or paired TCGA-UCEC cohort data sets and got equal conclusions(Figures 1B, Figures 1C). Moreover, we explored the protein expression of CDK6 in the Human Protein Atlas(HPA) database(www.proteinatlas.org). Interestingly, UCEC displayed negative to weekly positive CDK6 staining while. Images of normal endometrial (Patient IDs: 2361) and UCEC endometrial (Patient IDs: 167) are presented in Figure 1D-Figure 1G.

Subgroup analysis of the mRNA expression and prognostic significance of CDK6 in UCEC

To better understand the relevance of CDK6 expression in UCEC(Figure 2A), we used the TCGA cohort to analyze its underlying mechanism and correlate it with certain clinicopathological parameters. A Chi-square test was performed on samples of UCEC with qualified clinical information(Table 1). The results indicated that CDK6 was downregulated in different subgroups of UCEC, including subgroups of BMI, age, race, diabetes, histological types, histologic grades, clinical stages and surgical approaches(Figure 2B-Figure 2I). However, the serous type had a significantly higher CDK6 level compared with the endometrioid histological type. The same phenomenon was observed respectively in grades 3 and 1, stage IIIand I , stage III and II(Figure 2F-Figure 2H).

In addition, the down expression of CDK6 showed a significant association with OS, DSS, and PFI events in UCEC patients(Figure 2J-Figure 2L). What's more, we validated the prognostic value of CDK6 in UCEC using ULCAN and KM databases and the results were significant(Figure 2M-Figure 2N).

Table 1 Clinical characteristics of uterine corpus endometrial carcinoma(UCEC) patients.

	Low expression of CDK6	High expression of CDK6	p
n	276	276	
Clinical stage, n (%)			< 0.001
Stage I	189 (34.2%)	153 (27.7%)	
Stage II	29 (5.3%)	22 (4%)	
Stage III	46 (8.3%)	84 (15.2%)	
Stage IV	12 (2.2%)	17 (3.1%)	
Primary therapy outcome, n (%)			0.301
PD	10 (2.1%)	10 (2.1%)	
SD	4 (0.8%)	2 (0.4%)	
PR	3 (0.6%)	9 (1.9%)	
CR	225 (46.9%)	217 (45.2%)	
Race, n (%)			0.595
Asian	10 (2%)	10 (2%)	
Black or African American	49 (9.7%)	59 (11.6%)	
White	193 (38.1%)	186 (36.7%)	
Histological type, n (%)			< 0.001
Endometrioid	228 (41.3%)	182 (33%)	
Mixed	8 (1.4%)	16 (2.9%)	
Serous	40 (7.2%)	78 (14.1%)	
Residual tumor, n (%)			0.976
R0	201 (48.7%)	174 (42.1%)	
R1	12 (2.9%)	10 (2.4%)	
R2	9 (2.2%)	7 (1.7%)	
Histologic grade, n (%)			0.010
G1	57 (10.5%)	41 (7.6%)	
G2	70 (12.9%)	50 (9.2%)	
G3	145 (26.8%)	178 (32.9%)	
Menopause status, n (%)			0.314

	Low expression of CDK6	High expression of CDK6	p
Pre	15 (3%)	20 (4%)	
Peri	6 (1.2%)	11 (2.2%)	
Post	231 (45.7%)	223 (44.1%)	
Hormones therapy, n (%)			0.475
No	144 (41.9%)	153 (44.5%)	
Yes	26 (7.6%)	21 (6.1%)	
Diabetes, n (%)			0.471
No	169 (37.5%)	159 (35.3%)	
Yes	58 (12.9%)	65 (14.4%)	
Radiation therapy, n (%)			0.633
No	143 (27.1%)	136 (25.8%)	
Yes	121 (23%)	127 (24.1%)	
Surgical approach, n (%)			0.132
Minimally Invasive	97 (18.3%)	111 (20.9%)	
open	173 (32.6%)	149 (28.1%)	
OS event, n (%)			0.213
Alive	235 (42.6%)	223 (40.4%)	
Dead	41 (7.4%)	53 (9.6%)	
DSS event, n (%)			0.298
Alive	247 (44.9%)	240 (43.6%)	
Dead	27 (4.9%)	36 (6.5%)	
PFI event, n (%)			0.421
Alive	216 (39.1%)	207 (37.5%)	
Dead	60 (10.9%)	69 (12.5%)	
Age, meidan (IQR)	64 (57, 71)	64 (57, 71)	0.843
BMI, meidan (IQR)	32.57 (26.93, 38.06)	31.96 (25.84, 39.09)	0.912
Tumor invasion(%), meidan (IQR)	45 (17, 60)	41 (12.88, 65.25)	0.929

Enrichment Analysis of CDK6 Gene Co-Expression Network and Protein-Protein Interaction(PPI) network Analysis in UCEC

To further understand the biological significance of CDK6 in UCEC, we used the LinkedOmics database to analyze the CDK6 co-expression in UCEC. As shown in Figure 3A, 3495 genes are positively correlated with CDK6, and 1116 genes are significantly negatively correlated with CDK6 (FDR<0.05). The heat map shows the top 50 significant genes that are positively correlated (Figure 3C) and negatively correlated with CDK6 (Figure 3E), respectively. We use the R software package to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of CDK6 related genes. Under the condition of p.adj < 0.1, there are 69 biological processes (GO-BP), 9 cellular components (GO-CC), 23 molecular functions (GO-MF), and 2 KEGG. The bubble chart shows the first 15 pieces of information about GO and KEGG, including 5 pieces of BP, CC, and MF. GO function annotation shows that CDK6 co-expressions are mainly involved in extracellular structure organization, extracellular matrix component, cell adhesion molecule binding, and collagen-containing extracellular matrix (Figure 3B). KEGG pathway analysis showed that CDK6 co-expression is mainly related to the focal adhesion(P=0.063)(Figure 3D). To further understand the potential mechanism of CDK6, the STRING database was used to study the PPI network of CDK6(Figure 3F). The analysis showed that CDK6 was associated with Cyclin-dependent kinase inhibitor 1B(CDKN1B), Cyclin-dependent kinase inhibitor 1A(CDKN1A), RB transcriptional corepressor 1(RB1), Cyclin-dependent kinase inhibitor 2A(CDKN2A), Cyclin-dependent kinase inhibitor 2B(CDKN2B), Cyclin-dependent kinase inhibitor 2C(CDKN2C), Cyclin-dependent kinase inhibitor 2D(CDKN2D), Cyclin D1(CCND1), Cyclin D2(CCND2), Cyclin D3(CCND3). These proteins are necessary for the cell cycle regulating process[11].

The promoter methylation level of CDK6 in UCEC and subgroups

Compared with those in normal controls, the promoter methylation level of CDK6 was significantly higher in UCEC(Figure 4A). This conclusion was further validated in the subgroup analysis, including age, grade, stage, weight, TP-53 mutant status, race, and histology(Figure 5A-Figure 5G). Groups aged 21-40, endometrioid, grade1, stage1, and TP53 non-mutant had greater promoter methylation levels of CDK6. Furthermore, we explored the 25 top genes with hyper or hypomethylation promoters in UCEC and performed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses respectively(Figure 4 B-Figure 4E). GO function annotation and KEGG pathway analysis showed that the 25 top genes with hypermethylation promoters are mainly involved in Herpes simplex virus 1 infection. Meanwhile, the 25 top genes with hypomethylation promoters are mainly related to the detection of chemical stimulus involved in sensory perception of smell, olfactory transduction, and olfactory receptor activity.

Mutations of CDK6 in UCEC

We also evaluated the mutation frequency of CDK6 in UCEC in the cBio-Portal database. Five datasets (MSK, CPTAC, TCGA-Firehose Legacy, TCGA-Nature 2013 and TCGA-PanCancer Atlas), which included 1729 samples, were selected for analysis. The somatic mutation frequency of CDK6 in UCEC was 1.4%,

which was chiefly composed of missense mutations (Figure 6A). This mutation frequency was comparatively low, only 1.4 in 100 samples. Consequently, we failed to find a relationship between CDK6 mutation and the prognosis of UCEC patients. Additionally, the mutation types of CDK6 were further calculated in another database, COSMIC. For clarity, two pie charts of the mutation types are shown in Figure 6. In terms of proportion, missense substitutions were the most common, occurred in approximately 13.73% of the samples, followed by synonymous substitutions occurred in 4.92%, and frameshift deletions occurred in 0.66% (Figure 6B). The substitution mutations mainly occurred at G>A(30.35%), followed by C> T(28.86%), G>T(9.45%) and A>T (6.97%) (Figure 6C).

The association of CDK6 expression and immune infiltration in UCEC

It has been reported that tumor-infiltrating lymphocytes can predict the status and prognosis of cancer sentinel lymph nodes independently[30]. Thus, the TIMER database was used to investigate the association between CDK6 expression and immune infiltration in UCEC.

As seen in Figure 7A, The result indicated that CDK6 expression had a non-significant correlation with tumor purity (R=-0.065, P=2.64E-01). However, CDK6 expression correlated positively with CD8+T cells(r=0.275,P=2.13E-06),neutrophils(r=0.291,P=4.03E-07) and dendritic cells(r=0.248, P=1.79E-05). These results indicate that CDK6 plays a key role in the immune infiltration of UCEC. Moreover, we also found that CDK6 copy number variations(CNV) have a significant correlation with the infiltration level of CD8+T cells, CD4+T cells, and Dendritic Cells (Figure 7B). What's more, we further analyzed the correlations between CDK6 expression and related immune cell gene markers in UCEC. Correlation coefficients were adjusted by tumor purity(Table 2). The results showed that the expression level of CDK6 after adjustment of tumor purity was significantly correlated with most of the immune markers of different T cells in UCEC, including CD8A and CD8B of CD8+ T Cell, CXCR5 of Tfh, STAT4, and STAT1 of Th1 cells, QRSL1 and STAT5A of Th2 cells, STAT3 of Th17 cells, FOXP3 and STAT5B of Treg, PD-1 CTLA4 and LAG3 of exhausted T cells($P<0.05$, Table 2). It indicates that CDK6 may be involved in the T cell immune response in UCEC. We also found that the expression level of CDK6 was correlated significantly with the immune markers nitric oxide synthase 2(NOS2) and cyclooxygenase-2(CDX2) of M1 macrophage, CD163, VSIG4, and MS4A4A of M2 macrophage in UCEC($P<0.05$, Table 2). Furthermore, the tumor-associated macrophages(TAM) receptors-TYRO3, AXL, and MERTK were also verified to be correlated with CDK6 significantly. We also found that the expression of CDK6 was significantly correlated with immune markers of Monocyte, Neutrophil, and Dendritic cell in UCEC, including CD33, CD16, CD55, NRP1, and CD141 ($P<0.05$, Table 2). Collectively, these results indicate that the expression of CDK6 is related to immune cell infiltration in different ways in UCEC.

What's more, 552 tumor samples were divided into two groups based on the CDK6 expression, with 276 samples in the high-expression group and 276 samples in the low-expression group. The differential expression of 24 immune cells between different CDK6 expression groups was analyzed to determine whether the tumor immune microenvironment is different(Figure 7C). The results displayed that, compared with the low expression group, aDC, CD8+ T cell, Eosinophils, Macrophages, Mast cells, T

helper cells, Tcm, Tem, TFH, Tgd, Th1 cells, and Th2 cells increased in the high expression group of CDK6 (P<0.05), while the NK CD56bright cells, pDC, and Th17 cells decreased (P<0.05).

Table 2 Correlations between CDK6 and immune cells' gene markers in UCEC in TIMER.

Immune cell types	Gene markers	Non-adjusted		Purity-adjusted	
		Correlation	P-value	Correlation	P-value
B cell	CD19	0.059	1.67e-01	0.025	6.67e-01
	CD70	0.007	8.64e-01	-0.012	8.38e-01
CD8+ T Cell	CD8A	0.128	2.8e-03	0.116	4.81e-02
	CD8B	0.163	1.35e-04	0.141	1.56e-02
T cell(general)	CD3D	0.025	5.61e-01	0.013	8.30e-01
	CD3E	0.072	9.23e-02	0.059	3.16e-01
	CD2	0.083	5.4e-02	0.076	1.97e-01
Tfh	CXCR3	0.06	1.65e-01	0.05	3.95e-01
	CXCR5	0.152	3.66e-04	0.185	1.49e-03
	ICOS	0.294	8.66e-03	-0.011	9.24e-01
Th1	T-bet (TBX21)	0.084	4.9e-02	0.103	7.79e-02
	STAT4	0.182	2.03e-05	0.192	9.45e-04
	STAT1	0.334	1.6e-15	0.33	7.06e-09
	TNF-a	0.031	4.69e-01	0.025	6.64e-01
Th2	GATA3 (QRSL1)	0.252	2.37e-09	0.252	1.22e-05
	STAT6	0.06	1.63e-01	0.032	5.85e-01
	STAT5A	0.163	1.34e-04	0.155	8.03e-03
	IL13	0.05	2.46e-01	0.057	3.28e-01
Th17	STAT3	0.199	2.7e-06	0.179	2.11e-03
	IL17A	0.061	1.53e-01	0.025	6.7e-01
	IL23R	0.103	1.66e-02	0.095	1.06e-01
Treg	FOXP3	0.152	3.61e-04	0.164	4.88e-03
	CCR8	0.094	2.77e-02	0.089	1.29e-01
	STAT5B	0.268	2.29e-10	0.32	2.14e-08
T cell exhaustion	PD-1	0.129	2.54e-03	0.133	2.32e-02
	CTLA4	0.119	5.39e-03	0.146	1.22e-02
	LAG3	0.115	7.16e-03	5.28	5.28e-03

M1 Macrophage	NOS2	0.235	2.68e-08	0.280	1.08e-06
	IRF5	0.001	9.84e-01	-0.064	2.73e-01
	COX2(PTGS2)	0.167	8.63e-05	0.153	8.88e-03
M2 Macrophage	CD163	0.314	5.99e-14	0.298	2.07e-07
	VSIG4	0.255	1.53e-09	0.255	1.05e-04
	MS4A4A	0.297	1.76e-12	0.31	5.68e-08
Macrophage	ITGAM	0.099	2.1e-02	0.031	5.92 e-01
	CD68	0.167	9.43e-05	0.165	4.58e-03
TAM	TYRO3	0.159	2.04e-04	0.117	4.61e-02
	AXL	0.413	7.42e-24	0.398	1.46e-12
	MERTK	0.247	5.3e-09	0.21	2.86e-04
Monocyte	CD14	0.088	3.99e-02	0.067	2.53e-01
	CD33	0.146	6.44e-04	0.156	7.34e-03
Natural killer cell	KIR3DL1	0.037	3.95e-01	0.034	5.63e-01
	CD7	0.06	1.65e-01	0.064	2.76e-01
Neutrophil	CD16(FCGR3A)	0.265	4.03e-10	0.28	1.08e-06
	CD55	0.234	3.04e-08	0.203	4.27e-04
Dendritic cell	HLA-DPB1	0.069	1.09e-01	0.031	5.92e-01
	HLA-DQB1	0.042	3.26e-01	0.01	8.71e-01
	HLA-DRA	0.091	3.45e-02	0.041	4.84e-01
	BDCA-1(CD1C)	0.028	5.18e-01	0.067	2.51e-01
	BDCA-4(NRP1)	0.15	4.56e-04	0.143	1.43e-02
	CD141	0.272	1.33e-10	0.264	4.43e-06

Discussion

Aberrant CDK6 kinase activation often leads to uncontrolled cell proliferation and cancer development. Knockdown of CDK6 dramatically inhibited the proliferation and survival of tumor cells and reduced the expression level of drug resistance genes such as MRP and MDR[31]. Despite the evidence linking a wide variety of mutations to CDK4/6 activation, there are also reasons to suspect that CDK4/6 inhibition may not always be sufficient to prevent the growth of all cancer cells.

Our analysis suggested that the overall expression level of CDK6 in UCEC was significantly lower than that in adjacent normal tissues. Further subgroup analysis displayed a similar result that endometrioid histological type, grades 1 and stage 1–2 had reduced CDK6 expression compared to normal patients. However, inherent differences appeared among different cancer groups. We found that patients with serous carcinoma, or grade 3, or stage had significantly higher CDK6 levels and worse prognosis than those with endometrioid histological type, grades 1 or stage 1–2.

Notably, Ikeda et al found that high CDK4/6SA was robustly associated with shorter PFS, and this was an independent poor prognostic factor in the low-risk group. Inversely, in the intermediate-/high-risk group, patients with high CDK4/6SA tended a more favorable prognosis compared with patients with low CDK4/6SA[32]. The inconsistency of the results is likely attributable to heterogeneity in study designs and patient populations. This is an important finding in understanding the prognostic value of CDK6 in UCEC. According to ESGO/ESTRO/ESP guidelines[33], patients with serous carcinoma, or grade 3, or stage were assigned to an intermediate-/high-risk group, indicating that CDK6 was an important prognostic factor in the above groups instead of all the patients with UCEC. Collectively, these results indicated that CDK6 was differently expressed and had a prognostic value in UCEC, yet there were confounding factors among different histological types, grades, and clinical stages. A novel risk stratification system must be developed.

To better understand the differential expression of CDK6, the promoter methylation level was analyzed. The result demonstrated that the promoter methylation level of CDK6 was significantly higher in UCEC and this conclusion was further validated in subgroups aged 21–40, endometrioid, grade1, stage1, and TP53 non-mutant. DNA methylation of gene promoters is closely correlated with transcription suppression, although the mechanism for such suppression is unclear[34]. Numerous studies have demonstrated a negative association between the level of DNA methylation in promoters and the expression of downstream genes[35]. The aberrant promoter methylation at the CDK6 locus would most likely result in a low CDK6 expression in patients with low risk, which was consistent with the above conclusion. A pre-clinical study evaluated the response to palbociclib in endometrial malignancies in vitro and found that palbociclib treatment triggered shrinkage of endometrial lesions and reduced tumor cell proliferation[36].

To fully elucidate the underlying relationship between CDK6 and UCEC, Gene Co-Expression Network was enriched, which mainly involved extracellular structure organization and focal adhesion. These results suggested that CDK6 worked by participating in extracellular signaling, cell adhesion, and focal adhesion pathway in UCEC. Since cell adhesion molecules also support binding to the extracellular matrix, they represent excellent biosensors, which allow cells to modulate their behavior based on changes in the surrounding microenvironment[37]. In addition, PPI analysis validated that CDK6 was controlled by two distinct classes of regulatory subunits, the Cip/Kip family, comprising CDKN1A, CDKN1B, and CDKN1C, and the INK4 family, including CDKN2B, CDKN2A, CDKN2C and CDKN2D, which was consistent with the existing research[11]. For example, CDK4 and CDK6 associate with D-type cyclins (D1, D2, and D3), and the resulting complexes are required to enable the cell to pass from the G1 phase to the S phase.

Furthermore, GO function annotation and KEGG pathway analysis of the 25 top genes with hyper methylation promoters in UCEC showed that they mainly involved in Herpes simplex virus 1 infection, which contributes to the dysregulation of signaling pathways and disease onset[38]. Meanwhile, the 25 top genes with hypo methylation promoters are mainly related to olfactory receptor activity. Olfactory receptors (ORs) are not exclusively expressed in the olfactory sensory neurons, they are also observed outside of the olfactory system in all other human tissues tested to date, including the testis, lung, intestine, skin, heart, and blood[39]. Additionally, as ORs are highly expressed in different cancer tissues, they may have the potential to serve as diagnostic and therapeutic tools.

Various types of genomic aberrations accumulate in cancer genomes and play roles in the development and progression of the disease[40]. To fully elucidate the underlying relationship between CDK6 and UCEC, the mutation frequency of CDK6 was also evaluated. Unfortunately, The somatic mutation frequency of CDK6 in UCEC was comparatively low. Consequently, we failed to find a relationship between CDK6 mutation and the prognosis of UCEC patients.

At present, research on the function and mechanism of CDK6 in tumors mainly focuses on its cell cycle regulation. However, no study on the relationship between CDK6 and immune infiltration in UCEC has been reported. In this study, we used the TIMER database to reveal the relationship between CDK6 expression and the level of immune infiltration in UCEC. We found that CDK6 expression is significantly correlated with CD8 + T cells, neutrophils, and dendritic cells. In addition, we also found that CDK6 copy number variations(CNV) have a significant correlation with the infiltration level of CD8 + T cells, CD4 + T cells, and Dendritic Cells. What's more, we further analyzed the correlations between CDK6 expression and related immune cell gene markers in UCEC. The results after adjustment of tumor purity showed that the expression level of CDK6 was significantly correlated with most of the immune markers of different T cells in UCEC, including CD8 + T Cell, Tfh, Th1 cells, Th2 cells, Th17 cells, Treg, and exhausted T cells, which suggests that CDK6 plays an important role in regulating UCEC tumor immunity and probably involved in the T cell immune response. In addition, M1, M2 macrophage, and TAM were also verified to significantly correlate with CDK6. Macrophages play an important role in cancer development and metastasis. Proinflammatory M1 macrophages can phagocytose tumor cells, while anti-inflammatory M2 macrophages such as TAMs promote tumor growth and invasion[41]. Besides, the TAMs receptor family is essential for the efferocytosis of apoptotic material by antigen-presenting cells. It is expressed by virtually all cells of the tumor microenvironment and involves survival and therapy resistance[42].

We also found that the expression of CDK6 was significantly correlated with immune markers of Monocyte, Neutrophil, and Dendritic cell in UCEC. Furthermore, 24 immune cells between the high and low expression groups of CDK6 exhibited drastically different distribution patterns. Collectively, the above results indicate that the expression of CDK6 plays an important role in the regulation and recruitment of immune infiltrating cells in different ways in UCEC.

Nevertheless, there are several limitations to this study. First, clinical samples were required for further investigating the interactions and mechanisms of CDK6 in regulating the development and progression

of UCEC. Second, we should also pay attention to the activity and expressional alteration of CDK6. Third, more evidence is needed for explaining the relationship more accurately between CDK6 and immune infiltrating cells in vivo. However, we can conclude that CDK6 is a promising therapeutic target in the treatment of UCEC, particularly in intermediate-/high-risk patients.

Conclusion

In conclusion, CDK6 was differentially expressed in UCEC and significantly up-regulated in intermediate-/high-risk patients, which predicts a worse prognosis. Furthermore, CDK6's expression is correlated with immune infiltration levels which may contribute to a better understanding of the molecular basis of UCEC. Thus, CDK6 may serve as a novel genotype for UCEC and be included in the risk stratification system in the future. What's more, the development of CDK6-mediated UCEC therapies may be facilitated. However, further experimental studies are still needed to confirm our findings and promote the clinical application of selectively CDK6 inhibitor for UCEC.

Abbreviations

UCEC: Uterine Corpus Endometrial Carcinoma. GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; OS: Overall Survival; DSS: Disease Specifc Survival; PFI: progression-free interval; TCGA: The Cancer Genome Atlas; TIMER: Tumor Immune Estimation Resource; HPA: Human Protein Atlas; COSMIC: Catalogue of Somatic Mutations in Cancer.

Declarations

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Authors' contributions

YL, WY designed the study. YL, NZ performed the statistical analysis. YL, NZ, and JZ drafted the manuscript. WY supervised the experimental work. All authors read and approved the fnal manuscript. All authors reviewed the manuscript.

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Ethics approval and consent to participate

All methods were carried out in accordance with the Declaration of Helsinki. No ethics approval was required for this work. All utilized public data sets were generated by others who obtained ethical approval.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

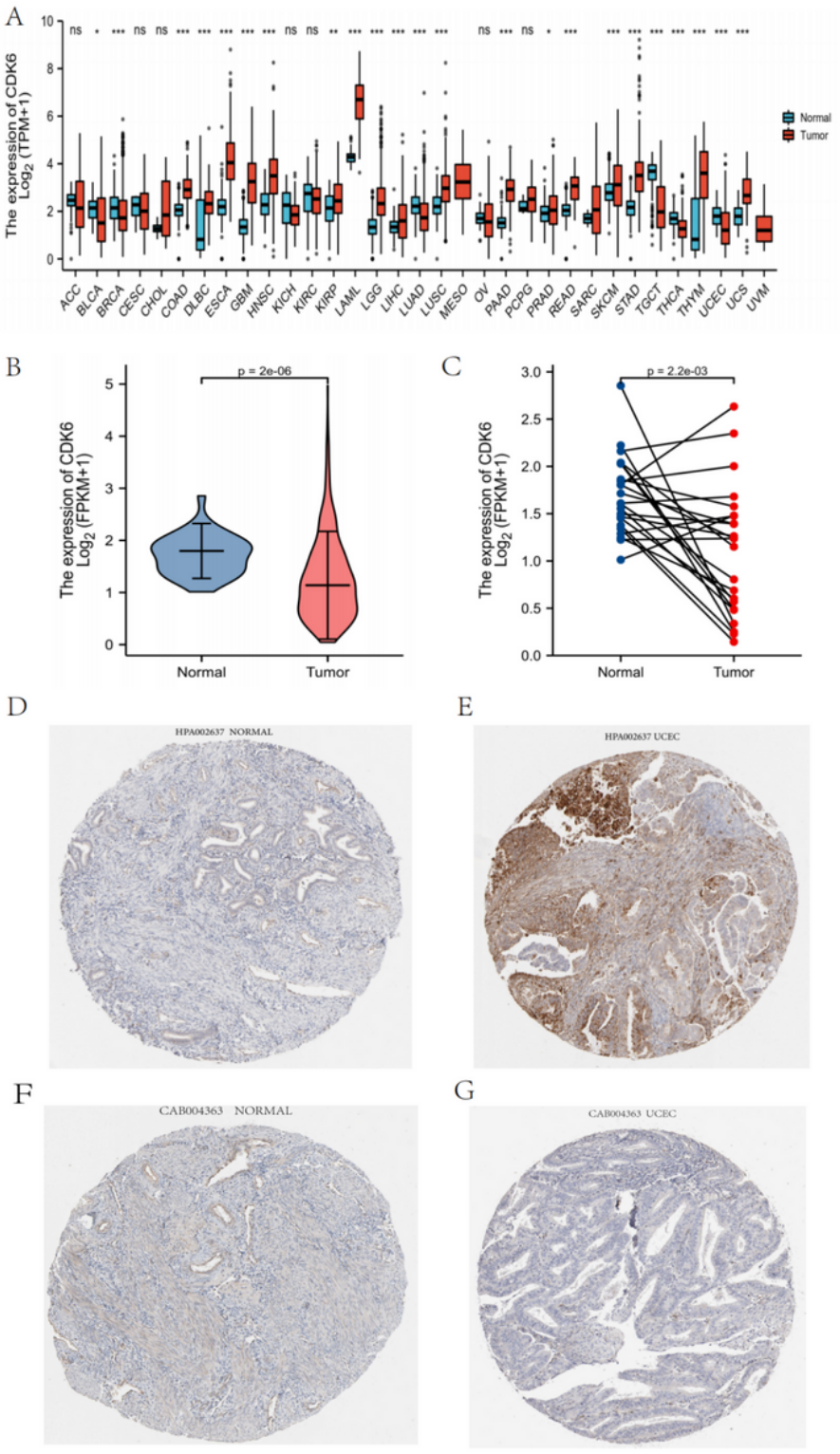

Figure 1

The expression of CDK6 in uterine corpus endometrial carcimoma (UCEC). CDK6 expression levels in different tumor types were measured using TIMER(A). TCGA cohort analysis of the expression level of CDK6 between normal and UCEC tissues(B,C). Protein expression of CDK6 in normal(D,F) and UCEC(E,G) endometrial tissue. (The Human Protein Atlas database).*p<0.05; **p<0.01; ***p<0.001; ****p <0.0001. ns, not significant.

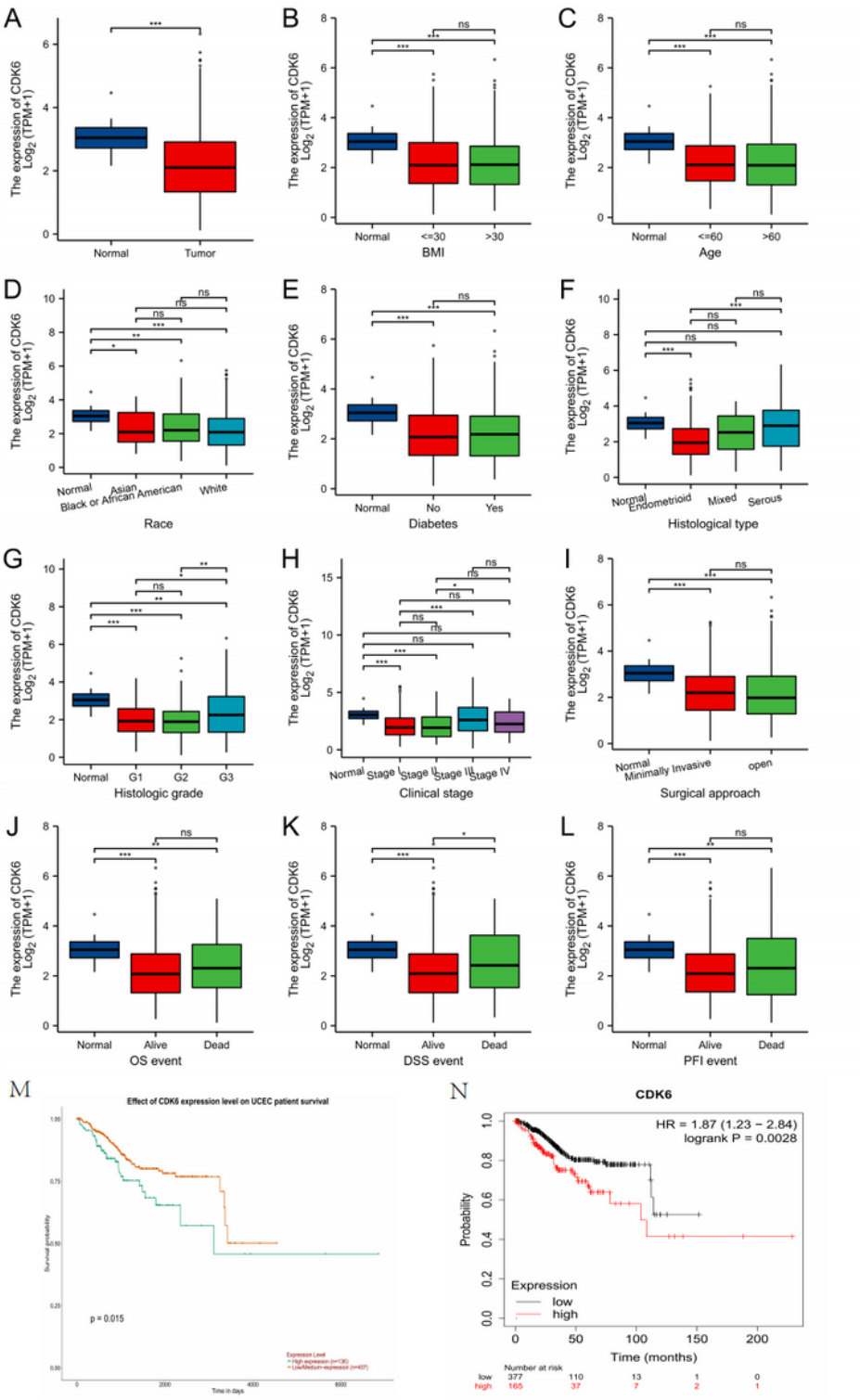

Figure 2

The expression levels of CDK6 mRNA in subgroups of UCEC. (A) mRNA expression of CDK6 in normal and UCEC patients. (B-E) CDK6 mRNA expression levels of UCEC patients with different BMI, ages, races, and diabetes status). (F-I) CDK6 mRNA expression levels of UCEC patients with different histological types, histologic grades, clinical stages, and surgical approaches. (J-L) CDK6 mRNA expression levels of UCEC patients in OS event, DSS event, and PFI event. OS, overall survival; DSS, disease-specific survival;

PFI, progression-free interval. The prognostic value of CDK6 was validated using the ULCAN(M) and KM(N) databases respectively(P<0.05).*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. ns, not significant.

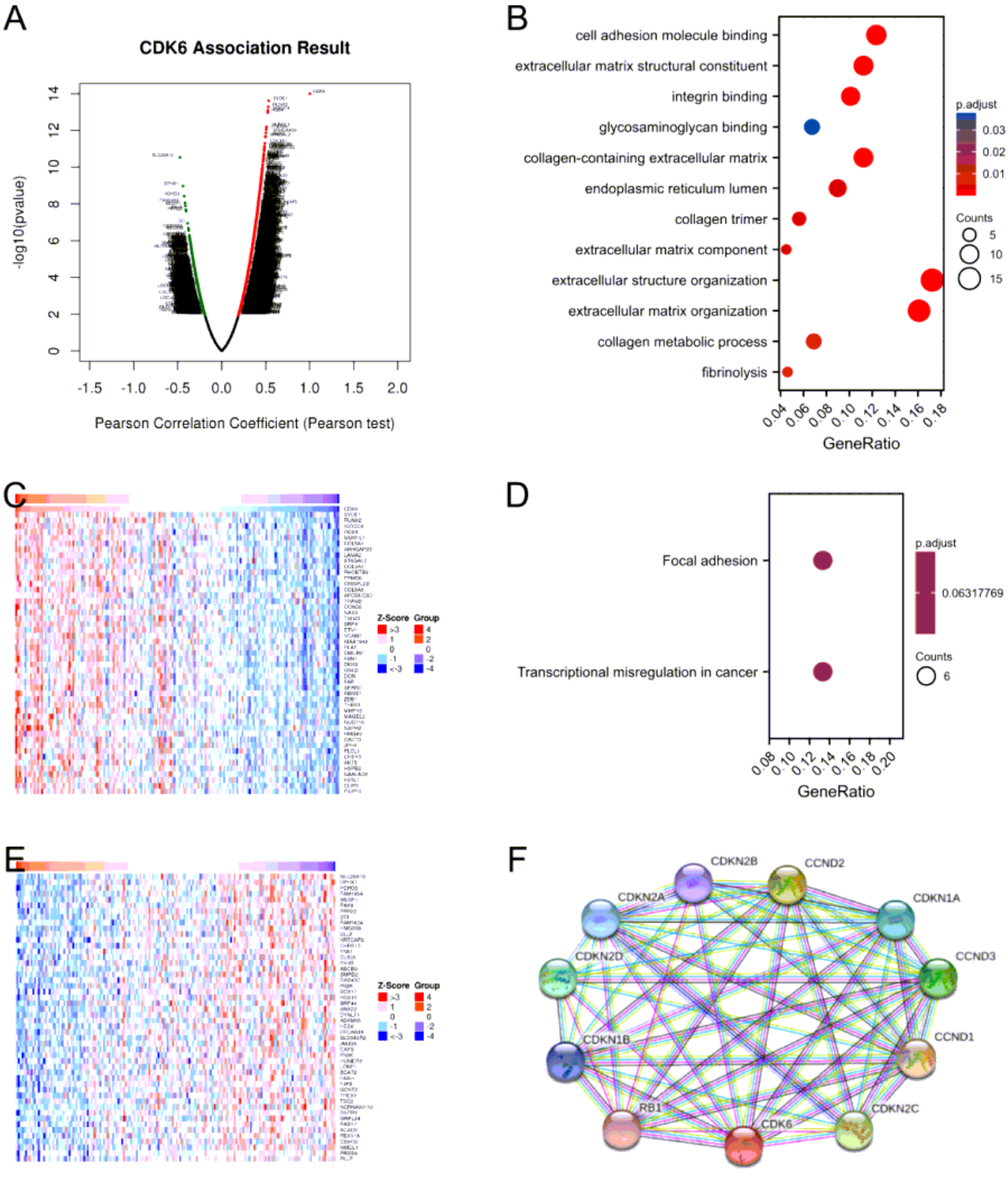

Figure 3

Enrichment analysis of CDK6 functional networks in uterine corpus endometrial carcinoma(UCEC). (A)Genes highly related to CDK6 were identified in the UCEC cohort by the Pearson test. (B)Enrichment of

gene ontology (GO) terms for genes related to CDK6. (C)The heat map shows the top 50 genes positively related to CDK6 in the UCEC cohort. (D)Enrichment of Kyoto Encyclopedia of Genes and Genomes (KEGG) terms for genes related to CDK6. (E)The heat map shows the top 50 genes negatively related to CDK6 in the UCEC cohort. (F)Protein-protein interaction network of CDK6.

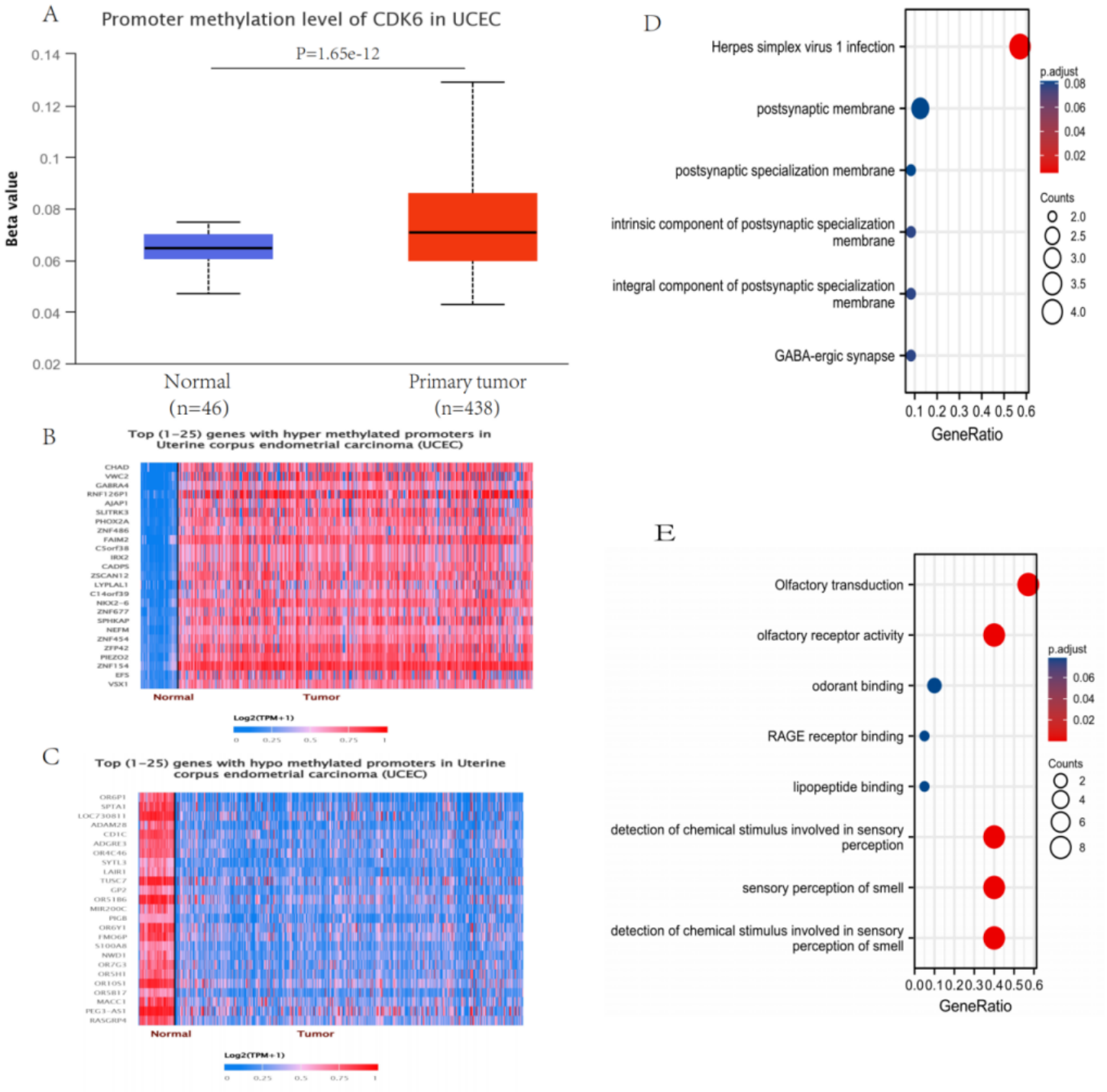

Figure 4

Analysis of the promoter methylation profile of UCEC. The promoter methylation level of CDK6 was significantly higher in UCEC(A). The heat map shows the top 25 genes with hypermethylation promoters

in UCEC(B). The heat map shows the top 25 genes with hypomethylation promoters in UCEC(C). GO and KEGG enrichment analysis of the top 25 genes with hyper/hypomethylation respectively(D/E). GO, Gene Ontology. KEGG, Kyoto Encyclopedia of Genes and Genomes.

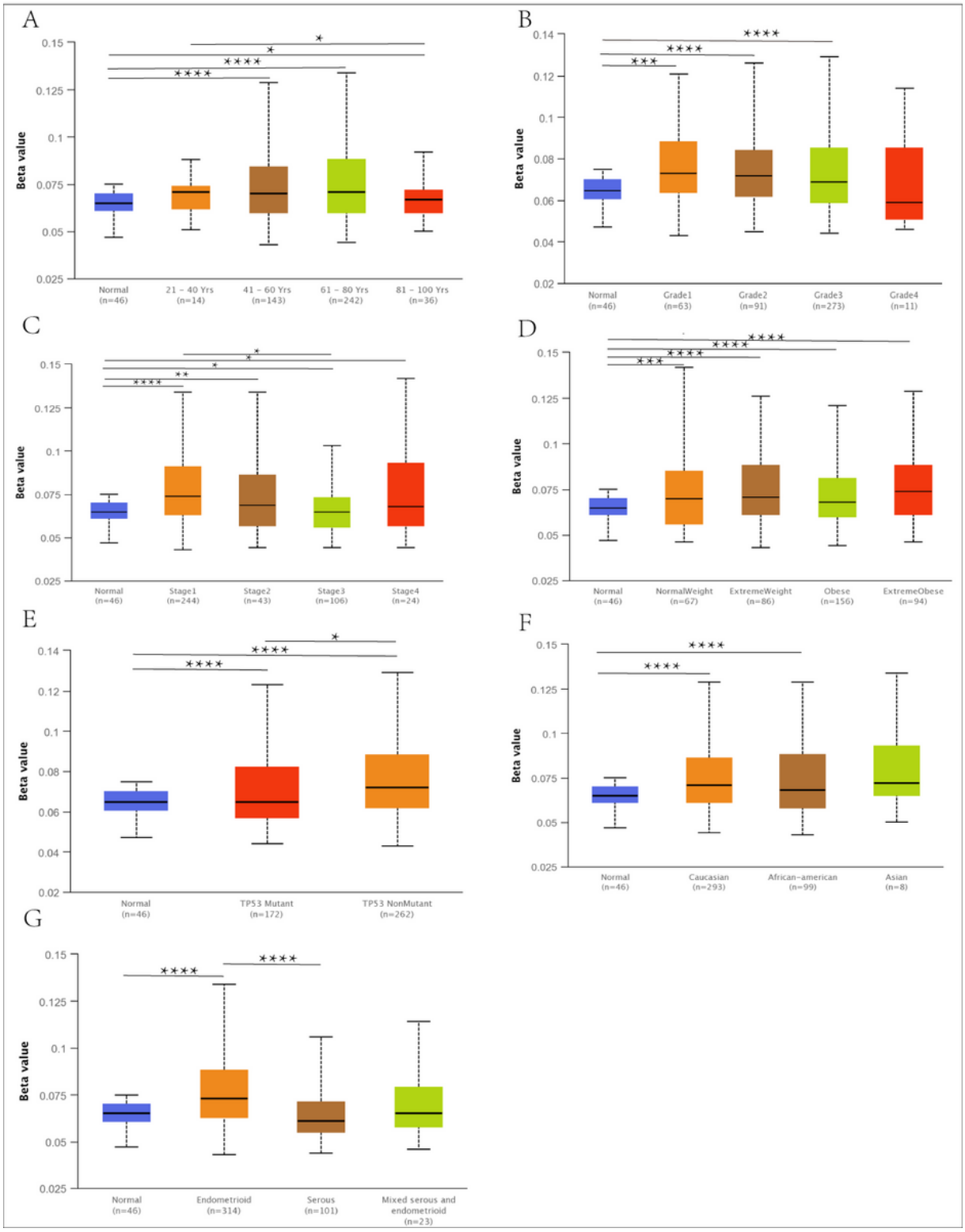

Figure 5

The promoter methylation levels of CDK6 in subgroups of UCEC patients. (A)Age; (B)Grade, (C)Stage; (D)Weight; (E)TP-53 mutant status; (F)Race; (G)Histology. Graphs are generated from the UALCAN database, *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. ns, not significant.

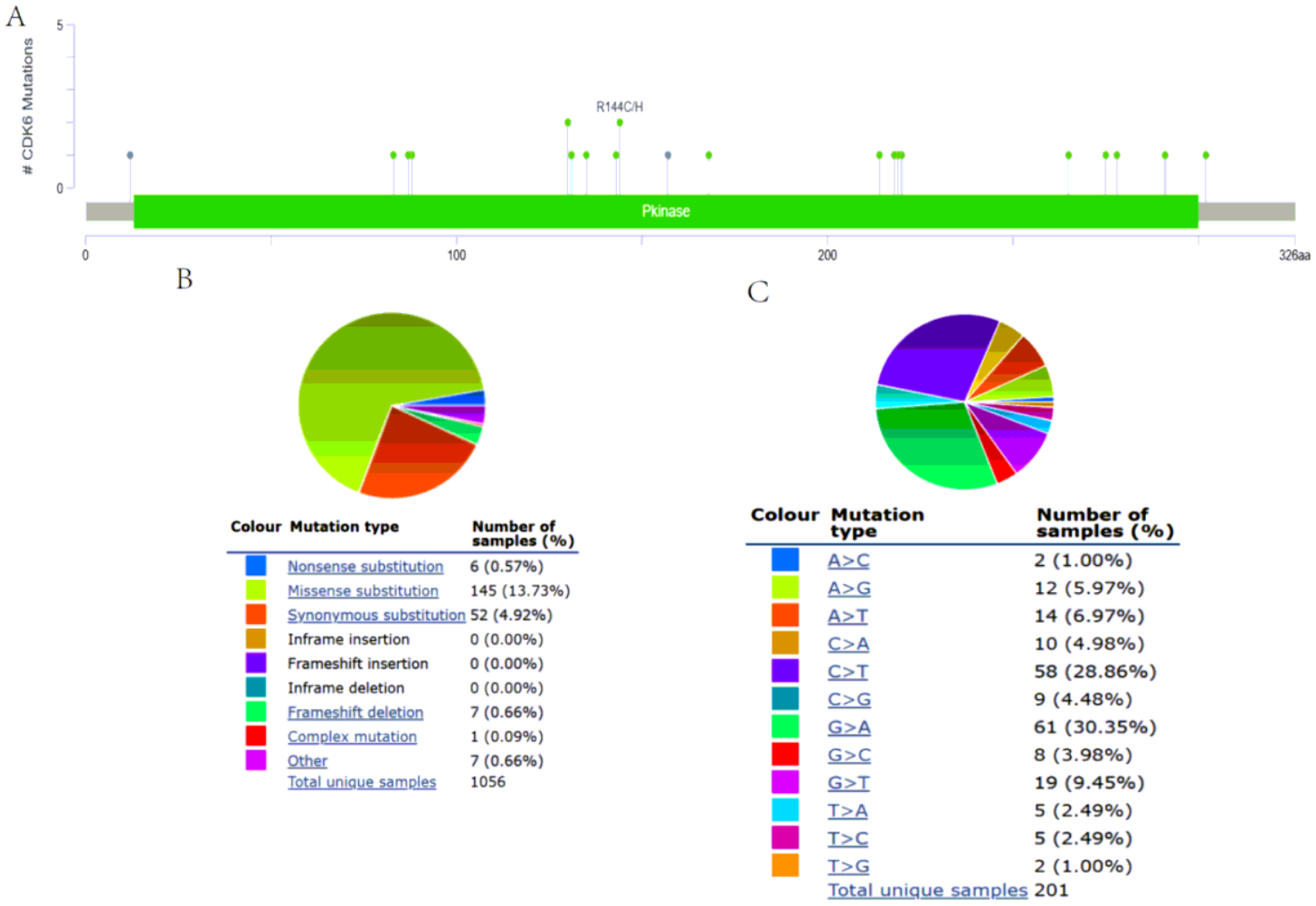

Figure 6

CDK6 mutations in UCEC. (A)The schematic representation of CDK6 mutations in UCEC(cBioPortal). (B,C)The mutation types of AGRN (%) in UCEC using the Catalogue of Somatic Mutations in Cancer (COSMIC) database.

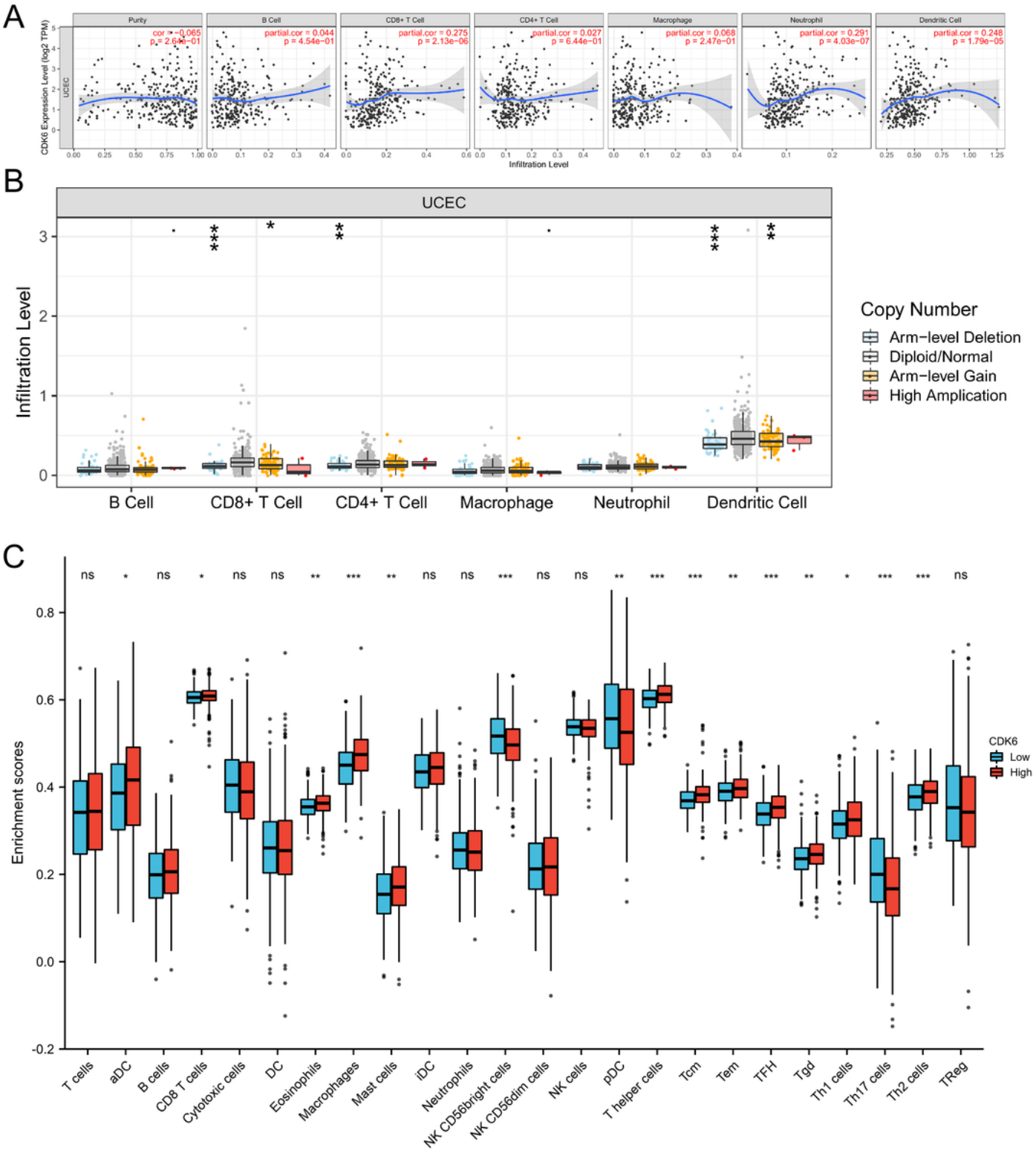

Figure 7

Correlations of CDK6 expression with immune infiltration level in UCEC. (A)The expression of CDK6 was correlated positively with infiltrating levels of CD8+T cells, neutrophils, and dendritic cells in UCEC. (B)CDK6 CNV affects the infiltrating levels of CD8+T cells, CD4+T cells, and dendritic cells in UCEC. (C)The change ratio of 24 immune cell subtypes in the high and low CDK6 expression groups in UCEC tumor samples.*p < 0.05; **p < 0.01; ***p < 0.001; ****p <0.0001. ns, not significant.