Large-Scale Analysis and Clinical Samples Reveals the Specific Clinical and Immune Features of KDM4B in UCEC

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Abstract

Background: Uterine Corpus Endometrial Carcinoma (UCEC) ranks fourth among female cancers in the world. Frustratingly, the 5-year survival rates for advanced patients are only 17%. KDM4B is overexpressed or dysregulated in a variety of cancers and could be associated with tumor progression and poor prognosis. Therefore, we performed bioinformatics analysis and in vitro assays to assess the role of KDM4B in UCEC. In addition, its relevance to immune cells in the tumor microenvironment was explored.

Methods: The mRNA level and protein level of KDM4B in UCEC was evaluated using the TCGA, HPA and GEO database. Immunohistochemistry and western blotting were used to verify the protein expression level of KDM4B in two batches of clinical samples. Kaplan-Meier curves, Univariate and multivariate analysis were used to assess the correlation between KDM4B expression and prognosis. GO and KEGG were used to predict the function and mechanism of KDM4B, and four immunity related database were used to explore their relevance to the tumor immune microenvironment.

Results: Firstly, the present study showed that KDM4B was significantly overexpressed in UCEC from several databases at the mRNA and protein levels, respectively. Immunohistochemistry and western blotting confirmed the abnormally overexpression of KDM4B. In addition, upregulation of KDM4B was associated with different clinicopathological prognostic factors. Secondly, overexpression of KDM4B was also associated with shorter OS and PFS. Univariate and multivariate analyses confirmed that KDM4B was an independent prognostic factor for poor prognosis. Then, GO and KEGG analysis revealed that KDM4B is enriched in biological processes and cellular signaling pathways closely related to immunity. Finally, KDM4B expression was closely associated with immune cell infiltration and immune checkpoints and it may be a new immune-related potential oncogene in UCEC.

Conclusions: KDM4B may be a new potential oncogene for UCEC patients. It is of clinical significance that KDM4B may not only be used to assess the clinical prognosis of UCEC patients but may also be a target for immunotherapy or gene targeted therapy.

Background

Uterine Corpus Endometrial Carcinoma (UCEC) ranks fourth among female cancers in the world. Its incidence is gradually increasing with increasing obesity and aging, affecting 300000 women worldwide each year(1, 2). Traditionally, it is divided into two histological types: estrogen-dependent (type I) and non-estrogen-dependent (type II)(3). In the era of molecular biology, it has been classified into four distinct molecular subgroups: DNA polymerases ε Hypermutated (POLE ultra-mutated), MSI (hypermutated), low copy number (CN low) and high copy number (CN high). Conventional treatments for UCEC include surgery, radiotherapy, chemotherapy and hormone therapy. It is frustrating that postoperative recurrence, resistance to chemotherapy and low response to hormone therapy in phase III and IV patients resulted in a 5-year survival rate of only 68% and 17% respectively(4). Therefore, it is crucial to determine the

prognostic biomarkers of UCEC and develop more effective and novel treatments for patients with advanced stages.

The KDM4 (lysine demethylase 4) subfamily consists mainly of four proteins (KDM4A-D), all harboring the Jumonji C structural domain (JMJC), but with different substrate specificities. The KDM4 protein is overexpressed or dysregulated in a variety of cancers, cardiovascular diseases and mental retardation, and is a potential therapeutic target(5). KDM4B is a newly identified member of the KDM4 subfamily, which is characterized by the JMJC domain. KDM4B specifically targets the trimethylated lysine 9 of histone H3 (H3K9) for demethylation at pericentric heterochromatin and euchromatin(6, 7). Recent studies have suggested that KDM4B facilitates in the regulation of PI3K(8), TGF-β, Notch, and Wnt/β-catenin pathways(9) during malignant transformation of cancer and researches also indicated that KDM4B was a potential biomarker for targeted therapy of these cancers(10, 11). Encouragingly, recent studies have reported that KDM4B-HOXC4-PD-L1 axis played an indispensable role in immune evasion of colorectal cancer cells(12). Therefore, the immune function of KDM4B in gynecological malignancies, especially in UCEC, has aroused great interest for us.

Leveraging sequencing technologies and bioinformatics to gain insight into unique molecular and genomic features of UCEC opens a new journey for its targeted therapy, immunotherapy and other precision therapies. In 2018, the US FDA approved anti-PD-1, which may benefit 20–30% of patients with advanced UCEC, thereby achieved milestone progress to immunotherapy in gynecologic tumors. In the era of precision medicine, the new classification of UCEC not only provides different prognostic information, but also potentially enables the selection of drug treatments based on the response rate of different subgroups. Due to its high mutation burden and immune invasion, immune checkpoint inhibition strategy is the most attractive candidate for the treatment of UCEC(13). Therefore, combining immunotherapy with gene targeting therapy is a new therapeutic prospect for UCEC, and there is an urgent need to find new biomarkers for the efficacy and prognosis assessment of immunotherapy and comprehensive treatment of UCEC.

Here, the TCGA and the GEO database were used for the first time for a pan cancer study of KDM4B. Meanwhile, we also tried to explore the potential molecular mechanisms of KDM4B in cancer initiation and clinical prognosis of UCEC from the aspects of gene expression, survival status, gene mutation, immune infiltration and related cellular signaling pathways. Most importantly, we collected clinical patient samples for validation and utilized multiple databases for comprehensive analysis of KDM4B gene expression level and immune cells infiltration in UCEC and correlated these data with clinical outcomes and prognosis of UCEC patients. Our results suggested that KDM4B is highly expressed in cancer tissues and affects the clinical prognosis of patients with UCEC. Excitingly, this study found that KDM4B expression was closely associated with the tumor immune microenvironment of UCEC, including immune cell infiltration and immune checkpoints, suggesting that it may serve as a new prognostic biomarker for UCEC patients and provide a basis for immunotherapy.

Methods

1. Data collection

The microarray data from the GSE36389 (UCEC = 13, Normal = 7) and GSE115810 (UCEC = 24, Normal = 2) dataset of Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) were used for subsequent studies. Gene expression and related clinical information data for 100 patients with UCEC and 31 normal controls were obtained from the Cancer Genome Atlas database (TCGA, https://portal.gdc.cancer.gov/) for follow-up studies. Based on the Human Protein Atlas database (HPA, https://www.proteinatlas.org/) which is an immunohistochemical database and the UALCAN database (http://ualcan.path.uab.edu/index.html), the differential expression of KDM4B between UCEC and normal controls were explored from the protein level. Finally, cBioportal (http://www.cbioportal.org/) is a powerful tool for studying different types of genetic alterations in tumors based on TCGA database and more than 200 published studies. We explored the KDM4B genomics data, including mutations and copy alterations, using this database.

2. Patients, Treatments and Follow-Up

128 patients who were diagnosed with UCEC were collected and analyzed in this research between August 2011 and September 2013 in the Harbin Medical University Cancer Hospital. Inclusion criteria: 1) patients with UCEC diagnosed pathologically, 2) all patients underwent standard staging surgery for UCEC, 3) all of them received standard postoperative adjuvant treatment based on the physician's recommendations. Exclusion criteria: 1) patients with UCEC unconfirmed pathologically, 2) patients who received preoperative radiotherapy or chemotherapy, 3) patients with incompletely documented UCEC. Specimens were taken from the operating room for surgical resection. This study was reviewed and ratified by the Ethics Committee of Harbin Medical University Cancer Hospital before starting the study and collecting samples. Written informed consent of all patients was obtained. The end of follow-up time was October 10, 2017, while among these 126 UCEC patients, two was lost to follow-up. The Histological type, FIGO stage, Histological grade, Lymph node metastasis and Muscular layer depth of Invasion of all tumor cases were estimated by skilled pathologists of Harbin Medical University Cancer Hospital. All relevant clinical characteristics were recorded and analyzed in the Table S3.

3. Immunohistochemistry

KDM4B expression at the protein level were detected by IHC (Immunohistochemistry). Cut the paraffin-embedded UCEC tissue into 4 μm-thick sections. After that, the tissue sections were deparaffinized in xylene and gradient ethanol. Rinse the sections with distilled water. Place the slices in a 0.3% hydrogen peroxide solution, shake and incubate for 10 minutes at room temperature to block endogenous peroxidase. The slices were placed in an EDTA solution (0.01mol/L, pH = 9.0), and then autoclaved for 4 minutes (121°C). KDM4B specific rabbit anti-human monoclonal antibody (dilution 1:200; Abcam, Cambridge) was added to the slices, and then incubated overnight in a refrigerator at 4°C. Then the sections were washed three times with PBS. Then add goat anti-rabbit secondary antibody (dilution 1:5000 Abcam, Cambridge) and incubate for 1 hour at room temperature on a shaker. Put the slices into DAB solution and add hematoxylin dye solution. Put it into a gradient of ethanol for dehydration and seal

with a neutral resin. After that, the specific staining of the sections, the percentage of positively stained tumor cells and the intensity of staining were observed under a light microscope.

4. Evaluation of Immunohistochemical Staining

Semi-quantitative analysis was performed on the protein expression level of KDM4B according to the total combined scores of staining intensity and percentage of positive-staining tumor cells. The staining intensity was graded as follows: 0, 1, 2 and 3 respectively represent no staining, weak staining, moderate staining and strong staining. percentage of positive-staining tumor cells was graded as follows: 0, 1, 2, 3 and 4 respectively represent < 5% positive cells, 5%-25% positive cells, 25%-50% positive cells, 50%-75% positive cells and > 75% positive cells. The scoring process of IHC was carried out twice by two experienced pathologists independently. Finally, the percentage of positive cells is added to the intensity score and recorded as the Total Score of the KDM4B protein expression level, which ranged from 0 to 7. All the scores were recorded in the correlation table, and then correlation analysis was done between the clinical characteristics and the KDM4B expression. In addition, the total score greater than or equal to 4 was defined as high expression, and a total score less than or equal to 3 was defined as low expression.

5. Western Blotting

Collected 30 cases of UCEC and 10 cases of normal endometrial tissue samples from the Harbin Medical University Cancer Hospital, from July 2016 to May 2017 and were used to detect and verify the protein expression of KDM4B by Western Blotting. 30 frozen UCEC tissue samples and 10 frozen normal endometrial tissues were lysed to extract the protein suspension. The mixture was then centrifuged at 12,000g for 15 minutes at 4ºC to collect the supernatant. The quantification of protein concentration was performed according to Bradford kit (Thermo Scientific, Waltham, USA). The protein was separated by electrophoresis in a 10% SDS-PAGE gel, and the protein was transferred to a PVDF membrane treated with methanol. Place the PVDF membrane in a TBS-T blocking solution containing 5% skimmed milk powder and shake on a shaker for 1 hour. The membrane was incubated with primary antibody, anti-KDM4B (Abcam, Cambridge, USA) at 4℃ overnight, which was diluted in buffer (Beyotime, CHINA). The membrane was washed, followed by addition of horseradish peroxidase-conjugated rabbit secondary antibodies to incubate for 1h at room temperature. Afterwards, the membrane was washed again. The experiment was conducted in triplicate. The blots were stained using chemiluminescent matrice and imaged with a charge-coupled camera LAS4000 (Fujifilm, Tokyo, Japan).

6. Immune databases (TIMER, CIBERSORT, TISIDB, EPIC)

Tumor immune estimation resource (TIMER, https://cistrome.shinyapps.io/timer) is a powerful tumor immunology and genetics-related database, including many types of information such as gene expression, mutation and copy number variation. The database is based on a total of 10,897 patient samples from 32 different types of cancers in the TCGA database, and further immune-related extended research has been done(14). In this study, via the TIMER database, the correlation between KDM4B expression and infiltration of six different immune cell types was evaluated (CD8 + T cells, CD4 + T cells,

B cells, macrophages, dendritic cells and neutrophils). The survival module can output a Kaplan Meier plot demonstrating the interrelationship of clinical survival outcome and immune cell infiltration or gene expression. Then, the three databases of CIBERSORT (http://cibersort.stanford.edu/.), TISIDB (http://cis.hku.hk/TISIDB/index.php), EPIC (https://gfellerlab.shinyapps.io/EPIC_1-1/) were used to verify, analyze and explore the relationship between KDM4B and immune infiltrating cells and immune checkpoint inhibitors.

7. KDM4B related gene enrichment analysis and Correlation analysis

Completed the Go (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis through DAVID (Database for Annotation, Visualization and Integrated Discovery, https://david.ncifcrf.gov/home.jsp). The positively correlated co-expressed genes and negatively correlated co-expressed genes of KDM4B calculated and obtained from the UALCAN database were uploaded to the DAVID database, and then analyzed by GO and KEGG. The R software (v.3.6.1 version) was used in this analysis. R packages such as "tidyr", "cnetplot" and "ggplot2" are used to visualize the results of GO and KEGG. Among them, Spearman correlation analysis was used to detect the correlation between the KDM4B gene and the enrichment pathway. A p value < 0.05 was considered statistically significant.

8. Statistical analysis

The R software (v.3.6.1 version) was used to perform statistical data analysis. Survival and clinical characteristics data were got from the TCGA database and clinically collected samples. Then, the overall survival of KDM4B was determined by COX regression and Kaplan-Meier. In addition, Wilcox or Kruskal was used to test the correlation between the clinical characteristics and the KDM4B expression level. The Univariate Cox and Multivariate Cox analysis were used to analyze the factors which may impact the prognosis of patients with UCEC. P value < 0.05 was considered statistically significant.

Results

1.The pan-cancer expression level of KDM4B and its mutation analysis

To probe into the possible role of KDM4B in tumors, the UALCAN database and the human protein atlas (HPA) database were fully utilized. The results showed that KDM4B was overexpressed in multiple malignancies (ACC, BLCA, BRCA, OV, STAD, TGCT, THCA and UCEC) at either the mRNA level or the protein level (Fig. 1A and 1B). Further, KDM4B was found to be highly expressed in gynecological malignancies. Then, the cBioPortal database was used to explore the mutation type and mutation site of KDM4B. In UCEC there are 7% of genetic alterations. KDM4B has a somatic mutation rate of 5.85% in UCEC, mainly in some areas: JmjN, JmjC, PHD2 and TUDOR. The above results suggested that KDM4B was abnormally

overexpressed in varieties of cancers and may have mutations and is a potential oncogene in patients with UCEC.

2.KDM4B expression level in UCEC and its relationship with the clinical characteristics

A total of 546 samples of UCEC patients with various clinical characteristics were obtained from the TCGA database. These clinical characteristics included Age at diagnosis, Histological type, FIGO stage and Histological grade. For one thing, we used GEO database (GSE36389, n = 20) and (GSE115810, n = 26) and TCGA database to verify the expression level and found that KDM4B is highly expressed in UCEC than normal endometrial tissue, which may play a role as an oncogene (Fig. 2A and B). Relevant information of the clinically collected samples were statistically analyzed and recorded in Table S3. These information included Age at diagnosis, Lymph node metastasis, Distant metastasis, Muscular layer depth of Invasion, FIGO stage and Histological grade for subsequent correlation analysis of the clinical characteristics and KDM4B expression level. In addition, we verified it through experiments, Fig. 2 (E-I) showed more detailed clinical feature information of patients with UCEC. The correlation between the expression level of KDM4B and the corresponding clinical characteristics was explored through the Wilcox and Kruskal tests method. The expression level of KDM4B in UCEC tissues from patients with Distant metastasis and Lymph node metastasis ($P < 0.05$). The KDM4B expression level increased with he increase of Histological grade and FIGO stage of UCEC ($P < 0.0001$). As for Muscular layer depth of Invasion, the gene expression level of patients with < 1/2 invasion was lower than that of patients with ≥ 1/2 invasion ($p < 0.001$). There was a close correlation between the KDM4B expression level and Histological grade, FIGO stage, Muscular layer depth of Invasion and Distant metastasis and Lymph node metastasis. However, there was no significant association between KDM4B immunoreactivity and other clinical characteristics (Age at diagnosis and Histological type). In summary, KDM4B was highly expressed in UCECs at both the protein level and nucleic acid level, and the high expression of KDM4B was closely associated with those clinical characteristics that affect the prognosis of patients with UCEC.

3.Validation of KDM4B in Our Clinical Samples

The KDM4B expression was localized in the cytoplasm of tumor cells. Moreover, the differences in expression levels of KDM4B protein in UCEC and normal tissue were assessed by Western blotting (Fig. 3A). The results showed that in 10 cases of normal endometrial tissue, the band of KDM4B was significantly shallower than that of 30 cases of cancer tissue (Fig. 3B). The KDM4B expression was lower in normal endometrium than in UCEC tissue (Fig. 3C). Notably, histogram statistical analysis revealed a significant difference in the two groups ($P < 0.05$). Our clinical follow-up information of 126 UCEC cases showed that elevated expression of KDM4B was associated with poor OS or PFS in patients with UCEC ($P < 0.001$, Fig. 3D-E). The Univariate Cox and Multivariate Cox analysis showed that the high expression of KDM4B was an independent prognostic factor for both PFS and OS (Table S1, Table S2, $P < 0.05$, respectively). Therefore, our results indicate that KDM4B may serve as an independent adverse prognostic biomarker for UCEC patients. In conclusion, KDM4B can be used as an independent risk factor

and a prognostic biomarker in UCEC. Immunohistochemistry and western blot showed that KDM4B was highly expressed and associated with poor prognosis in patients with UCEC.

4.Determination of KDM4B-related cellular signaling pathway and biological process by GO and KEGG

In order to in-depth explore the molecular mechanism that KDM4B may participate in regulation in the occurrence and development of UCEC, we tried to screen out a series of pathway and biological function enrichment analysis by targeting KDM4B binding proteins and KDM4B expression related genes. Figure 4A showed that KDM4B was significantly enriched in the following 4 Biological process: GO:0008152(metabolic process), GO:0009987(cellular process), GO:00065007 (biological regulation) and GO:0023052 (signaling). KEGG pathway enrichment analysis indicated that KDM4B was enriched in the following signaling pathways, including ErbB signaling pathway, FoxO signaling pathway, Central carbon metabolism in cancer, Metabolic pathways, Hedgehog signaling pathway and AMPK signaling pathway. Interestingly the Hedgehog signaling pathway is an immunity related pathway. In summary, by GO and KEGG we found that KDM4B is associated with immune-related biological processes and signaling pathways.

5.Genetic alteration analysis data (MSI, TMB, MMR)

Spearman correlation analysis of MSI and KDM4B gene expression can be seen in Fig. 6A. KDM4B is closely related to MSI in a variety of tumors, including UCEC, CESC, GBM, LGG, BRCA and LUAD. In UCEC, the expression level of KDM4B was positively correlated with MSI ($P < 0.00001$). Spearman correlation analysis of TMB and kdm4b gene expression is shown in Fig. 6B. KDM4B has a close correlation with TMB in multiple tumors, including UCEC, GBM, CESC, COAD, LGG and LUAD. Moreover, KDM4B expression levels were significantly correlated with TMB in UCEC ($P < 0.00001$). Clinically, mutations in MLH1, MSH2, MSH6, PMS2 are commonly found to cause dMMR, and the appearance of MSI in tumor tissue is an important hallmark of DNA mismatch repair deficiency(dMMR). Figure 6C shows that KDM4B is closely associated with MSH2($P < 0.05$) and PMS2($P < 0.01$). The expression level of KDM4B and the mutation status of MSH2 and PMS2 were significantly correlated. Overall, KDM4B is closely associated with MSI, TMB and MMR, and may serve as a promising genetic biomarker.

6.Immune infiltration and immune checkpoint inhibitor analysis data (TIMER, CIBERSORT, TISIDB, EPIC)

Tumor infiltrating lymphocytes (TILs) have been reported to be an important component of the tumor microenvironment and influence tumorigenesis and survival outcomes. To clarify the potential correlation between the expression level of KDM4B and immune cell infiltration in UCEC patients, we performed the following studies utilizing four different databases and algorithms: TIMER, CIBERSORT, TISIDB and EPIC. As shown in Fig. 7A, the timer database was searched to evaluate the correlation of KDM4B mRNA expression in Pan cancer with immune cell infiltration. Six immune infiltrating cells in the Timer database were significantly infiltrated in the pan cancer. As can be seen in UCEC, KDM4B expression was correlated

to CD4 + T cells (P < 0.01), Neutrophil cells (P < 0.001), Myeloid dendritic cell (P < 0.05) and B cell (P < 0.001). As illustrated in the Fig. 7B, the expression of KDM4B was negatively correlated with immune infiltration of CD4 + T cells (r = − 0.182, P = 1.87e-03) and B cells (r = − 0.135, P = 2.21e-02) and positively correlated with CD8 + T cells (r = 0.2,P = 6.46e-04).No association was discovered between KDM4B expression and Macrophage (r = 0.008,P = 8.92e-01), Eutrophils (r = − 0.105,P = 7.26e-02) or Dendritic cell (r = − 0.008,P = 8.96e-01) infiltration.

In addition, to validate the results of the Timer database, the CIBERSORT tool was further used to explore the relationship between the expression of KDM4B and immune infiltrating cells at the single-cell level. Figure 8(A-B) showed that high KDM4B expression was significantly associated with Monocyte cell (P < 0.001), Neutrophil (P < 0.05), B cell (P < 0.001), CD4 + T cell (P < 0.001).

Moreover, a combination of high-throughput screening and genomic profiling data from the TISIDB database was used to analyze the relevance of KDM4B for T cell killing or immunotherapy. Figure 9A showed that the expression of KDM4B has a significant correlation with the Tumor Infiltrating Lymphocytes (TILs) of pan-cancer. As for UCEC, the expression of KDM4B is negatively correlated with Act CD4 cell (r = − 0.309, P = 1.84e-13) and B cell (r = − 0.13, P = 0.00238), while it is positively correlated with CD56 (r = 0.105), P = 0.014) and Th17 (r = 0.102, P = 0.0169). This research showed that the expression of KDM4B has a significant correlation with pan-carcinoma immune checkpoint (Fig. 9B). In UCEC, the expression of KDM4B was positively correlated with immune checkpoint, including ADORA2A (r = 0.092, P = 0.03), CD96 (r = 0.086, P = 0.04), CD244 (r = 0.039, P = 0.03) and CTLA4 (r = 0.12, P = 0.04).

Eventually, we use the EPIC algorithm to find (Fig. 10A) that the expression of KDM4B is significantly correlated with the infiltration of immune cells in CD8 + T, CD4 + T, B cell, Endothelial cell and Macrophage (P < 0.001). Figure 10B showed that the expression of KDM4B in UCEC is positively correlated with CD4 + T cell (r = 0.337, P = 1.31e-03) and CD8 + T cell (r = 0.427, P = 3.27e-05). As can be seen in Fig. 10B, in UCEC, the expression of KDM4B was positively correlated with immune checkpoint, including SIGLEC15 (P = 0.01) and CTLA4 (P = 0.05).

7.Correlation analysis of KDM4B with immune-related copy number variation and immune infiltration-related survival rates

In UCEC patients, alteration in the copy number of KDM4B at different degrees results in altered infiltration of CD8 + T cells, Macrophages, Neutrophils, and Dendritic cells (Fig. 11A). This suggests that the expression of KDM4B is associated with immune infiltration in UCECs. In UCEC, the high expression of KDM4B is associated with poor OS. It is speculated that KDM4B affected the prognosis of patients through immune infiltration. Therefore, we further use Kaplan Meier curves to test the above hypothesis (Fig. 11B). The results showed that expression levels of KDM4B was associated with poor prognosis in patients with CD8 + T cell and B cell infiltration, but not with the other four kinds of cells. In conclusion, the above results indicated that the high expression of KDM4B gene could affect the prognosis of UCEC

patients through the immune infiltration of some immune cells and the immunosuppression of other immune cells.

Discussion

Patients with late-diagnosed and refractory UCEC including high-grade, recurrent and metastatic have a poor prognosis and are treated with traditional surgery and radiotherapy with limited success. Fortunately, harnessing the immune system through checkpoint blockade has greatly expanded the treatment options for advanced UCEC. The continued exploration of the oncogenic role of immune checkpoints and immune cell infiltration in the tumor immune microenvironment has provided new ideas for the development of new UCEC treatment options, such as a combination of immunotherapy and gene targeted therapy. Although significant advances have been made in immunotherapy and gene targeted therapies for UCEC, improvements in patient prognosis are yet to be achieved. Therefore, this study attempted to explore the relationship between the expression of KDM4B and the prognosis and tumor immune microenvironment of UCEC, using the potential oncogene KDM4B as an entry point. This study is dedicated to finding prognostic-related predictive biomarkers that can be used to identify subgroups that are particularly sensitive to immunotherapy, which is of great significance for improving the survival outcome of UCEC(15, 16).

Firstly, the expression level of KDM4B in UCEC and its value for prognostic assessment were explored. As seen in Fig. 2A and 2B, the use of the UALCAN database and the HPA database revealed significant overexpression of KDM4B at the protein and nucleic acid levels in various malignancies. In the light of the above pan-cancer results, we further explored the effect of KDM4B on the malignant biological process and prognosis of UCEC and explored its possible mechanisms. First of all, this study has been validated by TCGA data mining and GEO data. KDM4B was found to be significantly highly expressed in UCEC both at the protein level and at the mRNA level and mutated at multiple loci (Fig. 1C). Meanwhile, the use of IHC and WB further confirmed the respective expression levels of KDM4B in UCEC and normal control (Fig. 3A-C).Secondly, our experimental results also found that KDM4B is related to a variety of clinical factors that affect the prognosis of UCEC, such as the histological grade of the tumor, the FIGO staging, Muscular layer depth of Invasion, Lymph node metastasis and Distant metastasis, as shown in Fig. 2 (E-I) .Numerous studies have confirmed that FIGO stage III and IV, lymph node metastasis and deep muscle infiltration are high risk factors for poor prognosis in UCEC patients. Therefore, we boldly hypothesized that KDM4B contributed to the poor outcome of UCEC patients. Then, survival prognosis was explored by collecting clinical samples from 126 UCEC patients. Kaplan-Meier survival curves showed that patients in the high KDM4B expression group had shorter overall and progression-free survival than those in the low KDM4B expression group (Fig. 3D-E).However, in order to exclude the influence of accidental factors, we continue to rigorously conduct univariate and multivariate analysis to confirm that KDM4B can be used as an independent risk factor for poor prognosis in UCEC patients(Table S1and Table S2).A large number of previous studies have demonstrated that KDM4B contributes to the progression of various malignancies such as gastric, prostate and colorectal cancers, and is strongly associated with poor prognosis, which is highly consistent with our study(17–19). In summary, it is not difficult to find that

KDM4B can act as a new oncogene in UCEC leading to poor prognosis of patients, but the possible mechanism of its oncogenesis needs to be further explored.

To further understand the pathological mechanism of poor prognosis of UCEC due to KDM4B, GO annotation analysis and KEGG cell signaling pathway were used to perform enrichment analysis of KDM4B. In our study, many biological processes related to metabolism and immunity were found to be closely associated with high expression of KDM4B. (Fig. 5). The KEGG results suggested that KDM4B is significantly enriched in signaling pathways such as metabolic pathways and Hedgehog signaling pathways in UCEC. Interestingly, metabolic-related cellular signaling pathways play a pivotal role in the regulation of the tumor immune microenvironment(20). Of interest is that Hedgehog signaling pathway plays a crucial role in the progression of multiple tumors as well as in immune infiltration. Studies have shown that the Hedgehog signaling pathway is thought to be responsible for the formation of squamous cell carcinoma of the head and neck and leads to tumor formation(21). Second, Hedgehog signaling pathways and Innate Lymphocytes (ILCs) have important roles in immune responses to infection, cancer and autoimmunity(22). It has also been demonstrated that Hedgehog signaling pathway affect CD8 + T cell infiltration in primary squamous cell carcinoma of the head and neck(21). The above study gave us significant insight that KDM4B in UCEC may contribute to malignant progression and poor prognosis by altering the tumor immune microenvironment through immune-related biological processes and Hedgehog signaling pathways.

The tumor microenvironment (TME), consisting of tumor cells, mesenchymal cells and immune cells, is constantly being recognized, and the alteration of the TME is a fertile ground for malignant transformation of tumors. Among them, the immunologic suppression and immunologic escape play a decisive role in the unrestricted survival and development of tumor cells. By exploring the TME and new molecular biomarkers, the combination of both for clinical benefit is significant for the diagnosis and treatment of UCEC(23, 24). Therefore, we integrated four immune databases for the study of the TME. On the one hand, the molecular classification theory based on sequencing analysis has, to a certain extent, compensated for the limitations of traditional binary classification and opened up new horizons for prognosis evaluation and treatment guidance of patients. The correlation between KDM4B, a new potentially oncogene of UCEC discovered by the above research, and immune cell infiltration was explored finding that KDM4B expression was correlated to CD4 + T cells, Neutrophil cells, Myeloid dendritic cell and B cells (Fig. 7B). The relationship between the expression level of KDM4B and immune checkpoint was also explored showing that KDM4B was associated with SIGLEC15, ADORA2A, CD96, CD244 and CTLA4 (Fig. 8B and 9B). The present study demonstrated a significant positive correlation between KDM4B and CTLA4 and SIGLEC15 respectively, which may inhibit the immune response of UCEC patients through excessive immunosuppression leading to immunologic escape of malignant tumor cells resulting in poor prognosis of malignant tumor. Mesenchymal stem cell-derived extracellular vesicles overexpressing miR-15a limit immunologic escape in colorectal cancer (CRC) via the KDM4B/Hoxc4/PD-L1 axis, and this study confirms our findings(12). In addition, immune checkpoint blockade using anti-PD1/PD-L1/CTLA4 antibodies improved the prognosis of patients with refractory solid tumors. As a new player in the field of cancer immunotherapy, SIGLEC15 may be able to act as a novel

immunosuppressant with potential impact on anti-PD-1/PD-L1 resistant patients(25–28). On the other hand, since the Cancer Genome Atlas group (TCGA) performed extensive molecular genetic analysis, significant progress has been made in exploring the underlying molecular biology of UCEC. The molecular classification theory based on sequencing analysis has, to a certain extent, compensated for the limitations of traditional binary classification and opened up new horizons for prognosis evaluation and treatment guidance of patients. This study showed that the expression level of KDM4B in UCEC was significantly associated with representative signature genes evaluating immunotherapy response rates including high tumor mutation load, high microsatellite instability and mismatch repair deletion genes by bioinformatics analysis (Fig. 5A-C)(28). Since the crosstalk between the tumor and the immune system is complex and profound, the low response rate of cancer immunotherapy can be explained(29). Therefore, this study provides a solid foundation for further comprehensive study of the interaction between oncogenes (KDM4B) and immune cells, which will help to elucidate the pathogenesis of UCEC and improve the effectiveness of immunotherapy.

Immunotherapy is a promising approach for the treatment of gynecologic cancers. Current and ongoing researches were attempting to improve clinical prognosis through immunotherapeutic strategies. The newly identified potential oncogene KDM4B in this study is inextricably linked to immunity and may be a new hope for immunotherapy of UCEC. Recent advances in gene targeted therapy suggested that with appropriate biomarkers, gene targeted therapy has the potential to improve long-term survival of UCEC patients. However, the median progression-free survival of patients receiving single gene targeted therapy is less than 5 months(30). Thus, the KDM4B gene explored in this study is not only a potential oncogene that can be used for prognostic assessment, but also a possible therapeutic target for immunity(31). The cancer genetic sequencing data were analyzed in depth using advanced bioinformatics analysis, thus summarizing the genetic background of UCEC and opening new pathways to improve the survival outcome of patients with refractory advanced UCEC in the future. It also provides a theoretical background for the combination of gene targeted therapy and immunotherapy.

However, our study still has some limitations. Firstly, some clinical features were incomplete when studying the clinical correlation between KDM4B and UCEC in TCGA and GEO databases, such as the lack of data on TNM staging, Lymph node metastasis and Muscular layer depth of Invasion. But the lack of some specific clinical features is an inevitable drawback of public databases. So we compensated for the above limitation by collecting clinical samples. Secondly, the detailed mechanism about KDM4B and the alternation of TME needs more time and effort to continue our exploration at a later stage. However, it is gratifying to note that we conducted studies related to KDM4B at multiple levels (genomics and proteomics) and in multiple databases to ensure the comprehensiveness and reliability of our study. It is a solid foundation for further studies in the future to create more clinical benefits for UCEC patients.

Conclusions

Our study suggested that aberrantly expressed KDM4B may be a potential prognostic marker for UCEC and, more importantly, it may be associated with tumor immune microenvironment. Of clinical

significance, KDM4B may be used to assess the clinical prognosis of UCEC patients and may also serve as a target for immunotherapy or as a potential marker for checkpoint inhibitor-based immunotherapy.

Abbreviations

UCEC: Uterine Corpus Endometrial Carcinoma; KDM4B: lysine demethylase 4 B; JMJC: Jumonji C structural domain; H3K9: Trimethylated lysine 9 of histone H3; GEO: Gene Expression Omnibus; TCGA: The Cancer Genome Atlas; HPA: Human Protein Atlas; IHC: Immunohistochemistry; TIMER: Tumor immune estimation resource Go: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; dMMR: DNA mismatch repair deficiency; TILs: Tumor infiltrating lymphocytes; TME: The tumor microenvironment

Declarations

Ethics approval and Consent to participate The study protocol was approved by The Ethics Committee of the Harbin Medical University Cancer Hospital (Harbin, China). The use of patient samples conformed to the declaration of Helsinki. All patients provided informed written consent. Consent for Publication All authors of this study agree to the publication of this article. Availability of data and materials The data can be obtained through the email under reasonable request: zmj230530@163.com. Competing interests The author reports no conflicts of interest in this work. Funding This work was supported by grants from the National Natural Science Foundation of China (Grant Numbers: 82073239). Authors' contributions Xiuwei Chen designed the study. Mengjun Zhang and Yuan Liu reviewed the raw data and confirm the authenticity of all raw data. Siyu Hou performed the analysis. Yiru Wang and Can Wang collected data. Mengjun Zhang drafted the manuscript. Xiuwei Chen and Yin Yue revised the manuscript. All authors read and approved the final manuscript. Acknowledgements Thanks for the support of Harbin Medical University Cancer Hospital.

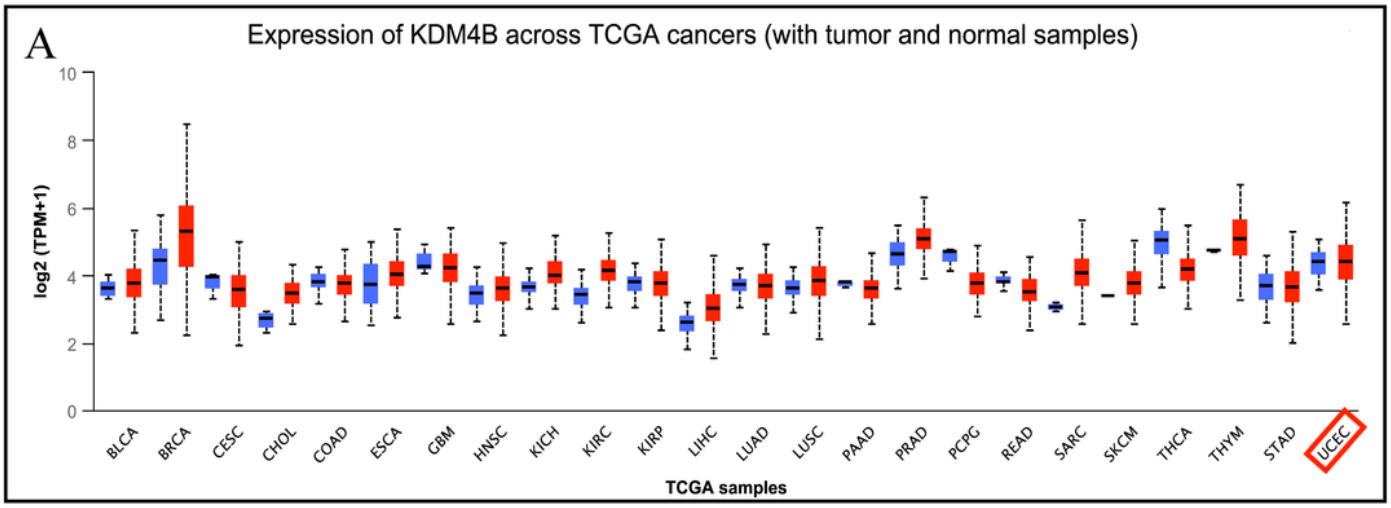
References

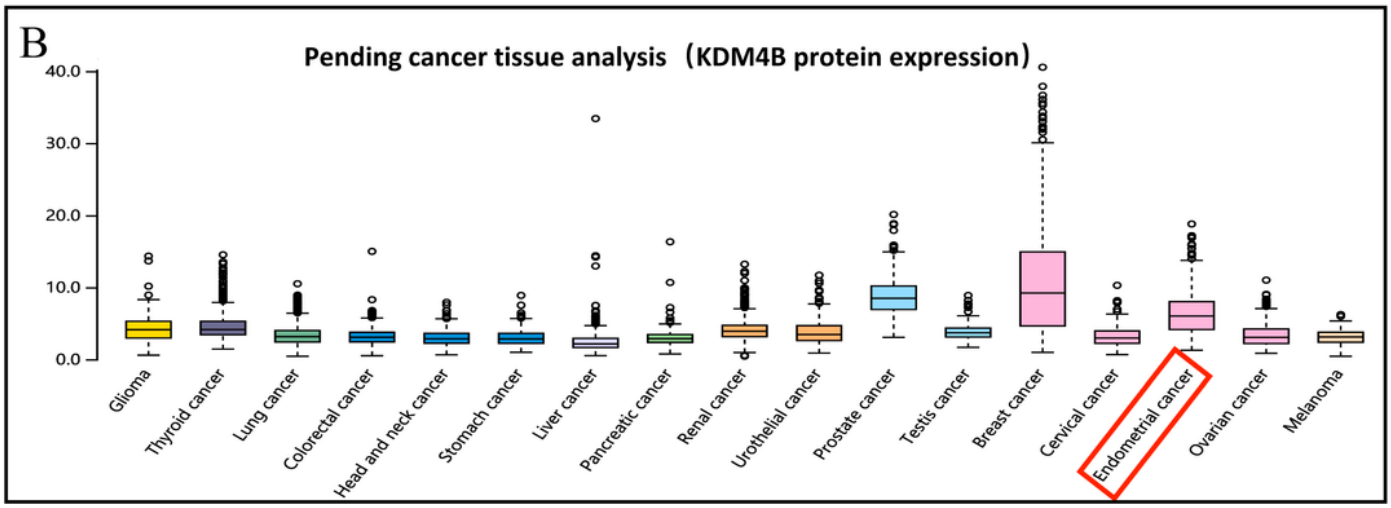
1. Cronin KA, Lake AJ, Scott S, Sherman RL, Noone AM, Howlader N, et al. Annual Report to the Nation on the Status of Cancer, part I: National cancer statistics. Cancer. 2018;124(13):2785 – 800.
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. Cancer J Clin. 2018;68(6):394–424.
3. Morice P, Leary A, Creutzberg C, Abu-Rustum N, Darai E. Endometrial cancer. Lancet. 2016;387(10023):1094–108.
4. Erkanli S, Ayhan A. Fertility-sparing therapy in young women with endometrial cancer: 2010 update. International journal of gynecological cancer: official journal of the International Gynecological Cancer Society. 2010;20(7):1170–87.

5. Lee DH, Kim GW, Jeon YH, Yoo J, Lee SW, Kwon SH. Advances in histone demethylase KDM4 as cancer therapeutic targets. FASEB journal: official publication of the Federation of American Societies for Experimental Biology. 2020;34(3):3461–84.
6. Wilson C, Krieg AJ. KDM4B: A Nail for Every Hammer? Genes. 2019;10(2).
7. Shi L, Sun L, Li Q, Liang J, Yu W, Yi X, et al. Histone demethylase JMJD2B coordinates H3K4/H3K9 methylation and promotes hormonally responsive breast carcinogenesis. Proc Natl Acad Sci USA. 2011;108(18):7541–6.
8. Li H, Lan J, Wang G, Guo K, Han C, Li X, et al. KDM4B facilitates colorectal cancer growth and glucose metabolism by stimulating TRAF6-mediated AKT activation. Journal of experimental clinical cancer research: CR. 2020;39(1):12.
9. Jing JC, Feng Z, Chen ZH, Ji BN, Hong J, Tang N, et al. KDM4B promotes gastric cancer metastasis by regulating miR-125b-mediated activation of Wnt signaling. Journal of cellular biochemistry. 2018.
10. Sha J, Han Q, Chi C, Zhu Y, Pan J, Dong B, et al. Upregulated KDM4B promotes prostate cancer cell proliferation by activating autophagy. Journal of cellular physiology. 2020;235(3):2129–38.
11. Wang W, Oguz G, Lee PL, Bao Y, Wang P, Terp MG, et al. KDM4B-regulated unfolded protein response as a therapeutic vulnerability in PTEN-deficient breast cancer. The Journal of experimental medicine. 2018;215(11):2833–49.
12. Liu L, Yu T, Jin Y, Mai W, Zhou J, Zhao C. MicroRNA-15a Carried by Mesenchymal Stem Cell-Derived Extracellular Vesicles Inhibits the Immune Evasion of Colorectal Cancer Cells by Regulating the KDM4B/HOXC4/PD-L1 Axis. Frontiers in cell developmental biology. 2021;9:629893.
13. Eggink FA, Van Gool IC, Leary A, Pollock PM, Crosbie EJ, Mileshkin L, et al. Immunological profiling of molecularly classified high-risk endometrial cancers identifies POLE-mutant and microsatellite unstable carcinomas as candidates for checkpoint inhibition. Oncoimmunology. 2017;6(2):e1264565.
14. Li T, Fan J, Wang B, Traugh N, Chen Q, Liu JS, et al. TIMER: A Web Server for Comprehensive Analysis of Tumor-Infiltrating Immune Cells. Cancer research. 2017;77(21):e108-e10.
15. Brooks RA, Fleming GF, Lastra RR, Lee NK, Moroney JW, Son CH, et al. Current recommendations and recent progress in endometrial cancer. Cancer J Clin. 2019;69(4):258–79.
16. Gargiulo P, Della Pepa C, Berardi S, Califano D, Scala S, Buonaguro L, et al. Tumor genotype and immune microenvironment in POLE-ultramutated and MSI-hypermutated Endometrial Cancers: New candidates for checkpoint blockade immunotherapy? Cancer treatment reviews. 2016;48:61–8.
17. Kim JG, Yi JM, Park SJ, Kim JS, Son TG, Yang K, et al. Histone demethylase JMJD2B-mediated cell proliferation regulated by hypoxia and radiation in gastric cancer cell. Biochim Biophys Acta. 2012;1819(11–12):1200–7.
18. Sun BB, Fu LN, Wang YQ, Gao QY, Xu J, Cao ZJ, et al. Silencing of JMJD2B induces cell apoptosis via mitochondria-mediated and death receptor-mediated pathway activation in colorectal cancer. Journal of digestive diseases. 2014;15(9):491–500.

19. Mak KH, Lam YM, Ng RK. Histone demethylase JMJD2B/KDM4B regulates transcriptional program via distinctive epigenetic targets and protein interactors for the maintenance of trophoblast stem cells. Scientific reports. 2021;11(1):884.
20. Baracos VE, Martin L, Korc M, Guttridge DC, Fearon KCH. Cancer-associated cachexia. Nature reviews Disease primers. 2018;4:17105.
21. Saloura V, Izumchenko E, Zuo Z, Bao R, Korzinkin M, Ozerov I, et al. Immune profiles in primary squamous cell carcinoma of the head and neck. Oral Oncol. 2019;96:77–88.
22. Kling JC, Blumenthal A. Roles of WNT, NOTCH, and Hedgehog signaling in the differentiation and function of innate and innate-like lymphocytes. J Leukoc Biol. 2017;101(4):827–40.
23. Ligorio M, Sil S, Malagon-Lopez J, Nieman LT, Misale S, Di Pilato M, et al. Stromal Microenvironment Shapes the Intratumoral Architecture of Pancreatic Cancer. Cell. 2019;178(1):160 – 75.e27.
24. Thorsson V, Gibbs DL, Brown SD, Wolf D, Bortone DS, Ou Yang TH, et al. The Immune Landscape of Cancer. Immunity. 2018;48(4):812 – 30.e14.
25. Yasunaga M. Antibody therapeutics and immunoregulation in cancer and autoimmune disease. Semin Cancer Biol. 2020;64:1–12.
26. Liu JN, Kong XS, Huang T, Wang R, Li W, Chen QF. Clinical Implications of Aberrant PD-1 and CTLA4 Expression for Cancer Immunity and Prognosis: A Pan-Cancer Study. Frontiers in immunology. 2020;11:2048.
27. Sun J, Lu Q, Sanmamed MF, Wang J. Siglec-15 as an Emerging Target for Next-generation Cancer Immunotherapy. Clin Cancer Res. 2021;27(3):680–8.
28. Goodman AM, Sokol ES, Frampton GM, Lippman SM, Kurzrock R. Microsatellite-Stable Tumors with High Mutational Burden Benefit from Immunotherapy. Cancer immunology research. 2019;7(10):1570–3.
29. Ribas A, Wolchok JD. Cancer immunotherapy using checkpoint blockade. Science. 2018;359(6382):1350–5.
30. Yen TT, Wang TL, Fader AN, Shih IM, Gaillard S. Molecular Classification and Emerging Targeted Therapy in Endometrial Cancer. International journal of gynecological pathology: official journal of the International Society of Gynecological Pathologists. 2020;39(1):26–35.
31. Polastro L, Closset C, Kerger J. Immunotherapy in gynecological cancers: where are we? Current opinion in oncology. 2020;32(5):459–70.

Figures





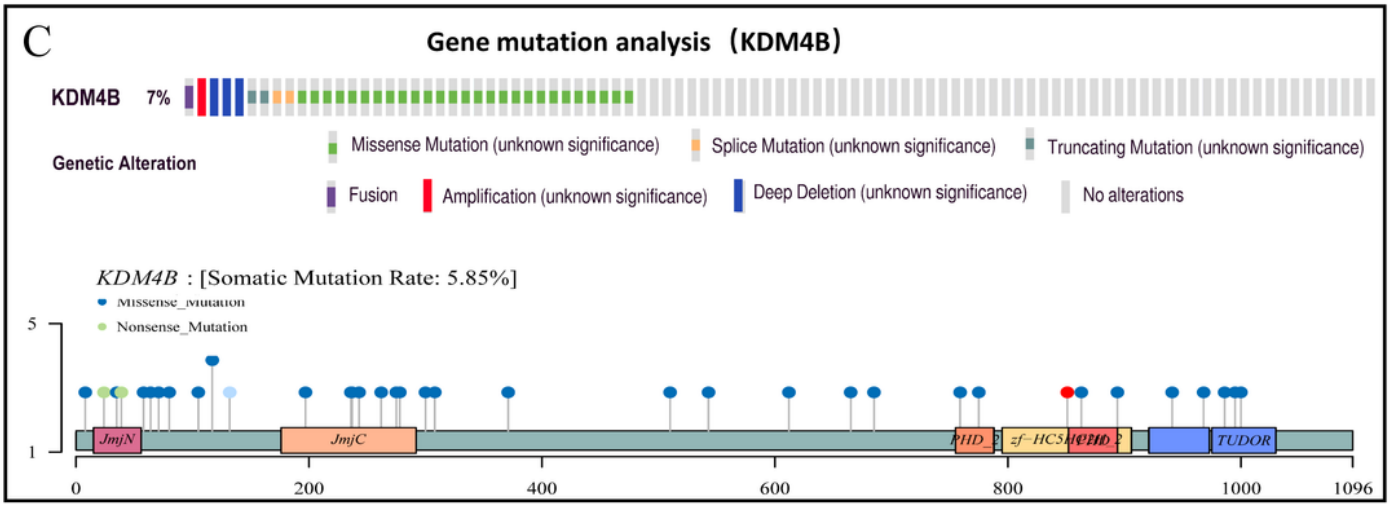


Figure 1

The expression of KDM4B in pan-cancer (mRNA and protein level). (A) The expression of KDM4B in different types of tumor tissues in the UALCAN database, the expression of KDM4B in UCEC (n=546) and normal uterine tissue (n=35), red and blue respectively represent the cancer and normal tissue in pan-cancer. (B) In the HPA database, KDM4B protein is expressed in pan-cancer. (C) Mutation feature of KDM4B in UCEC of TCGA. The mutation features of KDM4B was analyzed by the TCGA database using

the cBioPortal tool. The alteration frequency with mutation type and mutation site are displayed. In UCEC there are 7% of genetic alterations. KDM4B has a somatic mutation rate of 5.85% in UCEC, mainly in some areas: JmjN, JmjC, PHD2 and TUDOR.

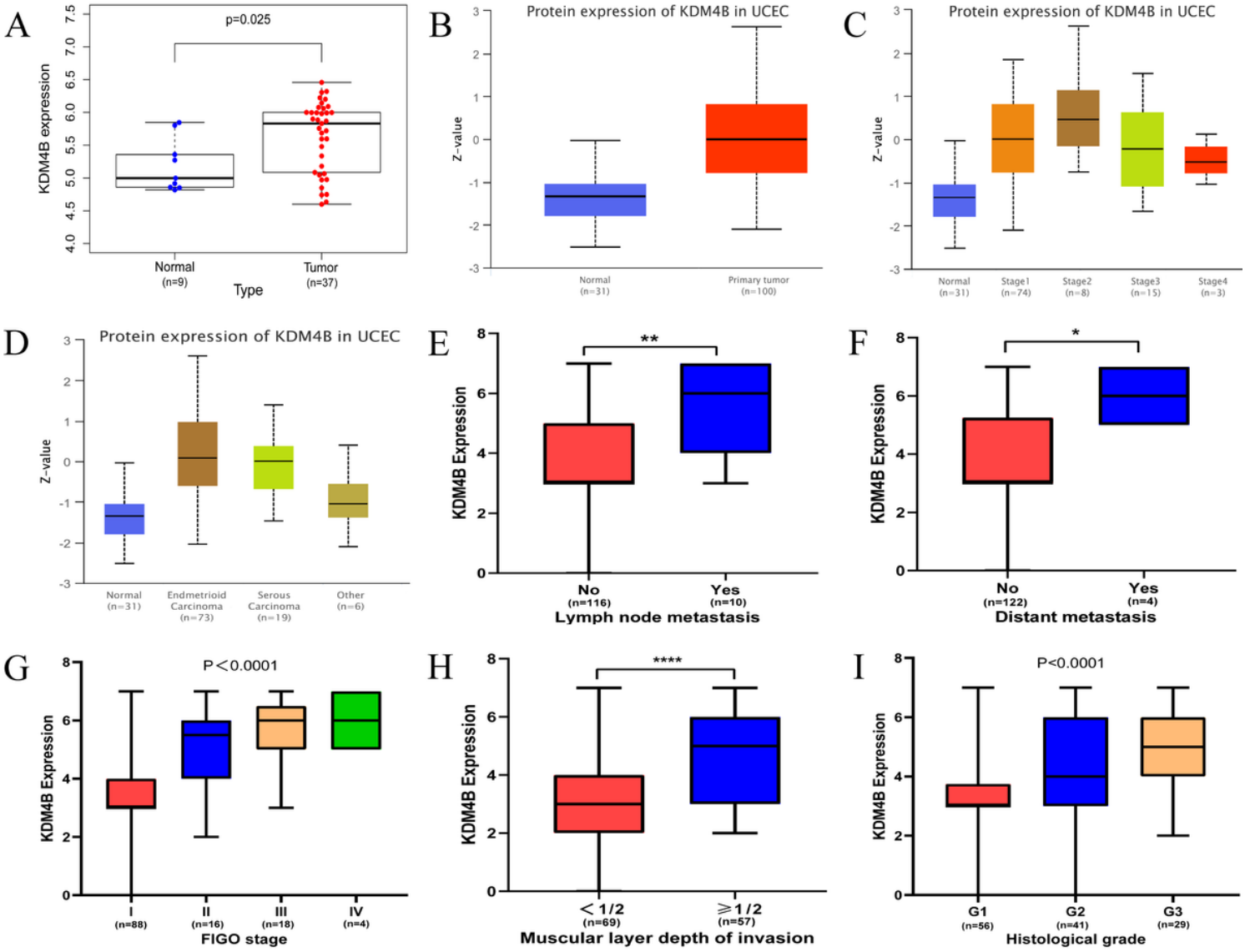


Figure 2

The expression difference of KDM4B and its correlation with different clinical characteristics. (A) The difference in mRNA expression of KDM4B in UCEC and normal endometrial tissues in GEO database (GSE36389 n=20 and GSE115810, n=26). (B) The difference of KDM4B protein expression between UCEC and normal endometrium in CPTAC database. (C) Differential KDM4B expression between UCEC and normal endometrial tissue in different FIGO stages in UCLCAN database. (D) KDM4B is differentially expressed between UCEC and normal endometrial tissue in different pathological types in the UCLCAN database. (E-I) Correlation between KDM4B and clinicopathological features in 126 endometrial cancer patients from clinic. (E) Lymph node metastasis (F) Distant metastasis (G) FIGO stage (H) Muscular layer depth of Invasion (I) Histological grade. (* P<0.05; ** P<0.01; *** P<0.001)

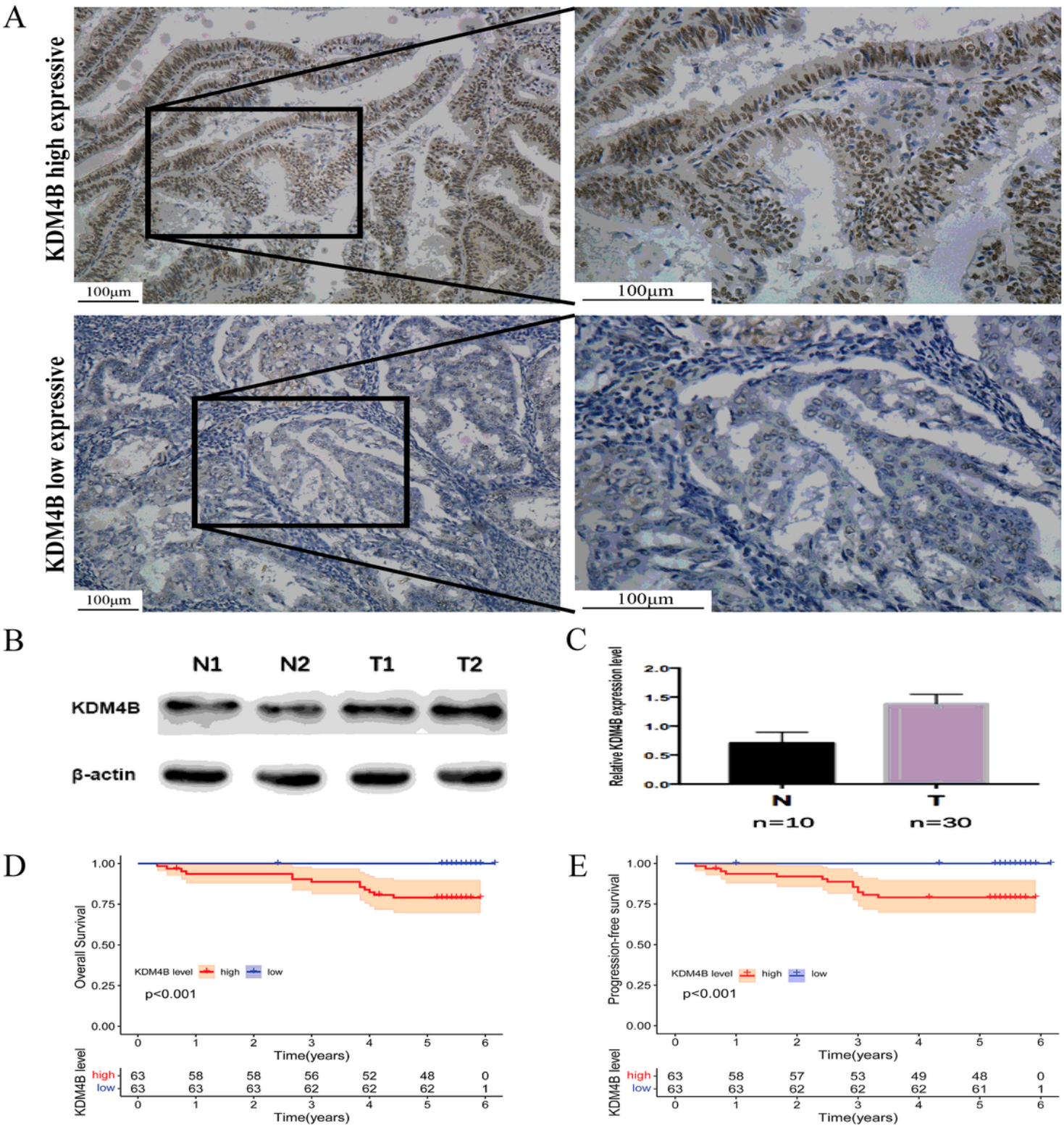


Figure 3

(A) Immunohistochemical staining of high and low KDM4B expression in UCEC specimens from clinic. KDM4B High expression and KDM4B low expression in UCEC (×100 original magnification, ×200 original magnification). (B) Protein samples obtained from frozen UCEC tissues(T) and normal endometrial tissues (N) were analyzed by Western blotting analysis. The levels of β-actin were used as an internal control. (C) Histogram of pooled data from N (n = 10) and T (n = 30). The KDM4B expression was

increased in UCEC tissues(T) compared with normal endometrial tissues (N) (P <0.05). (D-E) The correlation between KDM4B and the poor prognosis of patients with EC. The Kaplan-Meier survival curve reveals the high expression of KDM4B leads to a poor prognosis in UCEC. (D) The overall survival curve was based on clinical follow-up information of 126 patients. (E) The progression free survival curve was based on clinical follow-up information of 126 patients.

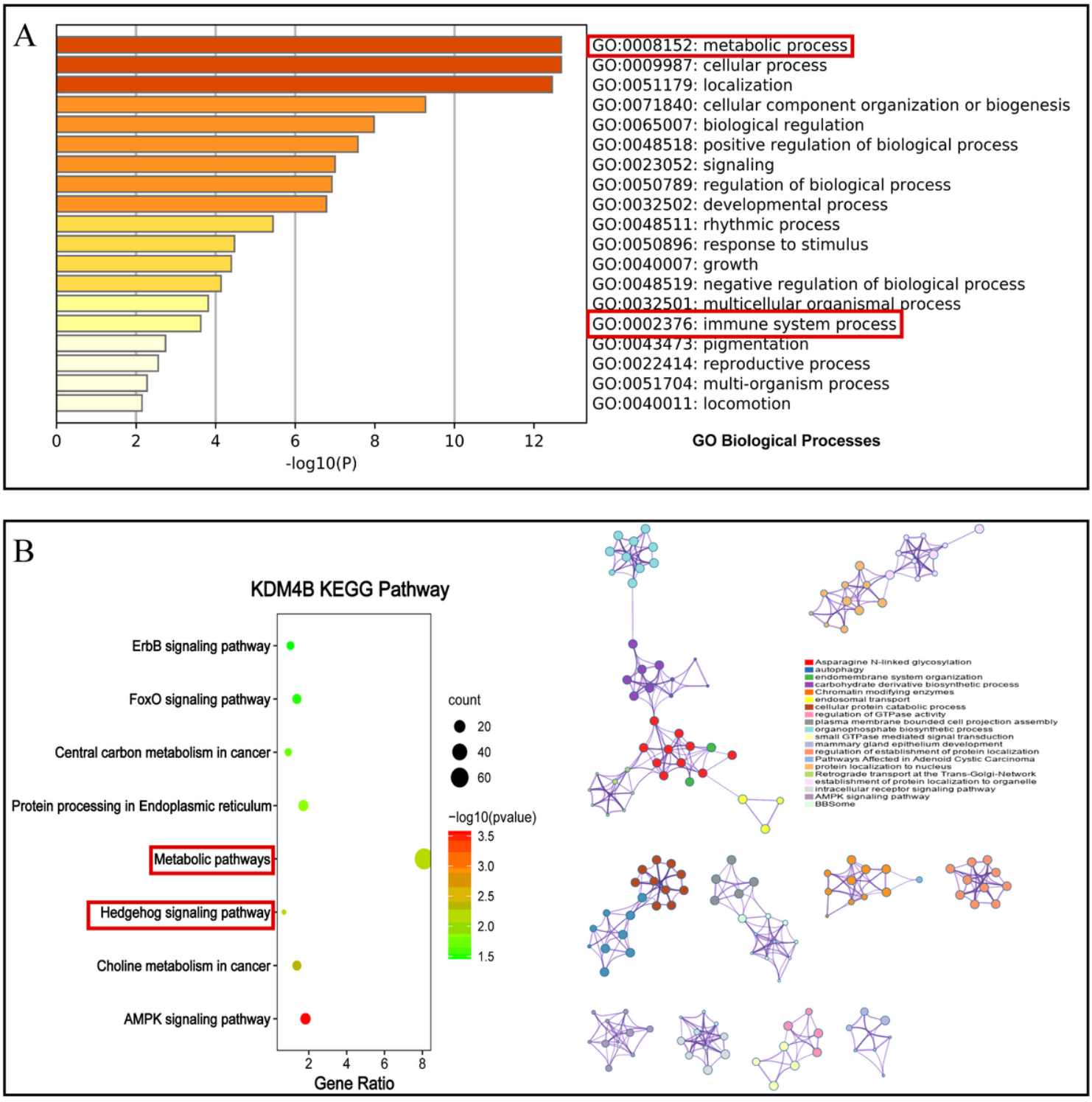


Figure 4

Go functional annotation and KEGG pathway enrichment analysis of KDM4B in UCEC. (A) Go functional annotation: Biological Process (metabolic process, cellular process, biological regulation, immune system process, etc) (B) KEGG pathway enrichment analysis (ErbB signaling pathway, FoxO signaling pathway, Central carbon metabolism in cancer, Metabolic pathways, Hedgehog signaling pathway and AMPK signaling pathway).

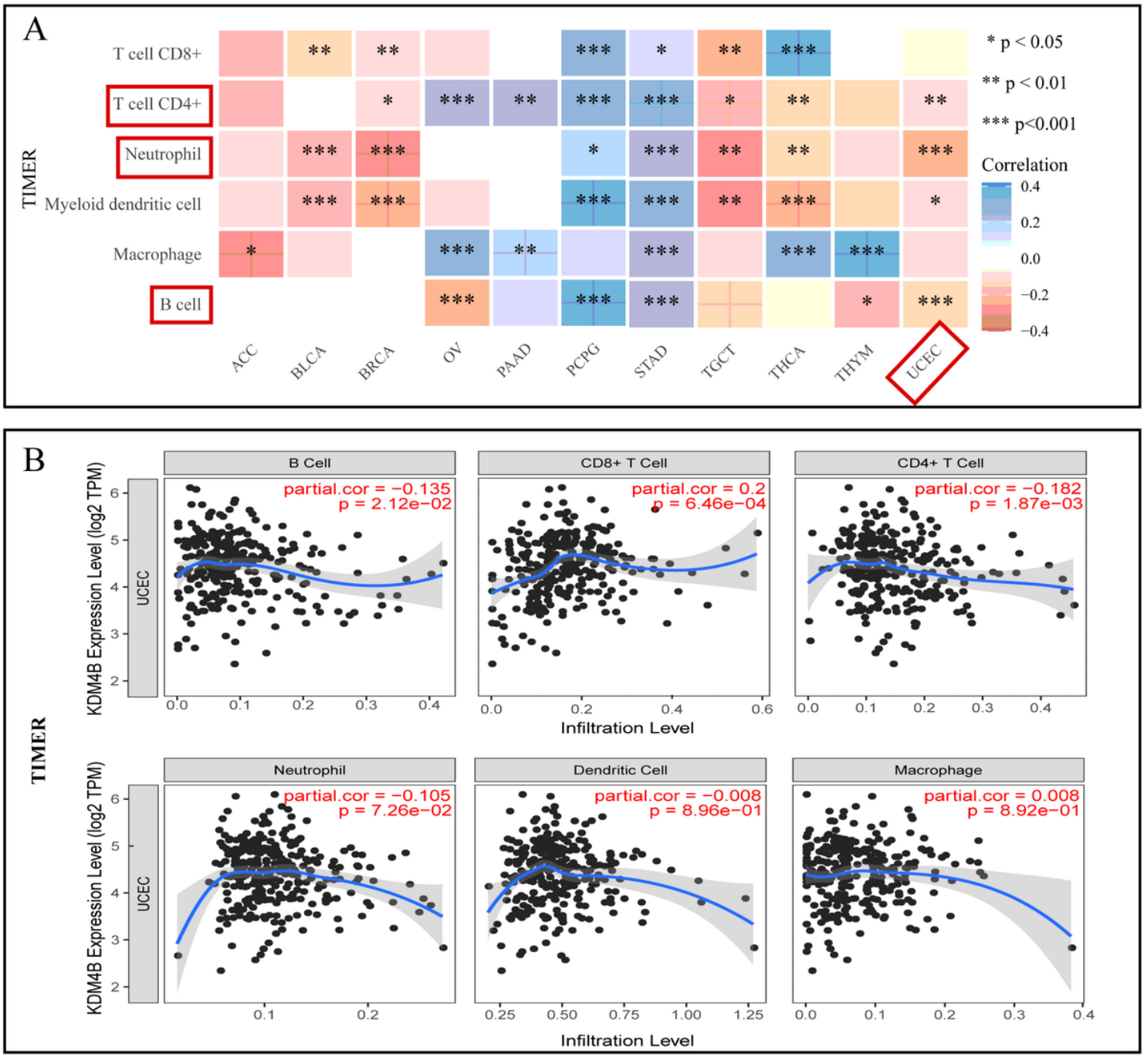


Figure 5

Relationship between expression of the KDM4B gene and proportion of immune infiltrates in TIMER database. (A) Spearman correlation analysis of tumor immune infiltration (B Cell, CD8+T Cell, CD4+T Cell, Macrophage, Neutrophil, Dendritic Cell) and KDM4B gene expression in Pan-cancer (ACC, BLCA, BRCA, OV, PAAD, PCPG, STAD, TGCT, THCA, YHYM, UCEC). (B) Correlation of KDM4B expression with infiltrating

levels of B cells, CD8+T cells, CD4+T cells, macrophages, neutrophils, and dendritic cells in UCEC are available on the TIMER database. Correlation coefficients (rho values) and p values were shown. (* P<0.05; ** P<0.01; *** P<0.001).

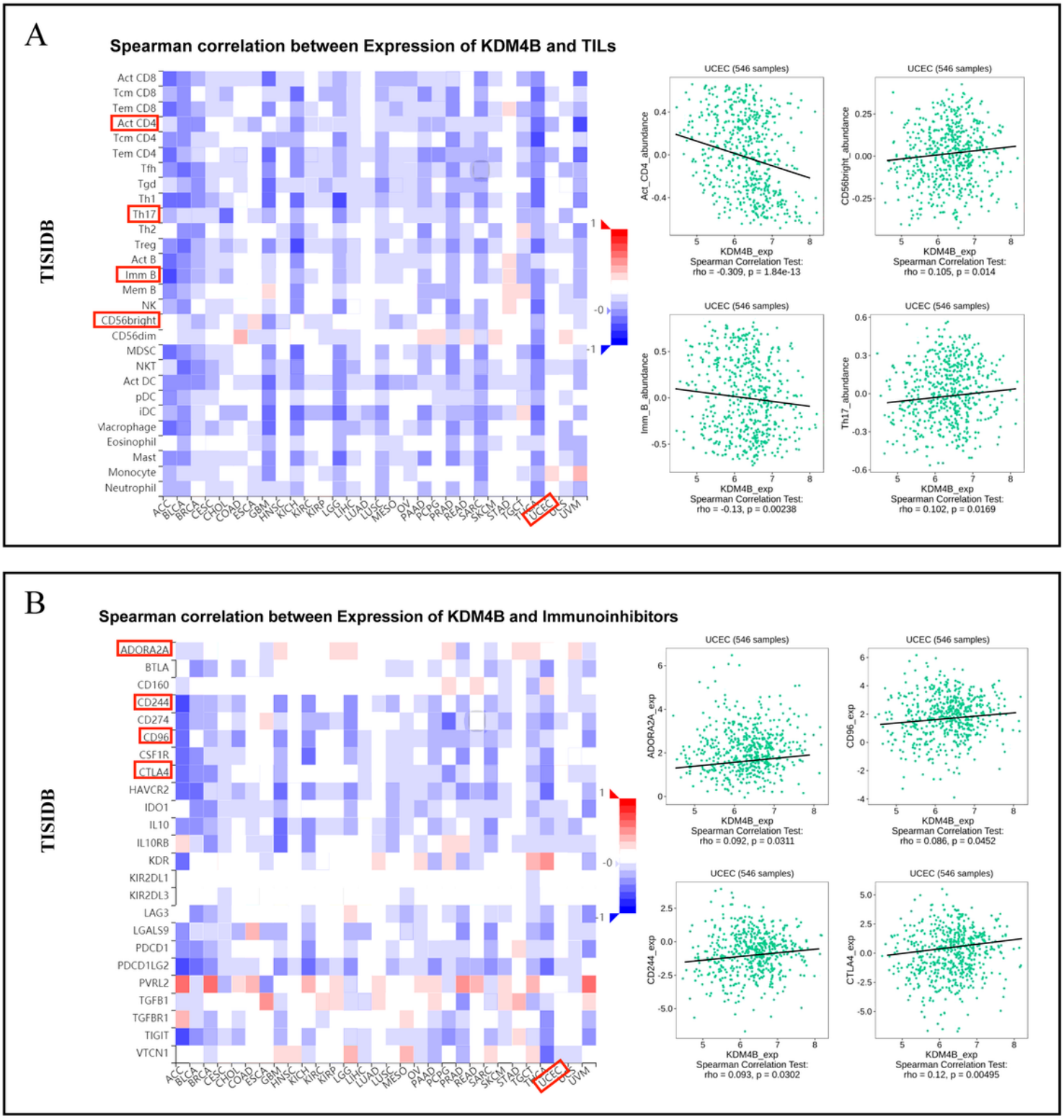


Figure 6

Immunological correlation of KDM4B in the TISIDB database. (A) Spearman correlation analysis of TILs (Tumor Infiltrating Lymphocytes: Act CD8, Act CD4, B Cell, NK, Macrophage, Th1, MDSC) and KDM4B

gene expression in Pan-cancer (ACC, BLCA, BRCA, OV, PAAD, CESC, STAD, TGCT, THCA, YHYM, UCEC). The abundance of act CD4, TEM CD4, Imm B and TCM CD8 in UCEC was correlated with the expression of KDM4B (P < 0.05). (B) Spearman correlation analysis of immune checkpoint (BTLA, CD274, CTLA4, IDO1, IL10, PDCD1, PDCD1LG2, TGFB1) and KDM4B gene expression in Pan-cancer (ACC, BLCA, BRCA, OV, PAAD, CESC, STAD, TGCT, THCA, YHYM, UCEC). The abundance of act CD274, PDCD1LG2, IDO1 and CTLA4 in UCEC was correlated with the expression of KDM4B (P < 0.05).

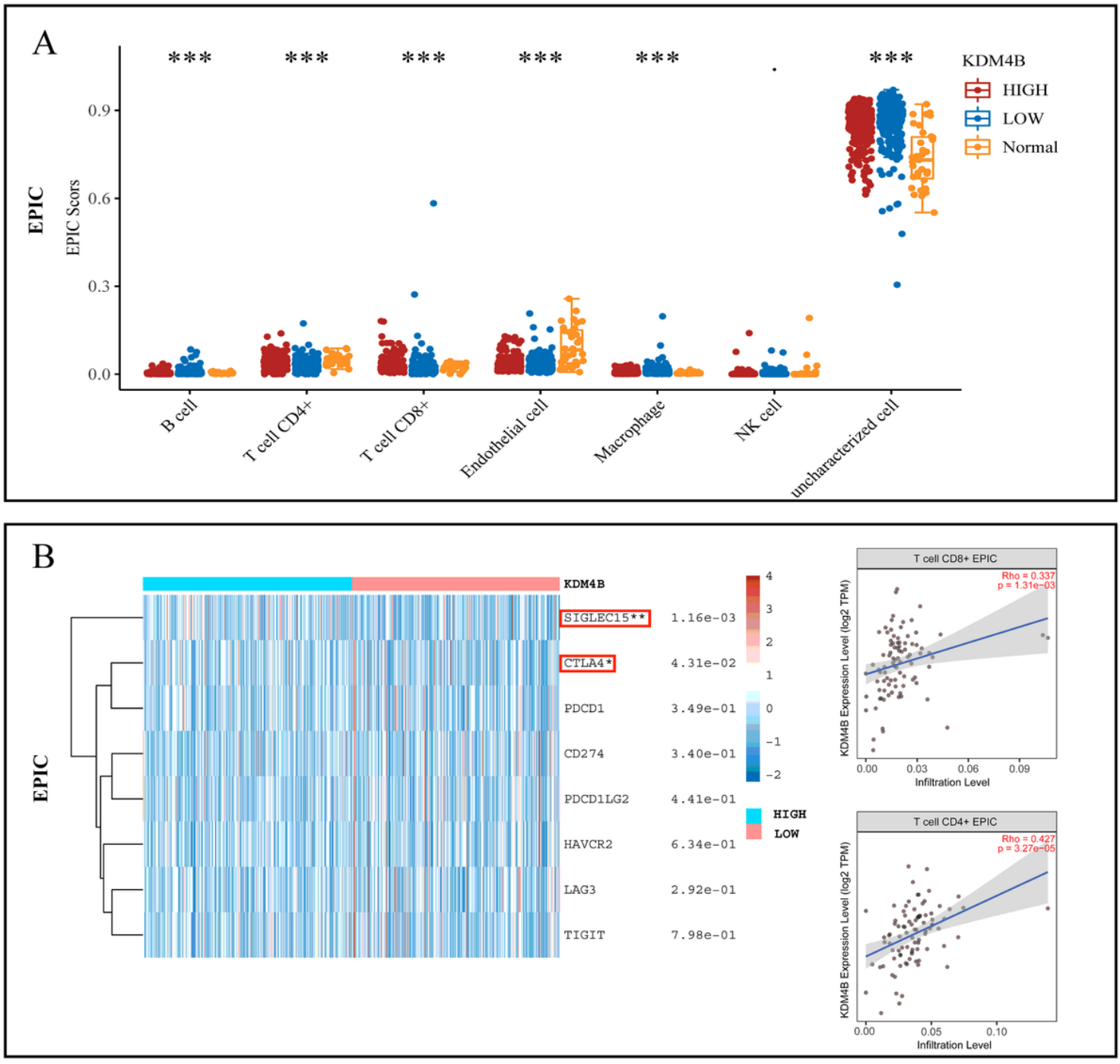


Figure 7

Immunological correlation of KDM4B in the EPIC database. (A) Relationship between different immune infiltrating cells and KDM4B gene expression levels in UCEC from EPIC database (* P<0.05; ** P<0.01; ***

P<0.001). (B) In EPIC database, the relationship between immune checkpoint immune checkpoint (SIGLEC15, CTLA4, PDCD1, CD274, PDCD1LG2, HAVCR2, LAG3, TIGIT) and KDM4B gene expression was analyzed by Heat map. The abundance of CD4+T Cell, and CD8+T Cell in UCEC was correlated with the expression of KDM4B (P < 0.05).

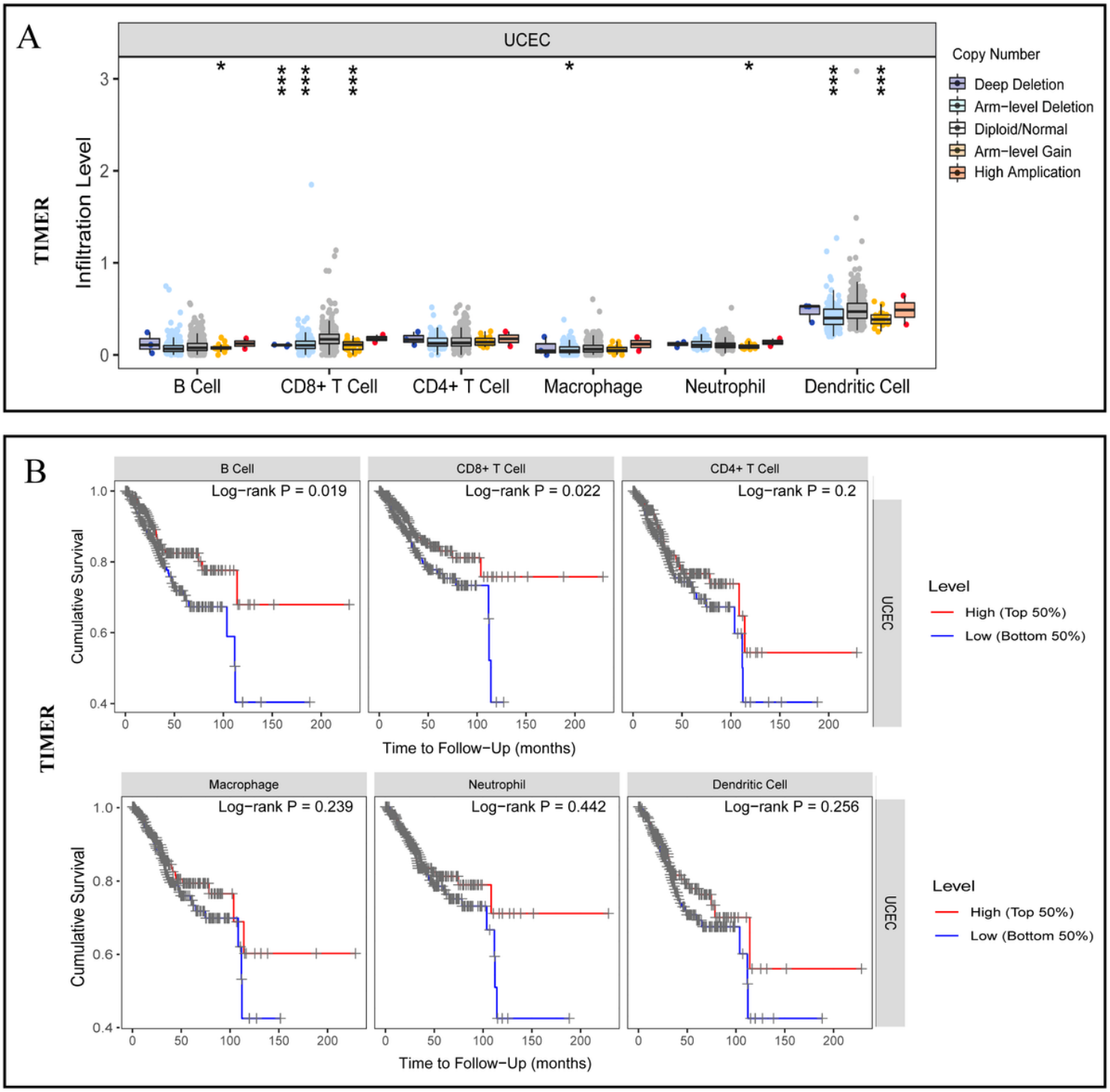


Figure 8

Relationship between KDM4B gene expression and copy number variation and prognosis in the TIMER database. (A) Association between KDM4B gene copy number and immune cell infiltration levels in UCEC

cohorts. (B) Kaplan-Meier plots were used to analyze the immune infiltration and overall survival rate of UCEC. *p<0.05, **p<0.01, ***p<0.001.

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