Overexpression of Pyruvate Dehydrogenase Kinase-3 Predicts Poor Prognosis in Urothelial Carcinoma

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Background: The mitochondrial pyruvate dehydrogenase complex (PDC) link glycolysis to the tricarboxylic acid cycle by decarboxylating pyruvate to acetyl coenzyme A irreversibly. Cancer cells are characterized by a shift in cellular metabolism from mitochondrial respiration to glycolysis. PDC activity inhibition mediated by phosphorylation via pyruvate dehydrogenase kinase (PDK) has been linked to cancer. However, the clinical significance of PDKs in urothelial cancer prognosis is not clear. We investigated the role and prognostic value of PDK3 expression in patients with upper urinary tract urothelial carcinoma (UTUC) and urinary bladder urothelial carcinoma (UBUC).

Patients and Methods: We retrospectively analyzed clinical data and pathological features. Formalin-fixed urothelial carcinoma (UC) tissues were collected and embedded in paraffin. The correlation of PDK3 expression with clinical characteristics, pathological findings and patient outcomes, including metastasis-free survival (MFS) and disease-specific survival (DSS) were analyzed by Pearson’s chi-square test, Kaplan–Meier analysis, and the multivariate Cox proportional hazards model.

Results: Data from 295 patients with UBUC and 340 patients with UTUC were evaluated. High PDK3 expression significantly correlated with several pathologic variables such as high T stage, lymph node metastases, high tumor grade, vascular invasion, and high mitotic rate (all \( P < 0.001 \)). High PDK3 expression was associated with poor disease-specific survival (DSS) (\( P < 0.0001 \)) and metastatic free survival (MFS) (\( P < 0.0001 \)) in a Kaplan–Meier analysis. Additionally, multivariate analysis demonstrated increased PDK3 expression is a significant predictive risk factor for DSS [hazard ratio (HR) in UBUC, 2.79, \( P = 0.009 \); in UTUC, 2.561, \( P = 0.03 \)] and MFS (HR in UBUC, 1.907, \( P = 0.024 \); in UTUC, 1.793, \( P = 0.044 \)). The gene co-expression analysis showed abundant PDK3 co-upregulated genes were involved in the processes of DNA replication and repair through the Gene Ontology classification system.
INTRODUCTION

The most common malignancy of the urinary system is urothelial carcinoma (UC), which includes UC of the urinary bladder (UBUC) and UC of the upper urinary tract (UTUC). Bladder cancer is the 10th most prevalent cancer globally, with about 573,000 new cases and 213,000 deaths in 2020 (1). In Western countries, urinary bladder tumors account for 90–95 percent of UCs, with UTUC accounting for another 5%–10% (2). In Taiwan, UTUC represents as many as 30% of affected cases, and ureteral tumors make up more than half of all UTUC cases (3). UTUC and UBUC are similar in biology and share risk factors; however, they represent different entities due to anatomical and practical differences. Approximately 70% of patients with organ-confined bladder cancer present with a disease confined to the mucosa (stage Ta and CIS) or submucosa (stage T1), referred to as non-muscle-invasive bladder cancer (NMIBC) (4). Despite the good prognosis, patients with low- and intermediate-risk NMIBC have 5-year recurrence-free survival rates of 43% and 33%, respectively, up to 21% of patients with high-risk features develop muscle-invasive bladder cancer (MIBC) (5, 6). In UBUC and UTUC, the gold-standard treatments are transurethral resection of the bladder tumor (TURBT) followed by intravesical chemotherapy and radical nephroureterectomy with bladder cuff excision (7). MIBC accounts for the remaining 30% of localized UC. Neoadjuvant chemotherapy followed by radical cystectomy or bladder preservation strategy with maximum TURBT followed by concomitant chemoradiation are the options for patients with MIBC. The 5-year overall survival rate ranges from 36%–48% (8). Clinical prognostic factors, such as pathological tumor stage, tumor grade, and lymph node metastases define high risk patients. However, a larger, follow-up research is needed to uncover more accurate genomics-based predictors of UC.

Cancer cells have been found to be able to sustain their growth by altering normal metabolic processes. Cancer cells favor aerobic glycolysis over mitochondrial pyruvate oxidation. The mitochondrial pyruvate dehydrogenase complex (PDC) decarboxylates pyruvate to acetyl coenzyme A (acetyl-CoA) irreversibly in normal cells. Pyruvate dehydrogenase kinase has four isoforms (PDK1-4) that can phosphorylate and inhibit the PDC. The affected cells prefer glycolysis over the tricarboxylic acid cycle for adenosine triphosphate (ATP) generation (9). In a variety of cancers, PDK has been linked to tumor aggressiveness, proliferation, and chemotherapy resistance. One study has reported PDK1 expression as an independent prognostic factor that affects overall survival of MIBC patients (10). Another study demonstrated that PDK4 is substantially upregulated in bladder cancer cell lines, and inhibition of PDKs combined with cisplatin therapy could further reduce tumor volume in vivo (11). Herein, we assess the genes related to the regulation of acetyl-CoA biosynthetic processes from pyruvate (GO:0010510) using a transcriptome dataset of UC (GSE31684). Among these genes, PDK3 was significantly upregulated in invasive UC compared with non-invasive UC. To date, the possible importance of PDK3 in UC has not been thoroughly investigated. In this work, we looked at PDK3 expression and its prognostic value in the UTUC and UBUC cohorts.

PATIENTS AND METHODS

Data Mining of the Gene Expression Dataset

The NCBI Gene Expression Omnibus (GEO) database yielded a transcriptome dataset (GSE31684), which included 93 UBUC patients who had major operation. We evaluated all probe sets without pre-selection or filtering and loaded the raw files into Nexus Expression 3 software to measure gene expression levels (BioDiscovery, El Segundo, CA, USA). By comparing tumor invasions (high stage vs. low stage) and metastasis (metastasis vs. non-metastasis), comparative studies were undertaken to examine differentially expressed genes associated to the regulation of acetyl-CoA biosynthetic pathway from pyruvate (GO:0010510). For further investigation, the genes expressed differentially (P < 0.05 and log2 ratio >0.3) were chosen.

To examine the roles of PDK3 in UC, the correlations between the PDK3 mRNA level and its co-expressed genes in The Cancer Genome Atlas (TCGA) database were examined using the cBioPortal online platform (http://cbioportal.org). The top 200 genes with a positive correlation or a negative correlation with PDK3 were further investigated using the Gene Ontology (GO) classification system (http://geneontology.org/) in agreement with biological processes or cellular components and ranked by fold enrichment for functional annotation.

Study Population

Between 1996 and 2004, the Chi Mei Medical Center enrolled 340 patients with UTUC and 295 patients with UBUC who had curative surgery. The institutional review board examined and approved this study (105–01–005). All of the subjects gave their informed consent. Retrospective data on demographics and
clinical information was recorded. Patients who had received preoperative chemotherapy or radiotherapy, had other malignancies, or had missing clinical data were excluded. The tumor stage was determined using the system developed by the American Joint Committee on Cancer (AJCC) in 2002. Using the seventh edition of the AJCC staging system, two pathologists assessed tumor specimens and categorized them as low- or high-grade. Cisplatin-based adjuvant treatment was given to all UBUC patients with pT3 or pT4 cancers or lymph nodes metastases.

**Immunohistochemistry and Scoring**

The formalin-fixed paraffin embedded samples from the study cohort were cut onto slides and prepared according to standard procedure. After antigen retrieval, sections were incubated with a primary antibody targeting PDK1 (Rabbit polyclonal, 10026-1-AP, 1:100, Proteintech) and PDK3 (Rabbit polyclonal, 12215-1-AP, 1:100, Proteintech), respectively, for 1 hour. The DAKO ChemMate EnVision Kit was used to detect primary antibodies (K5001, Carpinteria, CA, USA). Positive immunoreactivity was confirmed by the presence of brown chromogen in the cytoplasm of tumor cells. As positive controls, cell blocks made from cell lines known to express PDK1 and PDK3 were employed. As a negative control, a sample was incubated without the main antibody. Two pathologists used the H-score to estimate PDK1 and PDK3 immunoreactivity, which was calculated using the following equation: \( H\text{-score} = \Sigma Pi (i + 1) \), where \( Pi \) is the percentage of stained tumor cells of various intensities ranging from 0% to 100%, and \( i \) is the staining intensity (0 to 3+). In cases where there were scoring discrepancies, the two pathologists analyzed the slides at the same time and agreed on an H-score. Elevated expression was defined as tumors with H-scores equal to or greater than the median of all scored cases.

**Statistical Analysis**

Pearson’s chi-square test was used to analyze the relationship between PDK3 expression and several clinicopathological characteristics. The researchers looked at two end points: metastasis-free survival (MFS) and disease-specific survival (DSS).

The DSS was measured from curative surgery to the time of cancer mortality and the MSS was measured from curative surgery to the first metastasis. Relevant PDK3 expression and clinicopathological features were identified using univariate and multivariate analysis. The Kaplan–Meier method with a log-rank test was applied to create survival curves. To find the independent variables, all significant parameters from the univariate analysis were incorporated in the multivariate Cox proportional hazards model. For statistical analysis, SPSS Statistics V.17.0 software (IBM Armonk, NY, USA) was used. The threshold for statistical significance was set at \( P < 0.05 \).

**RESULTS**

**Upregulation of PDK3 Gene Links to Metabolic Reprogramming in the UBUC Transcriptome**

For data mining, we used a published UBUC transcriptome dataset (GSE31684) that includes 93 patients who had undergone radical cystectomy. A total of 78 patients were classified as having invasive disease (pT2–pT4), whereas 15 were classified as having noninvasive or superficial disease (pTa and pT1). We found 11 probes encompassing 5 transcripts linked to the regulation of acetyl-CoA biosynthetic process from pyruvate (GO:0010510). When invasive UC was compared to non-invasive UC, PDK3 was shown to be considerably upregulated (Table 1 and

| Probe        | Comparing invasive UC to non-invasive | Gene symbol | Gene title | Molecular function                                                                 |
|--------------|---------------------------------------|-------------|------------|------------------------------------------------------------------------------------|
| 202030_at    | 0.3824 0.0666                         | BCKDK       | branched chain ketoacid dehydrogenase kinase                                      |
| 206696_at    | -0.0323 0.8638                        | PDK1        | pyruvate dehydrogenase kinase; isozyme 1                                        |
| 226452_at    | 1.079 0.0748                          | PDK2        | pyruvate dehydrogenase kinase; isozyme 2                                        |
| 202935_at    | 0.2467 0.4089                         | PDK3        | pyruvate dehydrogenase kinase; isozyme 3                                        |
| 208347_at    | 0.1944 0.1994                         | PDK4        | pyruvate dehydrogenase kinase; isozyme 4                                        |
| **Table 1**  |                                       |             |            | Differential expression of genes related with the regulation of the acetyl-CoA biosynthetic process from pyruvate (GO:0010510) in the transcriptome of urothelial carcinoma of the urinary bladder (GSE31684) in relation to cancer invasiveness. |
Figure 1). Table 1 reveals upregulation of the PDK3 gene (Probe: 206348_s_at) in advanced UC with up to 0.4925-fold log ratios (P=0.0235). The goal was to find the most upregulated gene linked to tumor invasiveness, hence PDK3 was selected as a candidate for future testing. The PDK1 gene was found to be nonsignificant after analysis.

PDK3 Upregulation Is Linked to DNA Replication and Repair

A gene co-expression analysis was undertaken to better understand the role of PDK3 in UC. The top 200 genes from the TCGA database (n = 294) that were correlated positively (Supplementary Table 1) or correlated negatively (Supplementary Table 2) with PDK3 were examined. We found that, in terms of biological processes (Supplementary Figure 1) and cellular components (Supplementary Figure 2), abundant PDK3 co-upregulated genes were related to the processes of DNA replication through the GO classification system. The top terms associated with PDK3 upregulation comprised pre-replicative complex assembly involved in cell cycle DNA replication (GO: 1902299, fold enrichment: 49.75), DNA replication, synthesis of RNA primer (GO: 0006269, fold enrichment: 49.75), and DNA replication-dependent nucleosome assembly (GO: 0006335, fold enrichment: 39.8). With respect to cellular components, the top terms associated with PDK3 upregulation contained chromatin assembly factor 1 (CAF-1) complex (GO: 0033186, fold enrichment: 66.33), Elg1 replication factor C (RFC)-like complex (GO: 0031391, fold enrichment: 66.33), alpha DNA polymerase: primase complex (GO: 0005658, fold enrichment: 59.7), CMG complex (GO: 0071162, fold enrichment: 49.75), and DNA replication preinitiation complex (GO: 0031261, fold enrichment: 41.46). In addition, the biological process terms related to DNA repair, included double-strand break repair via break-induced replication (GO: 0000727, fold enrichment: 33.16), nucleotide-excision repair, DNA gap filling (GO: 0006297, fold enrichment: 25.95), and base-excision repair, gap-filling (GO: 0006287, fold enrichment: 17.06), were also associated with PDK3 upregulation. Collectively, the results revealed that the mRNA level of PDK3 and its co-upregulated genes are strongly correlated with DNA replication and repair, suggesting that PDK3 might be important in tumor proliferation and development.

In contrast, regarding the cellular components (Supplementary Figure 2), we identified the eukaryotic translation elongation factor 1 complex (GO: 0005853, fold enrichment: 82.16) as the most significantly associated with PDK3 downregulation and identified the eukaryotic translation elongation factor 1 alpha 1 (EEF1A1) gene (Spearman’s correlation: −0.23) to be related to this cellular component. In addition to its function in translation elongation, EEF1A1 has been reported to function as a pro-apoptotic factor (12). Furthermore, we observed that the transcripts of The Gene Expression Profiling Interactive Analysis database (http://gepia.cancer-pku.cn/detail.php?gene=EEF1A1) found that EEF1A1 levels in bladder tumor samples were lower than in matching normal tissues. However, the correlations among the levels of PDK3 and EEF1A1, cell apoptosis, and cancer progression in UC require further analysis.

Clinicopathological Features of Our UC Cohorts

A total of 340 UTUC and 295 UBUC patients were included in the study (Table 2). The median age was 65.8-year-old. In the UTUC cohort, 159 (46.8%) patients had high stage (pT2-4) tumors and 284 patients (83.5%) had high grade tumors. At the time of diagnosis, 28 patients (8.2%) had lymph nodes metastases. Vascular invasion (VI) and perineural invasion (PNI) were found in 106 (31.2%) and 19 (5.9%) of the patients, respectively. Furthermore, 167 lesions (49.1%) demonstrated a high mitotic rate. 123 patients (41.7%) in the UBUC cohort had muscle invasive bladder cancer (pT2-4). At the time of their initial diagnosis, the majority of patients (81%) had tumors with a high histological grade. Only 29 individuals (7.8%) had lymph node metastases.
when they were diagnosed. There were 156 lesions with a high mitotic rate (52.9%). Furthermore, VI and PNI were found in 20 (6.8%) and 49 (16.6%) of the tumors, respectively.

**Correlations Between PDK3 Expression and Pathological Features in UC**

Invasive UC had greater PDK3 immunoreactivity than non-invasive UC (Figure 2). Table 2 illustrates the correlation between PDK3 immunoreactivity and a variety of clinicopathological factors. In UTUC cohorts, PDK3 overexpression was associated with renal pelvis tumor (P = 0.009), T2-4 stage (P < 0.001), high histological grade (P = 0.018), vascular invasion (P < 0.001), and high mitotic rate (P < 0.001). In UBUC cohorts, high PDK3 immunoreactivity was associated with T2-4 stage, lymph nodes metastases, high histological grade, vascular invasion, perineural invasion, and high mitotic rate (all P < 0.001).

**Prognostic Significance of PDK3 Expression**

There was a total of 113 patients who died of UC, and 146 patients had subsequent tumor metastases. To investigate the predictive influence of PDK3 expression on cancer metastasis and patient survival in UC, we utilized univariate and multivariate analyses. In univariate analysis at UTUC (Table 3), tumor location, multi-focal tumors, vascular invasion, perineural invasion, PDK1 overexpression, and PDK3 overexpression were all found to be significantly associated with poor prognostic factors for DFS and MFS. Multivariate Cox regression analysis showed that lymph node metastases and perineural invasion were significant indicators for DFS, while multi-focal tumors, lymph nodes metastases, vascular invasion, and perineural invasion were significant indicators for MFS. PDK3 expression was significantly associated with poor DFS [hazard ratio (HR), 2.561; 95% confidence interval (CI), 1.070–3.934; P = 0.03] and MFS [hazard ratio (HR), 1.793; 95% confidence interval (CI), 1.017–3.163; P = 0.044]. In UBUC cohorts (Table 4), T stage, lymph nodes metastases, high tumor grade, vascular invasion, perineural invasion, high mitotic rate, PDK1, and PDK3 overexpression were significantly associated with poor prognostic factors for DSS and MFS in univariate analysis. T stage, vascular invasion, perineural invasion, and PDK3 overexpression [hazard ratio (HR), 1.907; 95% confidence interval (CI), 1.088–3.341; P = 0.024] were significantly associated with MFS.

**Survival Analysis in UTUC and UBUC**

In UTUC, a significant correlation between increased PDK3 expression and worse DSS (Figure 3A; P < 0.0001) and MFS
(Figure 3B; P < 0.0001) was found by Kaplan–Meier analysis. High PDK3 expression was similarly linked to worse DSS (Figure 3C; P <0.0001) and MFS (Figure 3D; P < 0.0001) in UBUC cohorts.

DISCUSSION

Cancer is caused by the uncontrolled expansion of aberrant or mutated cells in the body, and cancer cells are recognized by their ability to quickly divide and proliferate. Cancer cells acquire additional nutrients through multiple mechanisms to meet energy demands. The metabolic characteristics of cancer cells are different from those of normal cells due to their metabolic reprogramming (7). This “metabolic reprogramming” is characterized by their enhanced ability to survive, invade, and metastasize. This concept was originally conceived by Otto Warburg nearly 100 years ago (13). In normal cells, energy production is primarily accomplished via mitochondrial oxidative phosphorylation (OXPHOS) and the production of 36 ATPs. Cancer cells, on the other hand, have been found to preferentially use cytoplasmic aerobic glycolysis, which produces two ATPs even when oxygen is present. The mitochondrial pyruvate dehydrogenase complex (PDC) decarboxylates pyruvate to acetyl-CoA, and acts as a bridge between glycolysis and the TCA cycle. PDK1–4 can catalyze PDC phosphorylation, and afflicted cells prefer glycolysis to the tricarboxylic acid cycle for ATP generation (9). Aerobic glycolysis has been found to cause cancer chemoresistance through a variety of mechanisms, including enhanced ATP production and resistance to mitochondrial depolarization, both of which are required for cell death (14). PDK 1–3 functions as an oncogene by interacting with numerous signaling pathways that contribute in cancer growth. PDK4’s function is multifaceted, since it functions as both an oncogene and a tumor suppressor (15). PDK3 has highest affinity to the PDC and is least researched (16). Higher expression of PDK3 is associated with higher tumor stage in many cancers (17). PDK3 knockdown suppressed proliferation and caused apoptosis in gastric cancer cell lines (18) and the prostate cancer cell line (19). Furthermore, higher PDK3 expression has been associated with chemoresistance in gastric cancer cells (20) and colon cancer cells (17). It also has been associated with poor prognosis in cholangiocarcinoma (21), and AML (22). Studies showed mRNA expression of hypoxia inducible factor-1α (HIF-1α) is increased in UC, and it induced glycolytic genes, including PDK-1 and PDK-3 (17, 23). The HIF-1α genes are involved in many processes such as cellular metabolism, proliferation and survival, angiogenesis, invasion and metastasis of tumor cells (24). It has been known for over a decade that overexpression of HIF-1α is a common phenomenon in solid tumors and is highly associated with cancer metastases, aggressiveness and chemotherapy resistance (25). This may explain our conclusions that PDK3 expression associated with worse DSS and MFS.

It has recently been discovered that bladder cancer progression is linked to alterations in cell glycolytic characteristics (26) and manifests itself in a more aggressive stage (27). Several studies have found that distinct PDK isozymes are selectively upregulated in bladder cancer. In MIBC, PDK1 expression was linked to a high tumor grade and a high Ki67 index in one study. Another study demonstrated PDK4 is substantially activated in high grade bladder cancer cell lines, and inhibition of PDKs combined with cisplatin therapy could further decrease tumor volume in vivo (11). For accuracy, we identified 11 probes covering 5 transcripts (BCKDK, PDK1, PDK2, PDK3, PDK4) associated with the regulation of acetyl-CoA biosynthetic process from pyruvate through transcriptomic profiling. Among these genes, only PDK3 was significantly upregulated in invasive UC compared with non-invasive UC while PDK1 and PDK4 were not. Considering other studies (10)
### TABLE 3 | Univariate log-rank and multivariate analyses for disease-specific and metastasis-free survival in upper urinary tract urothelial carcinoma.

| Parameter | Category | Case No. | Disease-specific Survival | Metastasis-free Survival |
|-----------|----------|----------|---------------------------|--------------------------|
|           |          | No. of event | P-value | R.R. | 95% C.I. | P-value | No. of event | P-value | R.R. | 95% C.I. | P-value |
| Gender    | Male     | 158      | 28     | 0.8286 | –       | –       | –       | 32      | 0.7904 | –       | –       |
|           | Female   | 182      | 33     | –       | –       | –       | –       | 38      | –       | –       | –       |
| Age (years) | < 65   | 138      | 26     | 0.9943 | –       | –       | –       | 30      | 0.8470 | –       | –       |
|           | ≥ 65     | 202      | 35     | –       | –       | –       | –       | 40      | –       | –       | –       |
| Tumor side | Right  | 177      | 34     | 0.7366 | –       | –       | –       | 38      | 0.3074 | –       | –       |
|           | Left     | 154      | 26     | –       | –       | –       | –       | 32      | –       | –       | –       |
|           | Bilateral | 9        | 1      | –       | –       | –       | –       | 0       | –       | –       | –       |
| Tumor location | Renal pelvis | 141    | 24 | 0.0079* | 1 | – | 0.874 | 31 | 0.0669 | – | – | – |
|           | Ureter  | 150      | 22     | 0.928  | 0.498-1.728 | – | – | – | – | – | – |
|           | Renal pelvis & ureter | 49 | 15 | 1.316 | 0.363-4.775 | – | – | – | – | – | – |
| Multifocality | Single | 278      | 48     | 0.0026* | 1 | – | 0.212 | 52 | 0.0127* | 1 | – | 0.002* |
|           | Multifocal | 62 | 18 | 2.165 | 0.644-7.279 | – | – | – | 18 | 2.376 | 1.359-4.155 | – | 0.365 |
| Primary tumor (T) | Ta | 89 | 2 | <0.0001* | 1 | – | 0.151 | 4 | <0.0001* | 1 | – | 0.052 |
|           | T1      | 92       | 9      | 2.755  | 0.580-13.075 | – | – | – | 15 | 2.249 | 0.721-7.013 | – | 0.365 |
|           | T2-T4   | 159      | 50     | 4.274  | 0.923-19.784 | – | – | – | 51 | 2.151 | 0.662-6.989 | – | 0.001* |
| Nodal metastasis | Negative (N0) | 312 | 42 | <0.0001* | 1 | – | <0.001* | 15 | 2.994 | 1.591-5.632 | – | 0.004* |
|           | Positive (N1-N2) | 28 | 19 | 5.124 | 0.274-9.639 | – | – | – | 6 | 3.349 | 0.990-11.325 | – | 0.052 |
| Histological grade | Low grade | 36 | 4 | 0.0215* | 1 | – | 0.053 | 3 | 0.0027* | 1 | – | 0.002* |
|           | High grade | 284 | 57 | 3.073 | 0.986-9.580 | – | – | – | 67 | 3.349 | 0.990-11.325 | – | 0.004* |
| Vascular invasion | Absent | 234 | 24 | <0.0001* | 1 | – | 0.144 | 26 | <0.0001* | 1 | – | 0.003* |
|           | Present | 106 | 37 | 1.580 | 0.855-2.918 | – | – | – | 44 | 2.560 | 1.386-4.729 | – | 0.004* |
| Perineural invasion | Absent | 321 | 50 | <0.0001* | 1 | – | <0.001* | 61 | <0.0001* | 1 | – | 0.004* |
|           | Present | 19 | 11 | 4.059 | 1.898-8.679 | – | – | – | 9 | 3.089 | 1.437-6.369 | – | 0.004* |
| Mitotic rate (per 10 high power fields) | < 10 | 173 | 27 | 0.167 | – | – | – | 30 | 0.0823 | – | – | – |
|           | ≥ 10    | 167      | 34     | –       | –       | –       | –       | 40      | –       | –       | –       |
| PDK1 expression | Low | 170 | 19 | 0.0011* | 1 | – | 0.441 | 26 | 0.016* | 1 | – | 0.587 |
|           | High    | 170      | 42     | 1.246  | 0.712-2.179 | – | – | – | 44 | 1.159 | 0.689-1.950 | – | 0.044* |
| PDK3 expression | Low | 170 | 16 | <0.0001* | 1 | – | 0.030* | 18 | <0.0001* | 1 | – | 0.044* |
|           | High    | 170      | 45     | 2.561  | 1.070-3.934 | – | – | – | 52 | 1.793 | 1.017-3.163 | – | 0.004* |

*Statistically significant.
**TABLE 4** | Univariate log-rank and multivariate analyses for disease-specific and metastasis-free survival in urinary bladder urothelial carcinoma.

| Parameter | Category | Case No. | Disease-specific Survival | | | | | Metastasis-free Survival | | | |
|-----------|----------|----------|---------------------------|------|------|------|---------------------------|------|------|------|------|
|           |          |          | Univariate analysis | Multivariate analysis | | | | Univariate analysis | Multivariate analysis | | | |
|           | No. of event | P-value | R.R. | 95% C.I. | P-value | No. of event | P-value | R.R. | 95% C.I. | P-value |
| Gender    | Male     | 216      | 41  | 0.4446 | - | - | - | 60    | 0.2720 | - | - | - |
|           | Female   | 79       | 11  | - | - | - | 16 | - | - | - | - |
| Age (years)  | < 65     | 121      | 17  | 0.1136 | - | - | - | 31    | 0.6875 | - | - | - |
|           | ≥ 65     | 174      | 35  | - | - | - | 45 | - | - | - | - |
| Primary tumor (T) | Ta   | 84       | 1   | <0.0001* | 1 | - | <0.001* | 4 | <0.0001* | 1 | - | 0.007* |
|           | T1       | 88       | 9   | 4.856  | 0.488-48.362 | 23 | 4.705 | 1.304-16.983 | 49 | 7.487 | 2.059-27.080 |
|           | T2-T4    | 123      | 42  | 21.590 | 2.244-207.718 | 61 | <0.0001* | 1 | - | 0.070 |
| Nodal metastasis | Negative (N0) | 266 | 41  | 0.0002* | 1 | - | 0.773 | 15 | 1.785 | 0.953-3.343 | 5 | 0.0007* | 1 | - | 0.640 |
| Histological grade | Low grade | 29       | 11  | 1.111  | 0.542-2.279 | 5 | 1.785 | 0.953-3.343 | 71 | 0.765 | 0.249-2.348 |
|           | High grade | 239  | 50  | 0.793  | 0.155-4.055 | 49 | 5.401 | 1.600-16.620 | 22 | 0.906 | 0.490-1.678 |
| Vascular invasion | Absent | 246     | 37  | 0.0024* | 1 | - | 0.056 | 49 | 0.0001* | 1 | - | 0.754 |
| Perineural invasion | Present | 20       | 8   | 2.325  | 1.010-5.353 | 66 | 0.0007* | 1 | - | 0.198 |
| Mitotic rate (per 10 high power fields) | < 10     | 139      | 12  | <0.0001* | 1 | - | 0.047* | 23 | <0.0001* | 1 | - | 0.058 |
|           | >= 10    | 156      | 40  | 1.992  | 1.008-3.937 | 53 | 1.653 | 0.983-2.780 | 25 | 0.0044 | 1 | - | 0.879 |
| PDK1 expression | Low     | 147      | 13  | 0.0009 | 1 | - | 0.232 | 48 | 1.494 | 0.774-2.884 | 52 | 1.040 | 0.627-1.725 |
|           | High     | 148      | 39  | 1.494  | 0.774-2.884 | 25 | 1.494 | 0.774-2.884 | 52 | 1.040 | 0.627-1.725 |
| PDK3 expression | Low     | 147      | 13  | 0.0009 | 1 | - | 0.009 | 19 | <0.0001* | 1 | - | 0.024* |
|           | High     | 148      | 39  | 1.494  | 0.774-2.884 | 19 | <0.0001* | 1 | - | 0.024* |

*Statistically significant.
have shown PDK1 expression as an independent prognostic factor, we also performed a survival analysis for PDK1. Kaplan–Meier analysis indicated PDK1 overexpression was a poor prognostic indicator for DSS and MSS in UTUC (Supplementary Figures 3A, B) and UBUC, (Supplementary Figures 3C, D) respectively. However, it was not significant after multivariate analysis.

For patients with metastatic UC, platinum-based combination chemotherapy is the cornerstone of systemic therapy (28–31). Although response rate can be as high as 50%, it is not durable, as most patients eventually experience disease progression. Since May 2016, five programmed cell death-1 (PD-1) pathway inhibitors have been licensed for usage after platinum-based chemotherapy has failed (32–37). Immunotherapy plays an important role in UC treatment since 2016. Recent work has revealed that immune cells compete for nutrients in the microenvironment with cancer cells and other growing cells (38). Even though many tumor antigens for T cells to identify, the glycolytic activities of cancer cells have been shown to limit glucose intake by Tumor-infiltrating lymphocytes (TIL) T cells, resulting in T-cell depletion and immunological evasion, according to several recent investigations (39, 40). Moreover, glycolytic metabolites, such as high lactate level, low pH and hypoxia are likewise prevalent in the tumor microenvironment and can have a deleterious effect on immune function (41). Emerging evidence indicates glycolysis promotes the PD-L1 expression in tumors, and leads to T-cell inactivation (42). Thus, enhancing tumor immunogenicity by modifying these metabolic pathways could be promising to offset the negative consequences of energy competition between the tumor and the immune cells (43). Combining PDK pathway inhibitors and PD-1 pathway inhibitors may be a promising strategy and worth further

**FIGURE 3** | In patients with upper tract urothelial carcinoma (A, B, respectively) and urinary bladder urothelial carcinoma (C, D, respectively), Kaplan–Meier plots reveal PDK3 overexpression had a significant predictive influence on disease-specific survival and metastasis-free survival.
investigation. However, because of the various immune cell subtypes and metabolic reprogramming, simultaneously inhibiting tumor cells and activating T-cell function is challenging (44).

Intricate mechanisms are involved in maintaining DNA replication, and dysregulation may cause genome instability and tumor progression. Interestingly, we found many genes in this process co-upregulated with PDK3 (Supplementary Figures 1 and 2). When cells enter the G1 phase, a pre-replicative complex (GO: 1902299, fold enrichment: 49.75) is formed at the origin of replication. Upon entering the S phase, the MCM complex (GO: 0042555, fold enrichment: 44.22), GINS complex, and cell division cycle 45 (CDC45) are assembled to form the CMG (CDC45-MCM-GINS) complex (GO: 0071162, fold enrichment: 49.75), which is the active form of the replicative helicase (45). After the helicase unwinds the DNA duplex, the primase (GO: 0005658, fold enrichment: 59.7) generates short RNA primers for DNA synthesis. The ring-shaped sliding clamp, called proliferating cell nuclear antigen (PCNA), is then loaded onto the primer-template junction by the clamp loader/unloader RFC complex (GO: 0031391, fold enrichment: 66.33) (46). Once loaded, the DNA polymerases (GO: 0042575, fold enrichment: 31.42) work in pairs (Pol δ and Pol ε) to synthesize DNA with high fidelity. Additionally, during DNA replication, the deposition of newly synthesized histones is mediated by histone chaperones such as CAF-1 (GO: 0033186, fold enrichment: 66.33), which is known as a replication-dependent nucleosome assembly (GO: 0006335, fold enrichment: 39.8) (47). Aberrant DNA replication is leveraged by tumor cells to sustain proliferation. Recently, it has been suggested that DNA replication pathways are enriched in patients with UTUC derived xenografts. It is more prominent with invasive tumors, suggesting that the rate of cell proliferation is critical for UTUC progression (48). However, more research is needed to fully understand the molecular pathways driving PDK3’s proliferative action in UC.

In response to cellular requirements or exogenous stimulation, such as radiation or cytotoxic agents, damaged DNA undergoes repair to maintain normal function and survival. Inhibitors of DNA repair pathways have been shown to be efficacious when combined with DNA-damaging anticancer drugs (49). In addition, numerous DNA replication mediators have been reported to also play a crucial role in DNA repair pathways (50). Impressively, we found that several PDK3 co-upregulated genes that were involved in DNA replication and DNA repair (Supplementary Figure 1). The DNA repair pathways included double-strand break repair (GO: 0000727, fold enrichment: 33.16), nucleotide excision repair (GO: 0006297, fold enrichment: 25.95), and base excision repair (GO: 0006287, fold enrichment: 17.06).

The genes with functions of both DNA replication and repair are favorable for the cells in order to coordinate these two vital processes promptly and accurately. Targeting these genes may produce a more potent cytotoxic effect for UC therapy. Taken together, the role of PDK3 and glycolysis with DNA replication and repair and the overall contribution to cell survival in UC deserves further investigation.

In conclusion, we found that elevated PDK3 expression was linked to negative pathologic characteristics and poor oncological outcomes, suggesting that it could be used as a prognostic biomarker for UC. PDK3 mRNA levels and its co-upregulated genes are strongly correlated with DNA replication and repair, suggesting that PDK3 may play a key role in tumor proliferation and development. To our knowledge, this is the first study to demonstrate a role of PDK3 in UC. Clarification regarding the detailed mechanism and how to incorporate findings into daily practice needs further investigation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board (IRB10501-005) of Chi Mei Medical Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Y-HK, C-HH, and C-FL: study concept and design. T-CC and C-FL: acquisition of data. C-HH, H-YL, and C-FL: analysis and interpretation of data. Y-HK and C-FL: drafting of the manuscript. T-CC, H-YL, and C-FL: statistical analysis. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.749142/full#supplementary-material

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