

RESEARCH ARTICLE

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Understanding the Role of Pyruvate Dehydrogenase Kinase 3 in Gynecological Cancers

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Abstract

Objective: To investigate the role of pyruvate dehydrogenase kinase 3 (*PDK3*) in gynecological cancers through in silico analyses and in vitro experimental validation, and to assess its potential prognostic and therapeutic significance. **Methods:** • Analyzed gynecological cancer datasets from The Cancer Genome Atlas (TCGA) and Cancer Cell Line Encyclopedia (CCLE) databases to evaluate *PDK3* expression. • Performed KEGG pathway enrichment analysis to identify biological pathways associated with *PDK3*. • Kaplan–Meier survival analysis revealed cancer-type-specific associations between *PDK3* expression and overall survival in breast, cervical, and endometrial cancer. • Assessed the impact of *PDK3* expression on immune cell proportion and distribution in breast, cervical, and endometrial cancers. • Performed *PDK3* knockdown experiments in SiHa cells to evaluate its effects on cell proliferation and growth. **Results:** • *PDK3* was significantly upregulated in breast, cervical, and endometrial cancer samples compared with corresponding normal tissues. • KEGG pathway analysis showed significant enrichment of pathways related to cancer, viral carcinogenesis, and human papillomavirus (HPV) infection. • Elevated *PDK3* expression was associated with poorer overall survival in breast and cervical cancer patients. • *PDK3* upregulation influenced the proportion and distribution of immune cells in breast, cervical, and endometrial cancers. • Experimental knockdown of *PDK3* significantly inhibited the proliferation and growth of SiHa cells. **Conclusion:** *PDK3* appears to function as a potential oncogene in gynecological cancers. Its overexpression is associated with tumor progression, altered immune cell infiltration, and reduced survival in certain gynecological cancers. Therefore, *PDK3* may serve as a promising prognostic biomarker and therapeutic target in gynecological malignancies.

Keywords: *PDK3*, gynecological cancer, metabolic reprogramming, oncogene, proliferation, growth

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Introduction

The incidence of gynecological cancer (GC), a cancer affecting multiple organs of the female reproductive system, has substantially increased, contributing to 15.25% of all cancer cases reported and 15.77% of cancer-related deaths among women worldwide [1]. The global burden of gynecological cancer is predicted to increase by 49.61%, and the associated mortality is proposed to increase to 77.23% by the year 2050 [2]. Among all gynecological cancers, breast cancer continues to be the primary cause of cancer-associated death, followed by cervical cancer, ovarian cancer and endometrial cancer [1]. Metabolic reprogramming is an important hallmark of cancer, supporting the energy demand of rapidly proliferating and growing cancer cells. One such metabolic rewiring is the sustenance of a higher rate of glycolysis despite having sufficient oxygen, termed the Warburg

effect [3]. Cancer cells have been shown to display rewired metabolic pathways, perhaps shifting away from oxidative phosphorylation and toward glycolysis.

The pyruvate dehydrogenase kinase (PDK) family of proteins regulates the pyruvate dehydrogenase complex (PDHC), a critical regulatory enzyme that links glycolysis with the citric acid cycle. By controlling this complex, *PDK* plays a pivotal role in shifting cellular respiration toward the Warburg effect [4]. The PDK family consists of four isoforms, namely, *PDK1*, *PDK2*, *PDK3*, and *PDK4*, which share 70% homology and exhibit differential tissue expression, all of which are present in the mitochondrial matrix [5]. PDK inactivates PDHC by phosphorylating serine residues at three different sites (Ser293, Ser300, and Ser232) in its E1 α subunit [6]. The inactivation of PDHC results in altered metabolic pathways, which may shift away from oxidative phosphorylation and toward glycolysis, supporting the energy demand of rapidly

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proliferating and growing cancer cells [7].

The activity of PDK is proportional to its binding efficiency to the lipoyl domain of the E2 subunit in PDH. Among all the isoforms, pyruvate dehydrogenase kinase 3 (*PDK3*) has the highest affinity and is not affected by the presence of the substrate pyruvate [8]. Recent findings indicate a multifaceted role of *PDK3* in cancer progression [6]. *PDK3* is upregulated in cancers such as colon [9], acute myeloid leukemia [4], gastric [10] and urothelial carcinomas [11].

PDK3 promotes tumorigenesis by stimulating cell proliferation, angiogenesis, invasion, and glycolysis in multiple cancer types [12]. *PDK3* is overexpressed in colon cancer cells, where it impairs CD8⁺ T-cell-mediated cytotoxicity by upregulating programmed death-ligand 1 (PD-L1). Simultaneous inhibition of *PDK3* and PD-L1 significantly improved survival in murine models [13]. *PDK3* inhibits the T-cell-mediated immune response and suppresses the expression of PD-L1 by inhibiting the *Jak1/STAT1* pathway, thereby promoting cancer [13]. Induction of *PDK3* by hypoxia-inducible factor-1 promotes metabolic switch and drug resistance [8] and contributes to treatment failure in patients with high *PDK3* expression [9]. Similar findings have been reported. In glioblastoma multiforme, hypoxia enhances the expression of *HIF-1 α* and glycolysis-associated genes, including *PDK1* and *PDK3*. Inhibition of *HIF-1 α* with chrysin suppresses *PDK1* and *PDK3* expression and promotes glioblastoma cell death [14]. In addition, suppression of mitochondrial function via the Nucleus accumbens-1 (*NAC1*)/*HIF-1 α* /*PDK3* axis has implications in cancer, and silencing *NAC1* in the presence of the pro-oxidative agent elesclomol has antitumor efficacy [15]. *PDK3* overexpression reportedly contributes to chemoresistance and cancer recurrence through the combined action of *HIF- α* and heat shock factor 1 (HSF1) [16]. *PDK3* also regulates the PI3K-Akt pathway to promote tumorigenic characteristics [13]. Thus, *PDK3* could serve as a potential target in cancer due to its multifaceted tumor-promoting functions.

Nevertheless, the significance of *PDK3* in gynecological cancers remains poorly understood. To address this gap, we carried out a comprehensive analysis to understand the role of *PDK3* in gynecological cancers. We systematically studied the expression, regulation, clinical utility, interaction networks, biological functions, immune infiltration, and chemical activity of *PDK3* to target *PDK3* in gynecological cancers. Finally, we performed shRNA-mediated *PDK3* knockdown studies to assess the role of *PDK3* in cervical cancer.

Materials and Methods

Data acquisition and processing

PDK3 expression data were sourced from the Genotype-Tissue Expression (GTEx) and The Cancer Genome Atlas (TCGA) databases [17]. The GTEx database offers a detailed catalog of gene expression levels among various tissue types, facilitating the comparison of *PDK3* expression status across different tissue types. Box plots showing the differential expression of *PDK3* between normal and tumor samples were generated via

the TIMER2.0 database under the 'Gene DE' module [18]. The genomic, transcriptomic, and clinical data from TCGA were retrieved via UCSC Xena and GEPIA [19]. *PDK3* expression between tumors and normal tissues was tested for significance via the Wilcoxon rank-sum test. Statistical significance was set at a p value of <0.05. The SRPlot tool was used to represent the obtained result in box plot form [20]. Furthermore, *PDK3* expression data were cross-validated with datasets from the Cancer Cell Line Encyclopedia (CCLE) [21].

Exploration of genetic and epigenetic alterations

The IntOGen [22] database was analyzed to retrieve mutations connected with *PDK3* in the pancancer samples. The TCGA database was used to visualize mutations in different cancer types. The TIMER 2.0 platform was used to visualize the frequency and types of mutations. Additionally, copy number variations (CNVs), amplifications, and deletions linked to *PDK3* were examined via the GSCA database [23]. We next analyzed the association between DNA methylation and *PDK3* expression. *PDK3* promoter DNA methylation in normal and tumor samples was tested via the UALCAN database [24]. We specifically selected CpG sites located within 1000 base pairs upstream of the *PDK3* promoter. The correlation between methylation and expression was tested via Spearman correlation analysis [25].

To explore the posttranscriptional regulation of *PDK3*, we performed an extensive search of miRNAs that target *PDK3* via three miRNA target scanning tools, namely, miRNet [26], miRDB [27], and TargetScan [28]. The common miRNAs found in these three databases were identified via the InteractiVenn tool [29].

Coexpression and protein-protein interaction network construction

We used the GeneMANIA [30] and BioGRID databases [31] to construct the *PDK3* and its coexpressed gene network. The STRING web server was used to construct the protein-protein interaction network among coexpressed genes [32]. Functional linkages and interactions involving *PDK3* were interpreted by combining the interaction data with correlation coefficients from the TIMER2.0 database, which focused on gynecological cancer types. The Cancerhallmarks [33] tool was used to visualize the enriched cancer hallmarks related to *PDK3* coexpressed genes. This method allows for a thorough examination of *PDK3*'s regulatory network and possibly contributes to the development of cancer.

Transcriptional regulator identification and functional enrichment analysis

TFlink [34] was used to identify the transcription factors regulating *PDK3*. By combining information from chromatin immunoprecipitation (ChIP) investigations with computational predictions, TFlink offers information on putative transcription factors that may control genes of interest. The biological pathways linked to these regulators and the enrichment of gene ontology (GO) terms were evaluated via ShinyGO [35].

Clinical implications and survival outcomes

The correlation between *PDK3* expression and cancer progression stage was determined via the GDC-TCGA datasets accessible via GEPIA [36]. The prognostic significance of *PDK3* expression was evaluated via Kaplan–Meier plotter for survival analysis [37].

Immune infiltration and drug interaction analysis

The TISIDB tumor-immune system interaction database was used to analyze immune cell infiltration patterns and *PDK3* expression [38]. The relationships between *PDK3* expression and 24 distinct TIL subsets were examined via the TIMER 2.0 program. SRplot was used to create heatmaps from the data. Finally, the GSCA database was used to examine the relationship between *PDK3* expression and drug response [23]. Data on drug–gene correlations from the Cancer Therapeutics Response Portal (CTRP) and Genomics of Drug Sensitivity in Cancer (GDSC) databases are integrated by the GSCA. SRplot was used to create a bubble plot that represented the data. The various databases and their links used in the present study are listed (Supplementary Table 3).

Cell culture and transfection

SiHa cells were cultured and maintained according to ATCC guidelines. To generate the *PDK3* knockdown cell line, SiHa cells (2×10^6) were seeded in a 6-well plate and incubated at 37°C in a 5% CO₂ environment. After 24 hours, the cells were transfected with a shRNA against *PDK3* (sc-39029-SH; Santa Cruz). Control plasmid (sc-108060; Santa Cruz)-transfected cells served as controls. We used Lipofectamine 3000 (Thermo Fisher Scientific, USA) to carry out the transfections according to the manufacturer's protocol. *PDK3*-knockdown clones were isolated by culturing the cells in 2.0 µg/ml puromycin for 14 days [39].

Confirmation of PDK3 knockdown

Total RNA was isolated via the use of TRIzol reagent (Thermo Fisher Scientific, USA). *PDK3* Hs00178440_m1; Invitrogen) knockdown was confirmed via TaqMan-based qRT–PCR via the use of 25 ng of RNA, with β -actin as an endogenous control. Gene expression was calculated via the $\Delta\Delta Ct$ method, where $\Delta Ct = Ct$ (target gene) - Ct (reference gene) and $\Delta\Delta Ct = \Delta Ct$ test - ΔCt control. The fold change (FC) was calculated as $FC = 2^{(-\Delta\Delta Ct)}$ [40].

After being washed in ice-cold PBS, *PDK3* knockdown and vector-transfected cells were lysed in RIPA buffer containing a protease inhibitor. Using SDS–PAGE, approximately 40 µg of protein was separated and then blotted onto a nitrocellulose membrane. The membranes were incubated overnight at 4°C in the presence of primary antibodies against *PDK3* (1:2000 dilution, ABclonal, USA) and anti-ACTB (1:5000 dilution, ABclonal, USA). The membranes were then incubated for two hours at room temperature with an HRP-conjugated secondary antibody after being washed with TBST. To visualize the *PDK3* protein, Clarity Western ECL Substrate (Bio-Rad, USA) was utilized. An iBright imaging system (Invitrogen, USA) was used to capture images [39].

Proliferation assay

Trypsinization and trypan blue staining were performed on the cells every 24 hours, and a Neubauer chamber was used to count living cells under a microscope. An Omni calculator was used to determine the doubling times of the control and knockdown cells [40].

Colony formation assay

Approximately 500 cells were cultivated in a 6 cm cell culture plate for two weeks in full media. Following the disposal of the culture media and washing with PBS, the colonies were stained for ten to fifteen minutes with 0.5% crystal violet in methanol staining solution. After the extra stain was removed and the samples were washed with PBS, the number of stained colonies was calculated [41].

Results

PDK3 is overexpressed in gynecological cancers

We first examined the expression of *PDK3* in several tissue types via the GTEx database. *PDK3* was expressed at varying levels in various human tissues (Figure 1A). Pancancer analysis of *PDK3* expression via the TIMER 2.0 tool revealed that *PDK3* was upregulated in tumor samples compared with normal samples in the TCGA cohort (Figure 1B). We found that *PDK3* mRNA levels were significantly elevated in uterine corpus endometrial carcinoma (TCGA-UCEC, $n = 545$), cervical squamous cell carcinoma (TCGA-CESC, $n = 304$), and invasive breast carcinoma (TCGA-BRCA, $n = 1093$) (Figure 1C). Interestingly, *PDK3* expression did not significantly change in ovarian cancer (Figure 1D). The native expression of *PDK3* was evaluated using semiquantitative PCR in different cervical cancer (SiHa, HeLa, Caski, C33A) and breast cancer (MCF-7, MDA-MB-468, MDA-MB-231) cell lines along with immortalized human keratinocyte cells (HaCaT). Among the gynecological cancer models examined, SiHa and MCF-7 cells exhibited one of the highest levels of *PDK3* expression (Figure 1E). High expression of *PDK3* was observed in different gynecological cell lines (Supplementary Figure 1).

Regulation of PDK3 across gynecological cancers

Mutation analysis via IntOGen tool identified 129 mutations in *PDK3*, with missense mutations being the most frequent (60%), followed by synonymous (29%), truncating (8%), and splice-site (3%) mutations. Several hotspot mutations were observed, particularly within the functional domains of *PDK3*, suggesting that these mutations may affect *PDK3* function (Figure 2A). Analysis of TCGA datasets revealed that *PDK3* mutations were absent in breast cancer, occurred in 0.68% of cervical cancer cases, and were more common in endometrial cancer cases (6.7%) (Figure 2B). Overall, the low mutation frequency and upregulation of *PDK3* suggest a potential role for DNA methylation or miRNAs in regulating *PDK3* expression (Figure 2C).

To investigate mechanisms other than mutations in regulating *PDK3* expression, we analyzed copy number variations (CNVs) in endometrial, cervical, and breast cancers. A considerable proportion of patients

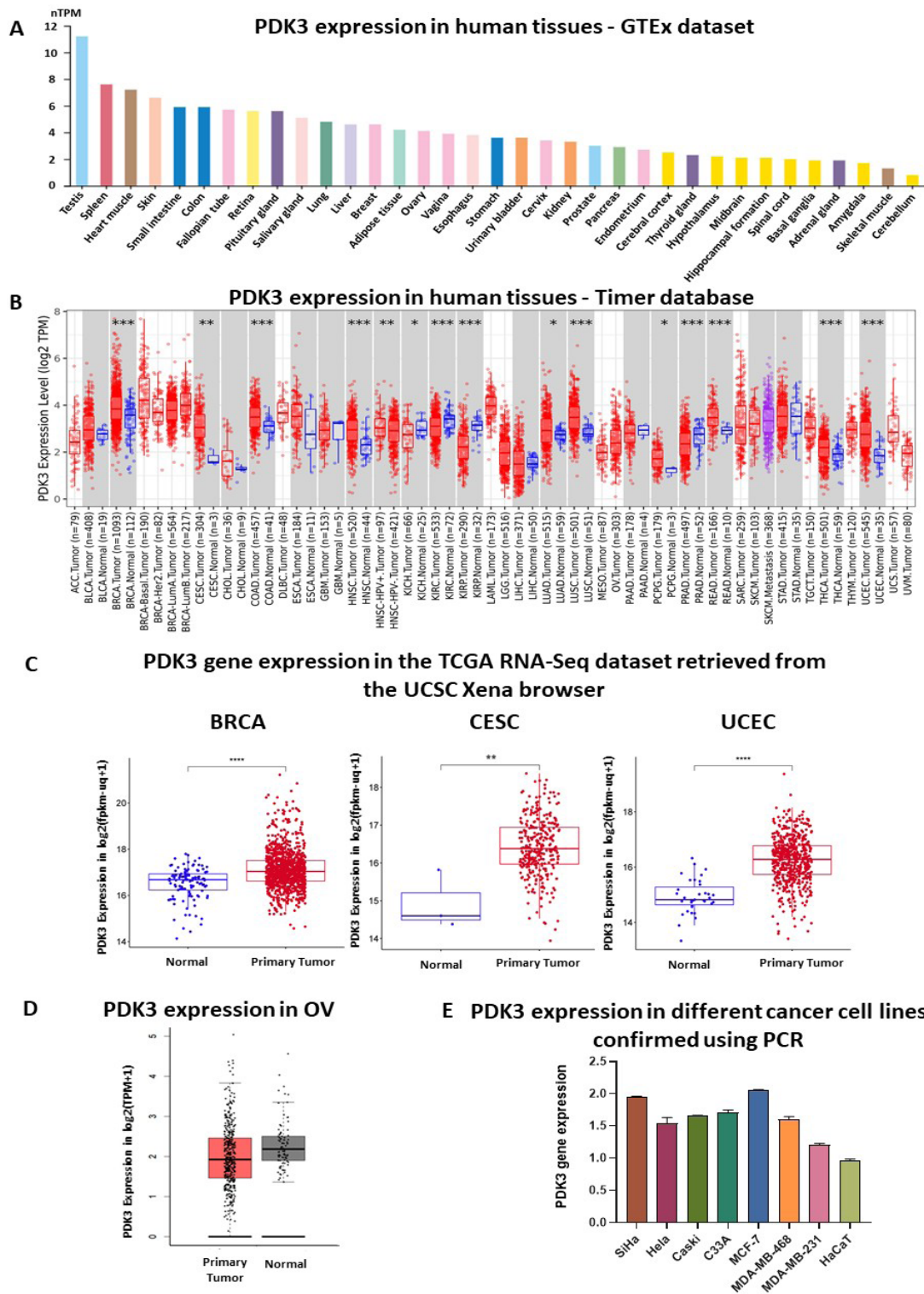


Figure 1. Gene Expression Analysis of *PDK3*. A *PDK3* expression across different human tissues retrieved from the GTEx dataset of the HPA database. B *PDK3* expression across different human tissues retrieved from the TIMER 2.0 database. C *PDK3* gene expression across different gynecological cancers retrieved from the UCSC Xena browser: Increased expression of *PDK3* in BRCA, CESC and UCEC was observed in tumors compared with normal tissues. D Cross-validation of *PDK3* expression in OV via the GEPIA database. E Semiquantitative PCR analysis of native *PDK3* expression in cervical, breast, and human keratinocyte cell lines.

presented copy number gains at the *PDK3* locus, with amplifications observed in approximately 17% of breast cancer patients, 16% of cervical cancer patients, and 14% of endometrial cancer patients (Figure 2D) (Table 1). We observed a positive correlation between CNVs with *PDK3* expression, especially in endometrial cancer (Spearman

R coefficient 0.19, p value < 0.05) and invasive breast carcinoma (Spearman R coefficient 0.09, p value < 0.001) (Figure 2E). The Spearman R coefficient of cervical cancer was 0.02, suggesting that the upregulation of *PDK3* in cervical cancer may be independent of CNV. These data indicate that CNVs may significantly contribute to the

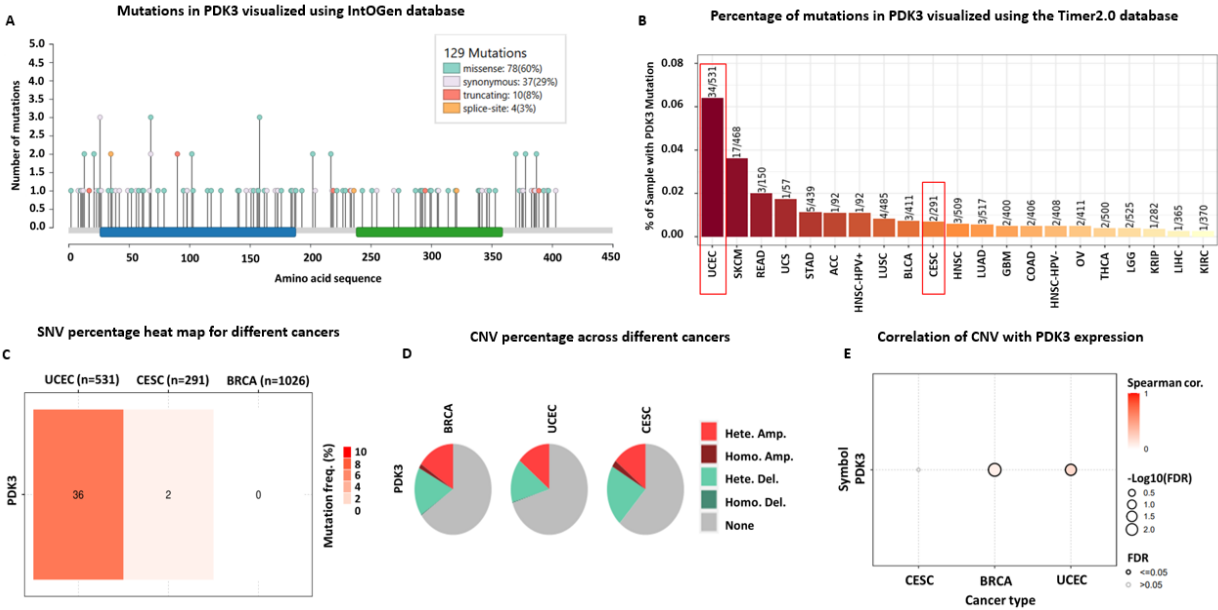


Figure 2. Types of Genetic Variation Observed at the *PDK3* locus: A Overall mutations in *PDK3* visualized via the IntOGen database. B Genetic variation analysis of the *PDK3* gene with the percentage of *PDK3* mutations visualized via the TImEr2.0 database. C & D Percentage and types of SNVs and CNVs observed at the *PDK3* locus in gynecological cancer types visualized via the GSCA database. E Correlations of CNV with *PDK3* expression in different cancers visualized through the GSCA database.

overexpression of *PDK3* in breast cancer.

We next evaluated epigenetic regulation by analyzing DNA methylation patterns at the *PDK3* promoter in several gynecological malignancies (Supplementary Figure 2A). Our analysis revealed significant methylation at most of the CpG sites (p value < 0.05) in breast cancer and uterine cancer (Supplementary Figure 2B) (Supplementary Table 1). Promoter methylation analysis indicated the methylation level below the hypermethylation threshold (a beta value cutoff of 0.7-0.5 indicated hypermethylation, and 0.3-0.25 indicated hypomethylation), suggesting that additional epigenetic elements, such as miRNAs, are involved in regulating gene expression (Supplementary Figure 2C). Correlation analysis revealed that *PDK3* expression and promoter methylation showed strong negative correlation in the TCGA-BRCA cohort and TCGA-CESC cohort (Supplementary Figure 2D).

Next, we investigated microRNAs (miRNAs) as potential regulators of *PDK3* expression. We previously reported that *PDK3* is a direct target of the chromosome 14 miRNA cluster [42]. Using the miRDB, miRNet, and TargetScan databases, we identified 11 miRNAs predicted to target *PDK3* (Figure 3A), with varying confidence scores (70–100, indicating high confidence) (Figure 3B). Among these, hsa-miR-525 and hsa-miR-369 were downregulated in all three cancers. In addition, hsa-miR-186 and hsa-miR-494 were downregulated specifically in TCGA-BRCA and TCGA-CESC, respectively, whereas hsa-miR-205 and hsa-miR-194 were downregulated only in breast and cervical cancers,

Table 1. The GSCA Database Showing Various Percentages and Types of A) SNVs and B) CNVs at the *PDK3* locus in different gynaecological cancers

A				
Percentages and types of SNVs at the <i>PDK3</i> locus in different gynecological cancers				
Cancer type	Effective mutations	Non-effective mutations	Sample size	Percentage
BRCA	0.0000	3.0000	1026.0000	0.0000
CESC	26.0000	1.0000	291.0000	0.6873
UCEC	36.0000	12.0000	531.0000	6.7797

B						
Percentages and types of CNVs at the <i>PDK3</i> locus in different gynecological cancers						
Cancer type	Total amplification (%)	Total Deletion (%)	Heterozygous amplification (%)	Heterozygous deletion (%)	Homozygous amplification (%)	Homozygous deletion (%)
BRCA	17.22222	17.50000	15.64815	17.12963	1.57407	0.37037
CESC	16.61017	21.69492	14.23729	21.69492	2.37288	0.00000
UCEC	14.10019	15.76994	14.10019	15.39889	0.00000	0.37106

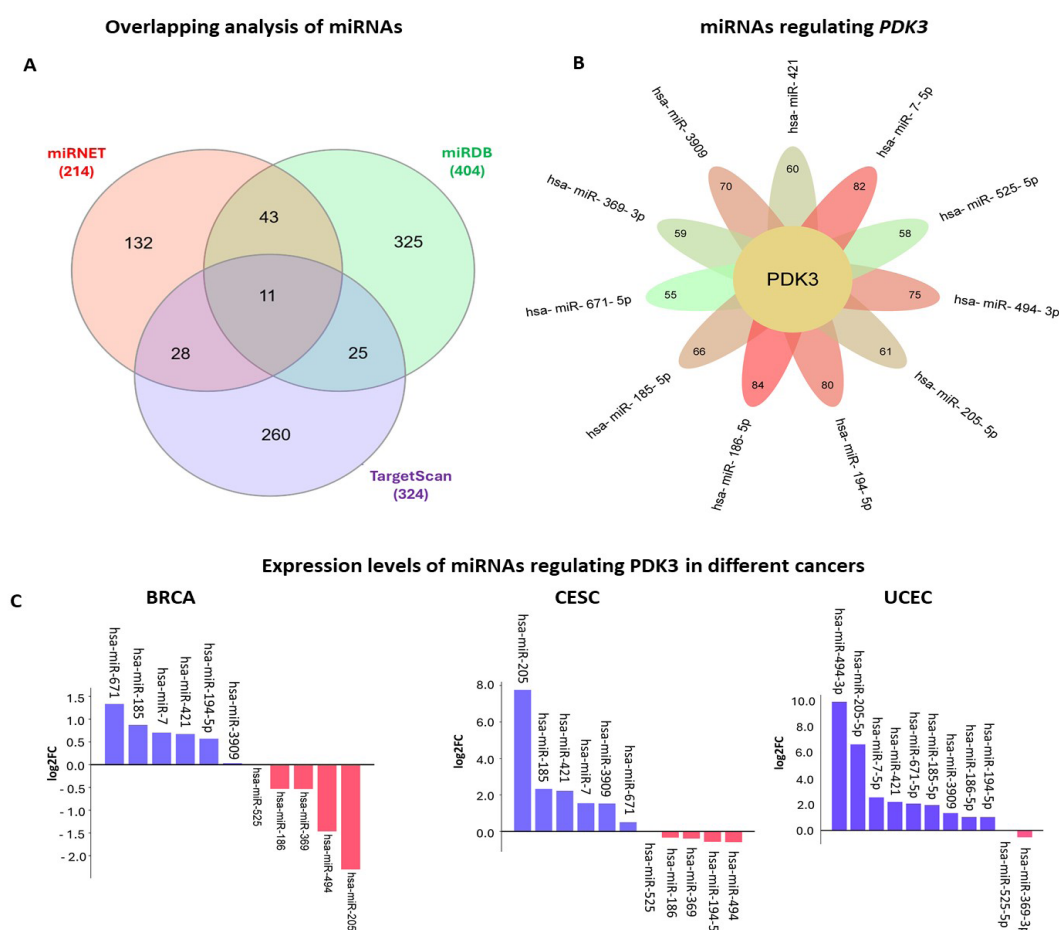


Figure 3. miRNA-Mediated Regulation of *PDK3* Gene Expression. A Overlap analysis of miRNAs targeting *PDK3* was conducted via three different databases, and the results were visualized via the InteractiVenn tool. B 11 miRNAs regulating *PDK3* expression with their miRDB target score, generated via SRplot. C Expression levels of all 11 miRNAs regulating *PDK3* in different cancers.

respectively (Figure 3C). The reduced expression of these miRNAs may weaken their suppressive effect on the *PDK3* gene.

Coexpression and interaction analysis of *PDK3*

We constructed a *PDK3* coexpression network via GeneMANIA (Figure 4A) and BioGRID (Figure 4B) and then generated a protein–protein interaction network (PPIN) via STRING (Figure 4C). The PPI network consisted of 14 nodes and 34 edges and showed significant interaction enrichment, indicating nonrandom protein interactions. Analysis of the overlapping genes revealed that *PDK3* directly interacts with eight proteins (Figure 4D) namely *DLAT*, *DLD*, *PDHA1*, *PDHB*, *PDHX*, *PDK1*, *PDK2*, and *PDK4*. Correlation analysis further supported these interactions, revealing positive correlations between *PDK3* and *DLAT*, *DLD*, *PDHA1*, *PDHB*, *PDHX*, and *PDK1* (Figure 4E). In addition, cancer hallmark enrichment analysis of *PDK3* coexpressed genes revealed a significant role for *PDK3* in metabolic reprogramming and energy metabolism (Figure 4F).

PDK3 modulates critical signaling pathways in cancer progression

In silico analysis revealed *PDK3* as a component

of several oncogenic signaling pathways. Using ChIP-seq data from the TFLink database, we identified 414 transcription factors with predicted binding sites near the *PDK3* gene (Supplementary Table 2). KEGG pathway enrichment analysis revealed that these upstream regulators are associated mainly with pathways related to cancer, cell cycle regulation, Wnt signaling, miRNAs in cancer, breast cancer, and HPV infection (Supplementary Figure 3A). Gene Ontology (GO) analysis revealed significant enrichment in biological processes related to RNA biosynthesis, transcriptional regulation, and macromolecule biosynthesis (Supplementary Figure 3B). Significant enrichment in transcription coregulator activity and different transcription factor binding activities was observed for *PDK3* targets at the molecular function (MF) level. Most of these functions were linked to cellular components that regulate DNA epigenetically (Supplementary Figure 3C & 3D).

Clinical significance of *PDK3* across gynecological cancers

The clinical relevance of aberrant *PDK3* expression in gynecological cancers was assessed via the GEPIA tool. Correlation analysis of *PDK3* expression with overall survival revealed significant prognostic implications in

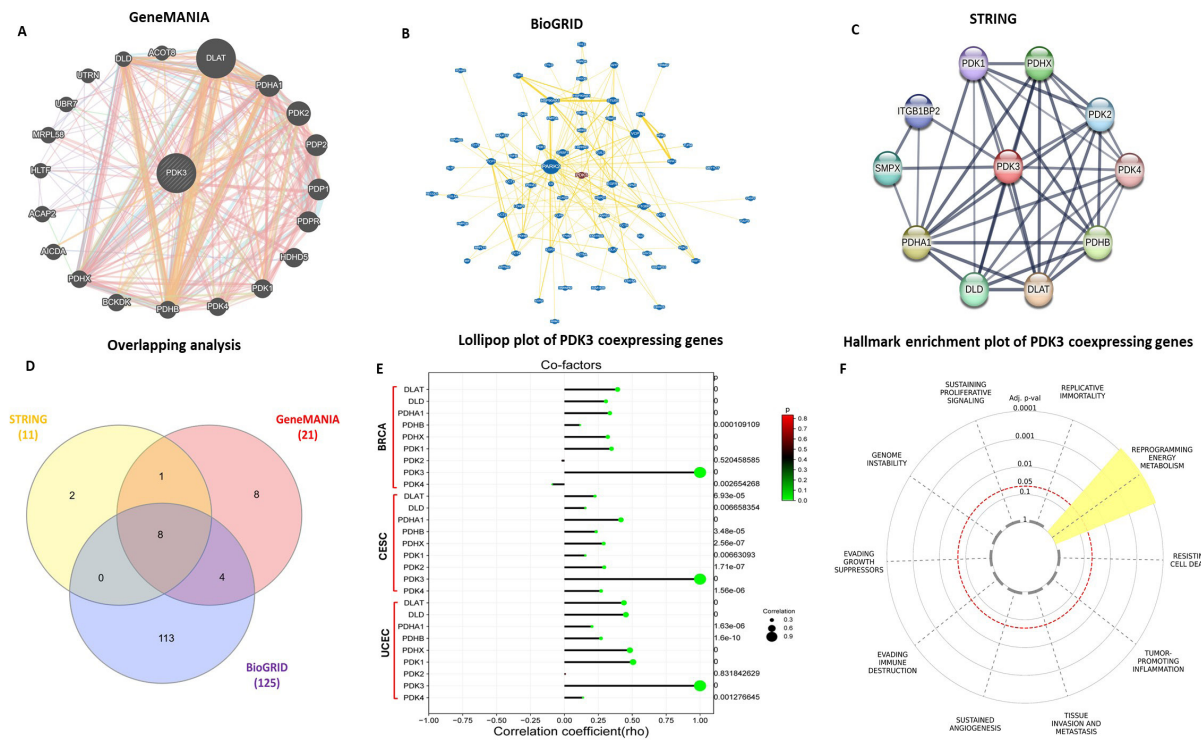


Figure 4. Analysis of Genes Coexpressed with *PDK3*. (A) Coexpression network of *PDK3* generated using GeneMANIA. (B) Coexpression network of *PDK3* generated using BioGRID. (C) Protein-protein interaction network of *PDK3*-associated proteins generated using STRING. (D) Overlap analysis of *PDK3*-associated genes identified using STRING, GeneMANIA, and BioGRID, visualized using the InteractiVenn tool. (E) Lollipop plot showing the correlation between *PDK3* and coexpressed genes across BRCA, CESC, and UCEC. (F) Hallmark enrichment analysis of *PDK3*-coexpressed genes

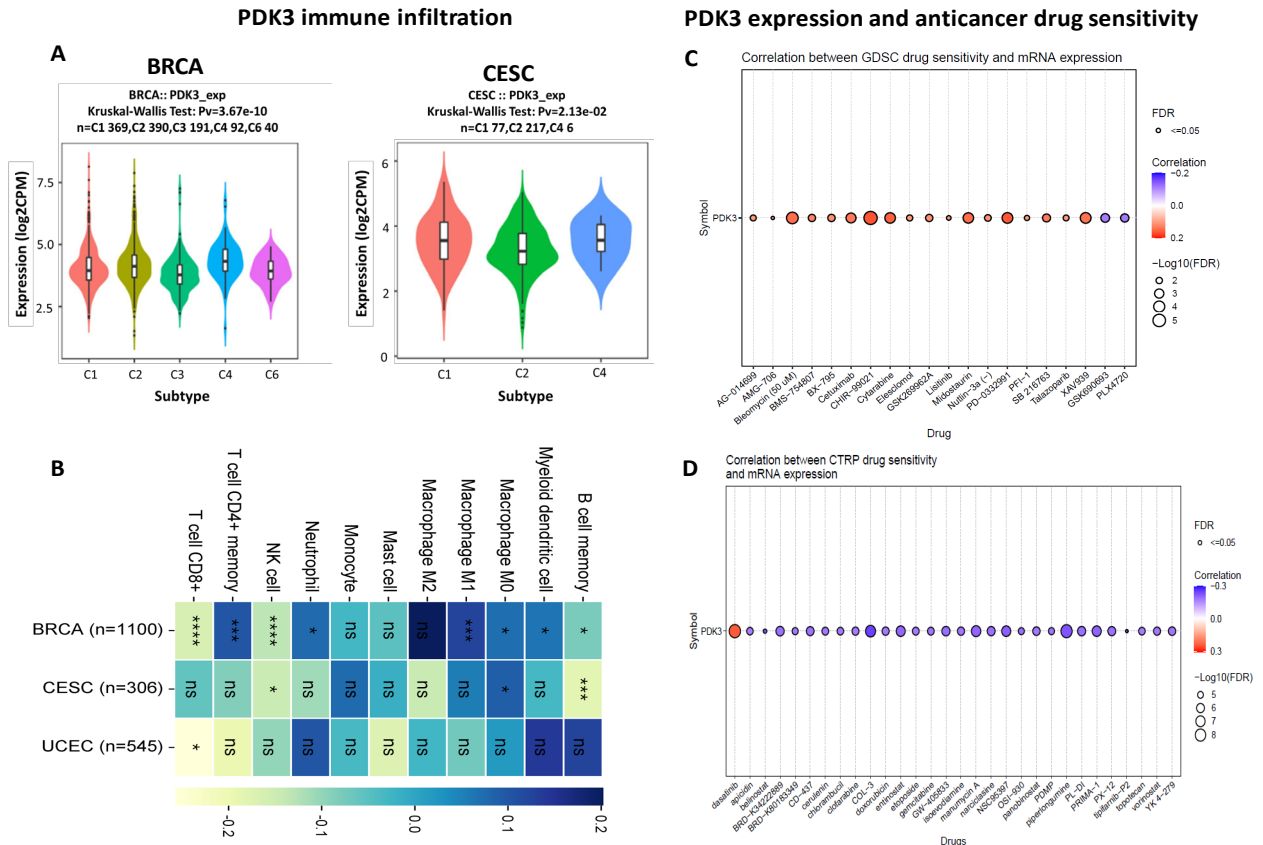


Figure 5. Immune Interactions of *PDK3*. A *PDK3* expression in different immune subtypes visualized in the TISIDB database. B Heatmap showing the correlation between *PDK3* expression and immune cells in different cancer types retrieved from the TIMER 2.0 database. C&D Bubble plot showing the correlation between *PDK3* expression and anticancer drug sensitivity from the GSCA database.

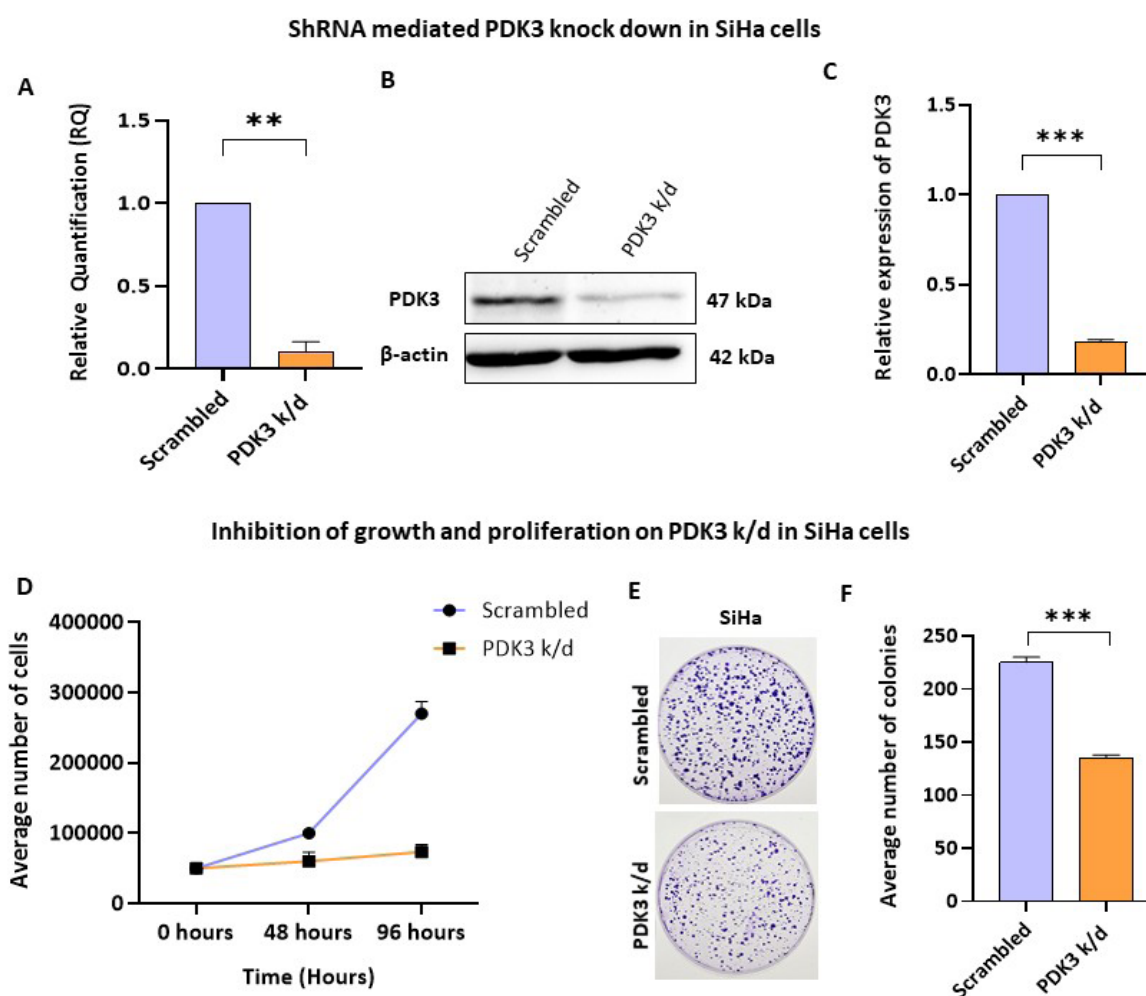


Figure 6. shRNA-mediated *PDK3* knockdown in SiHa cells. A qRT-PCR of colonies from transfected cells in which *PDK3* was knocked down. B Western blot analysis of colonies from transfected *PDK3*-knockdown cells. C Quantitative analysis of the western blot data shown in the panel. D The graph shows the rate of proliferation of scrambled and *PDK3* k/d SiHa cells. E Representative images of the colony formation assay. F The bar graph shows the results of the quantitative analysis of the colony formation assay presented in the panel. The graph represents the mean and SD * $p \leq 0.05$; ** $p \leq 0.01$ indicates statistical significance.

breast and cervical cancer patients. Notably, increased *PDK3* expression in the TCGA-CESC cohort ($n = 304$) and decreased *PDK3* expression in the TCGA-BRCA ($n = 1089$) and TCGA-UCEC ($n = 542$) cohorts correlated with improved survival outcomes ($p < 0.05$) (Supplementary Figure 4A). In contrast, no significant association was observed between *PDK3* expression and tumor stage in breast, cervical, or endometrial cancers (F value < 2.5) (Supplementary Figure 4B).

PDK3 expression can affect the abundance of immune cells

To investigate whether *PDK3* expression is linked to the immune context, we analyzed its expression across immune subtypes via TISIDB. We generated a violin plot to evaluate *PDK3* expression in different immune subtypes, classifying immune subtypes into six classes: wound healing (C1), IFN-gamma dominant (C2), inflammatory (C3), lymphocyte depleted (C4), immunologically quiet (C5), and TGF- β dominant (C6). In the TCGA-BRCA cohort, *PDK3* expression differed

significantly across five immune subtypes (C1, C2, C3, C4, and C6; $n = C1: 369, C2: 390, C3: 191, C4: 92, C6: 40$). In the TCGA-CESC cohort, significant differences were observed across three immune subtypes (C1, C2, and C4; $n = C1: 77, C2: 217, C4: 6$), indicating that *PDK3* expression varies according to the immune microenvironment (Figure 5A). TIMER 2.0 database analysis revealed an inverse relationship between *PDK3* expression and the numbers of NK cells and CD8 $^{+}$ T cells in the TCGA-BRCA cohort. In the TCGA-CESC cohort, unpolarized memory B cells were positively correlated with *PDK3* expression. Additionally, *PDK3* expression is positively correlated with antitumour M1 macrophages and negatively correlated with protumorigenic M2 macrophages. In addition, *PDK3* overexpression was significantly negatively correlated with the expression of memory B cells and NK cells. In the TCGA-UCEC cohort, a significant negative correlation between NK cells and *PDK3* was observed (Figure 5B).

PDK3-drug interactions

Drug sensitivity analysis revealed that increased *PDK3* expression was positively correlated with resistance to most GDSC drugs and one CTRP drug, dasatinib, suggesting that elevated *PDK3* levels may contribute to drug resistance. In contrast, a negative correlation of *PDK3* was observed with most of the CTRP drugs and two of the GDSC drugs, GSK690693 and PLX470, indicating that increased *PDK3* expression may increase sensitivity to these agents (Figure 5C&D).

PDK3 knockdown in SiHa cells

We previously reported that *PDK3* is significantly overexpressed in cervical cancer patient samples [42] qRT-PCR and western blot analysis revealed significant expression of *PDK3* in SiHa cells. Hence, we knocked down *PDK3* in SiHa cells to investigate its biological properties. *PDK3* knockdown in SiHa cells was confirmed by qRT-PCR, which revealed an 85% decrease in the expression of *PDK3* (Figure 6A). The knockdown effect was then confirmed by western blot analysis (Figure 6B & C).

PDK3 knockdown inhibits the growth and proliferation of SiHa cells

PDK3 knockdown in SiHa cells was confirmed by qRT-PCR and western blotting (Figure 6A–6C). The impact of *PDK3* on proliferation and growth was quantified via growth curve analysis and colony formation assays. The growth curve analysis revealed a significant reduction in cell number upon *PDK3* knockdown in SiHa cells. In contrast, the scrambled control-treated cells exhibited significant cell proliferation (Figure 6D). The cell doubling time in scrambled and *PDK3*-knockdown cells was 39.27 hrs. and 173.45 hrs., respectively. We next investigated the impact of *PDK3* knockdown on the growth of SiHa cells via a colony formation assay. Compared with scrambled treatment, *PDK3* knockdown significantly reduced the number of colonies (Figure 6E and F).

Discussion

The involvement of *PDK3* in cancer progression has gained significant attention because of its critical role in regulating cellular metabolism, especially in inhibiting PDHC. In this study, we comprehensively investigated the regulation, functional relevance, and clinical significance of aberrant *PDK3* expression in gynecological cancers via integrative in silico analyses and in vitro validation. Previous studies reported *PDK3* overexpression in multiple malignancies, including gastric [10], colorectal [9], lung [43], cervical [42], breast [44], and the urinary bladder [11] cancers, where it contributes to tumor progression and metabolic adaptation [16]. Despite its aberrant expression in multiple cancers, its role in gynecological cancers has not been well investigated. Our In silico analysis of the TCGA-BRCA, TCGA-UCEC, and TCGA-CESC datasets revealed notable overexpression of *PDK3* in cancer samples compared with normal samples. The Cell Line Encyclopedia (CCLE) also confirmed

relatively high *PDK3* expression in gynecological cancer cell lines, further emphasizing the oncogenic potential of *PDK3*.

Genetic and epigenetic analyses of *PDK3* revealed that somatic *PDK3* mutations were relatively uncommon in all the cancer samples examined. Considerable CNV gains were observed in the TCGA-CESC, TCGA-UCEC, and TCGA-BRCA samples. Spearman correlation analysis revealed a positive correlation of *PDK3* expression only in the TCGA-UCEC cohort and TCGA-BRCA cohort. Kaplan–Meier survival analysis showed that *PDK3* overexpression was associated with better overall survival in TCGA-CESC, whereas lower *PDK3* expression was associated with better overall survival in TCGA-BRCA and TCGA-UCEC. CNV correlation was observed; however, experimental confirmation was not performed in the current study. This finding provides the scope for further investigation of the cause of *PDK3* overexpression in the TCGA-CESC cohort.

Aberrant DNA methylation has been shown to influence gene expression, with hypermethylation contributing to gene silencing and hypomethylation contributing to gene upregulation [40, 45, 46]. Although our DNA methylation studies revealed significantly increased methylation in the normal tissues of all three cancers at specific CpG sites, the findings were inconsistent across all the CpG sites. The beta value cutoff indicated that neither significant hypermethylation nor hypomethylation was present in the tumor samples. However, according to the Spearman correlation analysis, the TCGA-BRCA and TCGA-CESC cohorts presented significant negative correlations with promoter methylation and *PDK3* expression. We previously reported that *PDK3* is an upregulated gene in cervical cancer [42]. More detailed studies are needed to understand the relationship between promoter DNA methylation and *PDK3* expression.

MicroRNAs (miRNAs) represent another critical layer of posttranscriptional gene regulation. Our integrative miRNA network analysis identified several miRNAs predicted to target *PDK3* in gynecological cancers, including hsa-miR-186-5p, hsa-miR-194-5p, hsa-miR-7-5p, and hsa-miR-494-3p. hsa-miR-186-5p downregulates *PDK3* expression in glioma [47], whereas hsa-miR-7-5p has been studied for its role in suppressing *PDK3* expression in TCGA-HNSC [48]. Notably, our earlier work demonstrated that hsa-miR-494-3p directly targets *PDK3* in cervical cancer, leading to metabolic reprogramming and reversal of tumorigenic phenotypes [42]. These findings collectively suggest that cancer type-specific miRNA regulation plays a key role in fine-tuning *PDK3* expression to support distinct metabolic demands.

Coexpression analysis further revealed strong associations between *PDK3* and genes involved in mitochondrial metabolism, including *PDK1*, *PDK2*, *PDK4*, *PDHX*, *PDHB*, *PDHA1*, *DLAT*, and *DLD*. The interaction of *PDK3* with core components of the PDH complex and associated enzymes highlights its central role in regulating metabolic flux between glycolysis and oxidative phosphorylation. Hence, more thorough mechanistic research is needed to understand how *PDK3* and its coexpressed genes contribute to metabolic

reprogramming and the development of cancer. Prognostic markers are clinical indicators used in oncology to determine a patient's risk of disease recurrence following initial therapy. Previous studies have shown that *PDK3* influences patient survival in urothelial carcinoma [11], colon cancer [9], and cholangiocarcinoma [49]. Thus, we explored the prognostic significance of *PDK3* in breast, cervical, and endometrial cancer using the TCGA dataset. In TCGA-UCEC patients, the overexpression of *PDK3* indicated a poor survival outcome. However, *PDK3* overexpression contributed to better overall survival in the TCGA-BRCA and TCGA-CESC datasets.

Immune cells and the tumor microenvironment are decisive in determining tumor progression and metastasis [50]. Tumor-infiltrating immune cells have been reported to influence patient survival. Tumor-associated macrophages and neutrophils exhibit either protumorigenic (M2 and N2) or antitumorigenic (M1 and N1) effects on the basis of their polarization status [51, 52]. Natural killer (NK) cells cause potent cytotoxicity and antitumor activity in cancer cells [53]. Similarly, CD8⁺ and CD4⁺ T cells exhibit increased antitumor activity through granular exocytosis [54]. We analyzed the link between *PDK3* expression and immune cell abundance via TCGA datasets. In the TCGA-BRCA cohort, significantly lower numbers of NK cells and CD8⁺ T cells were observed, which might be responsible for the poor survival outcome. Similarly, in TCGA-UCEC patients, the reduced survival can also be attributed to the significant downregulation of CD8⁺ T cells. The TCGA-CESC dataset revealed significantly increased numbers of unpolarized macrophages and significant downregulation of NK cells. These preliminary observations on tumor infiltration and its involvement in tumor prognosis upon *PDK3* expression need to be explored in detail. Previous studies reported the role of *PDK3* in therapy resistance, such as in colon cancer [9], gastric cancer [10], and melanoma [55]. Many drugs, such as doxorubicin, etoposide, and COL-3, are negatively correlated with *PDK3* expression. More detailed studies are needed to understand the role of *PDK3* in the development of therapeutic resistance to these drugs.

Finally, we investigated the biological functions of *PDK3* in SiHa cells. The knockdown of *PDK3* in SiHa cells inhibited both the proliferation and growth of SiHa cells. Our study revealed that *PDK3* may promote tumor growth by increasing cell proliferation through a decrease in the cell doubling time. Our findings are consistent with previous reports in other cancers, such as urothelial carcinoma [11] and colon cancer [9]. Overall, we report a procarcinogenic function of *PDK3* in gynecological cancer.

In conclusion, our study investigated the role of *PDK3* in gynecological cancers through extensive computational analysis followed by experimental validation. The gene expression data from multiple databases consistently revealed its overexpression, which emphasizes its role as a potential oncogene. Furthermore, genomic and epigenomic changes have been shown to influence *PDK3* expression. *PDK3* expression may influence immune cell abundance and clinical outcomes in breast, uterine, endometrial and cervical cancers. Our study identified

potential drugs that can be used to treat cancers that overexpress *PDK3*. The results of the computational analysis followed by experimental validation suggest an oncogenic role for *PDK3* in cervical cancer. These findings clarify the role of *PDK3* as a promising biomarker in gynecological cancers. In our *In silico* analysis across multiple gynecological cancers, we aimed to perform initial functional validation in one representative model to understand the oncogenic role of *PDK3*. Cervical cancer (SiHa) was selected as a suitable model for *PDK3* knockdown studies, allowing us to assess its effects on proliferation and growth with clear interpretability. However, validation in breast and endometrial cancer cell lines was not performed which limits our broader understanding of *PDK3* in gynaecological cancers. In future studies, functional assays in MDA-MB-231 (breast) and Ishikawa or AN3CA (endometrial) cell lines could be performed to confirm the role of *PDK3* across gynecological cancers.

Author Contribution Statement

Dr. Shama Prasada Kabekkodu: Conceptualization, project administration, resources, supervision, visualization, writing – review and editing. Medha Bairy: Formal analysis, Investigation, Software, Validation, Writing – original draft, Visualization. Aishath Shaheeda: Formal analysis, investigation, software, validation, writing – original draft, and visualization. Padmanaban S. Suresh: Writing – review and editing. Roshan Mascarenhas: Writing – review and editing.

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Ethics approval

The authors declare that they have no ethical statements to disclose since the research does not include any animal or human studies.

Data availability statement

The authors declare that all other data supporting the findings of this study are available within the article and its supplementary information files.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships

that could have appeared to influence the work reported in this paper.

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