Original Article

Phosphoinositide-dependent Kinase-1 (PDPK1) regulates serum/glucocorticoid-regulated Kinase 3 (SGK3) for prostate cancer cell survival

Geetha Nalairndran1 | Azad Hassan Abdul Razack2 | Chun-Wai Mai3,4 | Felicia Fei-Lei Chung5 | Kok-Keong Chan6 | Ling-Wei Hii3,4,7 | Wei-Meng Lim3,4,7 | Ivy Chung1,8 | Chee-Onn Leong3,4

1Department of Pharmacology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia
2Department of Surgery, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia
3Center for Cancer and Stem Cell Research, Institute for Research, Development and Innovation (IRDI), International Medical University, Kuala Lumpur, Malaysia
4School of Pharmacy, International Medical University, Kuala Lumpur, Malaysia
5Mechanisms of Carcinogenesis Section (MCA), Epigenetics Group (EGE), International Agency for Research on Cancer World Health Organization, Lyon, France
6School of Medicine, International Medical University, Kuala Lumpur, Malaysia
7School of Postgraduate Studies, International Medical University, Kuala Lumpur, Malaysia
8Faculty of Medicine, University of Malaya Cancer Research Institute, University of Malaya, Kuala Lumpur, Malaysia

Abstract
Prostate cancer (PCa) is the most common malignancy and is the second leading cause of cancer among men globally. Using a kinome-wide lentiviral small-hairpin RNA (shRNA) library screen, we identified phosphoinositide-dependent kinase-1 (PDPK1) as a potential mediator of cell survival in PCa cells. We showed that knock-down of endogenous human PDPK1 induced significant tumour-specific cell death in PCa cells (DU145 and PC3) but not in the normal prostate epithelial cells (RWPE-1). Further analyses revealed that PDPK1 mediates cancer cell survival predominantly via activation of serum/glucocorticoid-regulated kinase 3 (SGK3). Knock-down of endogenous PDPK1 in DU145 and PC3 cells significantly reduced SGK3 phosphorylation while ectopic expression of a constitutively active SGK3 completely abrogated the apoptosis induced by PDPK1. In contrast, no such effect was observed in SGK1 and AKT phosphorylation following PDPK1 knock-down. Importantly, PDPK1 inhibitors (GSK2334470 and BX-795) significantly reduced tumour-specific cell growth and synergized docetaxel sensitivity in PCa cells. In summary, our results demonstrated that PDPK1 mediates PCa cells’ survival through SGK3 signalling and suggest that inactivation of this PDPK1-SGK3 axis may potentially serve as a novel therapeutic intervention for future treatment of PCa.
INTRODUCTION

As the most prevalent form of non-cutaneous cancer, prostate cancer (PCa) is the second-highest incidence of cancer in male globally. In 2018, approximately 1.3 million new patients were diagnosed with PCa, and nearly 360,000 deaths occurred globally. In the early stages, PCa is mainly regulated by androgen, thus, androgen deprivation therapy (ADT) has become routine in clinical practice. However, about 10%-20% of patients inevitably fail this therapy and progress to castration-resistant PCa (CRPC) with a median survival ranges between 15 and 36 months.

Despite the fact that our understanding of the clinical, molecular and pathologic characteristics of PCa is incomplete, the androgen receptor (AR), which is regarded as the primary oncoprotein in PCa and CRPC, is regularly expressed in a heterogeneous way, even in the context of AR gene amplification. In AR-positive PCa, hormonal treatment resistance can arise via clonal selection, intracrine mechanisms or adaptation to decreased androgen (e.g., mutation, AR phosphorylation and bypass of the AR pathway).

While some of the early studies indicated that CRPC depends on AR activity, numerous new evidence suggests that other mechanisms have the capability to promote CRPC progression in a manner that is independent of AR activation. For example, several studies have shown that phosphoinositide 3-kinase (PI3K) signaling is adequate for CRPC survival when AR activity is reduced or not present.

In contrast to primary PCa, AR gene expression signatures were found to be inversely correlated with cell proliferation signatures in a subset of CRPC patients. Furthermore, AR activities have also been shown to possess a tumor and metastasis suppressor function, suggesting PCa disease progression can be driven by AR-independent mechanisms.

In order to identify genes and pathways that modify PCa growth in the context of suppressed AR signaling, we conducted in vitro high-throughput RNA interference (RNAi) screening using a lentiviral shRNA library designed to target the whole kinase against the AR-negative PCa DU145 cell line.

Here, we describe the results of our screen that identified the 3-phosphoinositide-dependent protein kinase 1 (PDPK1) as an essential kinase critical for the proliferation and viability of a subset of PCa cells.

MATERIALS AND METHODS

Cell lines and cell cultures

The human PCa cells (DU145, PC3 and LNCaP) and normal prostate epithelial cell (RWPE-1) were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). DU145 and PC3 are AR-negative while LNCaP harbors an androgen-responsive AR mutant T877A. All PCa cells were maintained in RPMI 1640 supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). The RWPE-1 normal prostate cell line was grown in keratinocyte serum-free medium consisting of 5 ng/mL of recombinant epidermal growth factor and 0.05 mg/mL of bovine pituitary extract (Invitrogen, Carlsbad, CA, USA). All cells were cultured at their logarithmic growth in a humidified 37°C, 5% CO₂ incubator.

Lentiviral human kinase shRNA library screen

The MISSION LentilExpress™ Human Kinases shRNA library (Sigma-Aldrich, St Louis, MO, USA) was used to screen for candidate protein kinases mediating the growth of PCa cells. Briefly, the AR-negative DU145 cells were seeded in a 384-well plate overnight, followed by transduction of lentiviral particles at multiplicities of infection (MOI) of 1 in the presence of 7.5 μg/mL polybrene (Sigma-Aldrich, St Louis, MO, USA). After 18h incubation, the medium containing the lentivirus particles was replaced with complete medium, and the cell viability was evaluated using the CellTiter-Glo® assay (Promega, Madison, WI, USA) at 72 hours post-transduction. Lentiviral particles carrying an empty vector (pLKO.1-puro), a non-target shRNA (NS) or a GFP expressing lentiviral construct were included as controls to examine transduction efficiency and well-to-well variation. All data were normalized against NS controls and hits were considered when shRNA targeting a specific gene achieved a Z-score of less than –2.

Quantitative real-time PCR (QPCR) analysis

The total RNA extraction and first-strand cDNA synthesis were conducted using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and High Capacity RNA to cDNA Master Mix (Applied Biosystems, Carlsbad, CA, USA), respectively. Gene expression levels were quantified by CFX96 PCR detector system (Bio-Rad, Richmond, CA, USA) in the presence of FastStart Universal SYBR Green Master reagent (Roche Diagnostics, Indianapolis, IN, USA). The specific forward and reverse primer sequences are shown in Table S1. The qPCR condition that applied for all samples were: 94°C for 3 minutes, followed by 40 cycles of 94°C for 40 seconds, 60°C for 40 seconds and 72°C for 30 seconds. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was served as housekeeping gene for normalization.
2.4 | Western blot analysis

All cell protein lysates were harvested using ice-cold lysis buffer (1% NP-40, 1 mM DTT, protease inhibitors, and phosphatase inhibitor I and II cocktails in PBS) as previously described.\textsuperscript{14,15} A total of 50 µg protein was loaded for immunoblotting. Monoclonal antibodies against PDPK1 and β-actin were purchased from Santa Cruz Biotechnology, CA, USA. Primary antibodies against AR, PDPK1, p-PDPK1 (S241), PTEN, AKT, p-AKT (S473), p-AKT (T308), SGK3, p-SGK3 (T320), SGK1 and p-SGK1 (S78) were obtained from Cell Signalling Technology, MA, USA.

2.5 | Lentiviral production and transduction

Lentiviral non-targeting shRNA (NS) and shRNA constructs targeting PDPK1, CAMKV and CKS1B were purchased from Sigma-Aldrich, MO, United States, with target sequences shown in Table S2. To produce the lentiviral particles of interest, the target shRNA constructs were co-transfected into HEK-293T cells with lentiviral packaging plasmids, psPAX2 (Addgene plasmid #12260) and envelope plasmids, pMD2.G (Addgene plasmid #12259) as described previously.\textsuperscript{16-18} The lentiviral particles were then collected and added with 7.5 µg/mL polybrene (Sigma-Aldrich, St Louis, MO, USA) for transduction.

2.6 | Detection of apoptosis by annexin V flow cytometry

All floating and attached cells were stained for cell apoptosis assay using a PE Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) as described previously.\textsuperscript{19,20} The samples were quantitated using a FACSCalibur flow cytometer and analysed by CellQuest Pro software (version 5.1.1; BD Biosciences, San Jose, CA, USA).

2.7 | Transfection

Plasmids for constitutively active myristoylated AKT and SGK3-S486D mutant were obtained from Addgene (Addgene plasmid # 9008) and Gene Universal (Newark, DE, USA), respectively. Plasmids were transfected into target cells using X-tremeGENE HP DNA transfection reagent (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer’s protocol.

2.8 | Drug combination analysis

Drug combinatory effects were analysed using the Chou-Talalay method and Highest Single Agent (HSA) models as described previously.\textsuperscript{21,22} Briefly, cells were plated at 2.5 × 10\(^3\) cells/well in 96-well format and treated with docetaxel and/or PDPK1 inhibitors (GSK2334470 and BX795) alone or in combination. The plates were terminated by MTT cell proliferation assay at 72 hours after treatment.\textsuperscript{23,24} CalcuSyn 2.1 software (Biosoft, Cambridge, UK) was used to generate combination assay (CI) based on Chou-Talalay method,\textsuperscript{19,25} in which CI < 1, = 1 and >1 indicates synergy, additive and antagonism effect, respectively (Table S3). The dose-response surface curves with levels of HSA synergy were plotted by Combenefit software (Cancer Research UK Cambridge Institute).\textsuperscript{26}

2.9 | Statistical analysis

All results were presented as mean ± standard deviation (SD) from at least three independent experiments. SPSS (version 19.0 INC, Chicago, IL) was used to evaluate the statistical significance based on Student’s independent t test. A P-value < 0.01 was considered statistically significant.

3 | RESULTS

3.1 | Kinome-wide shRNA library screen identifies PDPK1 as putative target mediating PCa cell survival

To identify genes and pathways that modify PCa growth, we conducted in vitro high-throughput RNA interference (RNAi) screening using the RNAi Consortium (TRC) kinome shRNA library consisting of 3197 lentiviruses carrying shRNA sequences targeting 506 human kinase genes. Each gene is represented by at least 3–5 individual constructs, targeting different regions of the gene sequence. A total of 45 kinases (8.9%) in the TRC kinome library were identified to induce significant growth inhibition (Z-score < -2) in DU145 PCa cells (Figure 1A and B, Table S4). Among them include a number of proto-oncogene (ERBB3, ERBB4, RET, SRC and YES1), pro-survival genes involved in the PI3K signalling (AKT1, AKT3, GSK3A, GSK3B, PDPK1 and SGK3) and MAPK signalling (MAP3K4, MAPK1, MAPK4, MAPK13 and MAPK15). Importantly, knock-down of AKT1,\textsuperscript{27-29} AKT3,\textsuperscript{27-29} GSK3A,\textsuperscript{30-32} GSK3B,\textsuperscript{30-35} MAPK1 (also known as ERK2),\textsuperscript{36,37} MAPK4 (also known as ERK4)\textsuperscript{29} and ROCK2\textsuperscript{38-40} have been shown to inhibit PCa cell growth, independently validated our results.

Next, we selected 3 candidate kinases (PDPK1, CAMKV and CKS1B) for further study based on the novelty and the magnitude of effect by shRNA knock-down. Indeed, depletion of the endogenous PDPK1, CAMKV and CKS1B significantly reduced DU145 cell survival, consistent with the results obtained in the primary screen (Figure S1).

Recent studies have shown that the locus containing PDPK1 gene (16p13.3) is more frequently amplified in lymph node metastases and castration-resistant PCa, compared to primary tumours,\textsuperscript{41} we decided to focus on understanding the mechanism underlying PDPK1 mediated cell survival in PCa cells. We first
evaluated whether PDPK1 is expressed in a panel of AR-negative (DU145 and PC3) and AR-positive (LNCaP) PCa cell lines, as well as non-transformed prostate epithelial cells (RWPE-1). Real-time qPCR shows that PDPK1 mRNA was highly expressed in all the PCa and normal prostate epithelial cells (Figure 1C). The level of gene expression correlated well with the PDPK1 protein expression as PDPK1 proteins were highly expressed in LNCaP, PC3, RWPE-1 and DU145 (Figure 1D). Interestingly, PDPK1 proteins were found to be phosphorylated in cells which express them, suggesting that PDPK1 proteins are constitutively active in these cells.

3.2 | Depletion of PDPK1 induces tumour-specific cell death PCa cells

To determine whether depletion of endogenous PDPK1 has any effect on the proliferation and survival of PCa cells that exhibit active PDPK1, we performed lentiviral shRNAs-mediated knock-down of PDPK1 in a panel of PCa and non-transformed prostate epithelial cells. Efficient knock-down of PDPK1 in all prostate cell lines by two independent shRNA constructs was demonstrated in Western blotting (Figure 2A). Significant reduction in cell viability of at least 80% in DU145 and PC3 was observed while no effect was observed in LNCaP and RWPE-1 (P < .05, Student’s t test) (Figure 2B and Figure S2). Consistent with the findings from cell viability assay, depletion of endogenous PDPK1 also induced significant amount of apoptosis in DU145 and PC3 cells (P < .01, Student’s t test) (Figure 2C), corroborated with the induction of caspase 3 and 9, but not caspase 8 activity (Figure 2D). In contrast, no such effects were observed in RWPE-1 nor in LNCaP cells. These results suggest that PDPK1 is required for the survival of the AR-negative DU145 and PC3 cells, but not for the AR-positive LNCaP cells.

3.3 | Depletion of PDPK1 inhibits SGK3 phosphorylation

PDPK1 is known to phosphorylate AKT that regulates several signalling pathways altered in cancer. However, recent studies have also shown that PDPK1 can activate many other members of AGC kinase family such as p70S6K, SGK, p90RSK and the members of PKC family, independent of AKT. To evaluate whether the pro-survival effects of PDPK1 in PCa cells are mediated through aberrant activation of downstream AKT or SGK pathways, we analysed the effects of PDPK1 knock-down on the expression and phosphorylation of these targets. As shown in Figure 3, knock-down of PDPK1 in DU145 and PC3 cells significantly reduced phosphorylation of SGK3 but not the phosphorylation of AKT or SGK1. The total expression of SGK3, SGK1 and AKT remained unchanged. In stark contrast, no such effects were observed in RWPE-1 or LNCaP cells.
3.4 | PDPK1 mediates the survival of DU145 and PC3 cells via SGK3 signalling pathway

To test whether the pro-survival effects of PDPK1 is mediated through SGK3, we transfected a constitutively active SGK3 S486D mutant in DU145 and PC3 cells followed by PDPK1 depletion. As shown in Figure 4, ectopic expression of SGK3 S486D significantly rescued the apoptosis induced by PDPK1 depletion (P < .01, Student’s t test). In contrast, no such effects were observed in cells transfected with a constitutively active myristoylated AKT (Myr-AKT), suggesting that PDPK1 regulates cell survival in PCa cells through activation of SGK3 signalling (Figure S3).

3.5 | Inhibition of PDPK1 enhances docetaxel sensitivity in PCa cells

Since PDPK1 up-regulation and activation have been recently shown to confer chemoresistance in breast, glioblastoma and neuroblastoma and pancreatic cancer cells, we asked whether inhibition of PDPK1 might enhance chemotherapy sensitivity in PCa cells. To test this hypothesis, we first evaluated the effects of docetaxel, a commonly used chemotherapeutic agent for PCa, and PDPK1 inhibitors (GSK2334470 and BX795) on a panel of PCa cells (DU145, PC3 and LNCaP). As shown in Figure 5A and Table S5, the AR-positive LNCaP was more sensitive to docetaxel (IC50 of 7.49 ± 2.45 nM) as compared to the AR-negative DU145 and PC3 (IC50 of 20.00 ± 5.68 nM and > 100 nM, respectively). In contrast, both DU145 and PC3 were more sensitive to GSK2334470 (IC50 of 12.74 ± 1.22 µM and 9.16 ± 0.59 µM, respectively) and BX795 (IC50 of 11.49 ± 5.49 µM and 5.10 ± 0.45 µM, respectively) as compared to LNCaP (IC50 >100 µM), consistent with the results obtained from the PDPK1 knock-down.

Next, we investigated whether the inhibition of PDPK1 could synergize docetaxel sensitivity in PCa cells. As shown in Figure 5B, inhibition of PDPK1 enhances docetaxel sensitivity in DU145 and PC3 but not LNCaP cells, suggesting that PDPK1 inhibitors might potentiate sensitivity of refractory PCa cells to chemotherapy (Tables 1 and 2).
In this study, we identified 45 kinases that mediate PCa cell survival. These include previously identified proto-oncogene such as ERBB3, ERBB4, RET, SRC and YES1; and pro-survival genes involved in the PI3K and MAPK signalling such as AKT1, AKT3, GSK3A, GSK3B, PDK1, SGK3, MAP3K4, MAPK1 (also known as ERK2), MAPK4 (also known as ERK4), MAPK13, MAPK15 and ROCK2. Particularly, knock-down of PDK1 induced tumour-specific cell death in both DU145 and PC3 AR-negative cells, but not in the AR-positive LNCaP cells nor in the RWPE-1 non-transformed prostate epithelial cells.

Amplification or overexpression of PDK1 has been implicated in tumourigenesis and cancer cells survival in many human cancers including breast cancer, 50,51 esophageal squamous cell carcinoma, 52 melanoma, 53 gastric carcinoma, 54 hepatocellular carcinoma 55 and acute myeloid leukaemia. 56 A recent study identified the locus containing PDK1 gene (16p13.3) is more frequently amplified in lymph node metastases and CRPC, compared to primary tumours, suggesting PDK1 may also support cancer metastasis. 41

PDK1 is known to function downstream of PI3K and is required for the full activation of AKT serine/threonine kinase 1 (AKT1) and other AGC kinases such as protein kinase C (PKC), p70 ribosomal protein S6 kinase (S6K), p90 ribosomal protein S6 kinase (RSK), polo-like kinase 1 (PLK1) and serum glucocorticoid-dependent kinase (SGK). 57

Upon activation, PDK1 binds to phosphatidylinositol 3,4,5 triphosphate (PIP3), the product of PI3K, via its pleckstrin homology (PH) domain at the plasma membrane. This in turn leads to the phosphorylation AKT at T308 and activates AKT signalling. In the case of PDK1 substrates that do not possess a PH domain (eg S6K, RSK and SGK), PDK1 can still interact and bind to the hydrophobic motif of the target kinase via the PDK1-interacting fragment (PIF) to activate the downstream signalling pathways. 40,56
In an effort to define the mechanisms of PDPK1-mediated regulation of PCa cell survival, we observed that depletion of PDPK1 reduced SGK3 phosphorylation, but have no effects on AKT phosphorylation in cell line models with low- and hyperactivated background levels of AKT activity (DU145 and PC3 respectively). Furthermore, ectopic expression of a constitutively active SGK3 significantly abrogated the apoptotic effects induced by PDPK1 depletion, while no such effects were observed in cells expressing a myristoylated AKT. These data are consistent with accumulating evidence indicating that PDPK1 can contribute to cancer through...
activation of SGK3, independent of AKT. For instance, it was reported that human cancers with PIK3CA mutations where AKT activity is deficient, SGK3 serves as the main PDPK1 effector to drive tumour cells survival. Similarly, SGK3 was also found to be a key mediator of PDPK1-dependent melanomagenesis and a driver for tumour formation in breast cancer cells in both PIK3CA wild-type and mutated cells in an AKT-independent manner. Thus, our data strongly suggest that PDPK1 is mediating the survival of the AR-negative DU145 and PC3 PCa cells through activation of SGK3, independent of AKT signalling.

Finally, our study further demonstrates that pharmacological inhibition of PDPK1, using 2 distinct chemical compounds (GSK2334470 and BX795), strongly reduced cancer cell growth in the AR-negative DU145 and PC3 PCa cells, but not in the AR-positive LNCaP or the RWPE-1 non-transformed prostate epithelial cells (data not shown). Importantly, we also report that both GSK2334470 and BX795 synergize docetaxel sensitivity in DU145 and PC3 cells, but not in LNCaP cells. Indeed, it has been observed that SGK3 can substitute for AKT in activating mTORC1, which in turn has been implicated in docetaxel resistance in PCa. To
date, the role of PDPK1 in driving cancer and chemoresistance has been outlined in multiple cancers, including acute myeloid leukaemia, breast cancer and ovarian cancer.44 These findings further underscore the potential of PDPK1 as a therapeutic target, as they indicate that PDPK1 or SGK3 can act as druggable targets in the treatment of hormone-refractory PCa as single agents or in combination with chemotherapeutics as components of a multitargeted therapy regimen.

5 | CONCLUSIONS

In conclusion, we identified PDPK1 as a novel potential therapeutic target in PCa and demonstrated PDPK1 is mediating PCa cells' survival through activation of SGK3 in an AKT-independent manner. Our data further suggest that combination of PDPK1 inhibitors with docetaxel enhances their anti-cancer activity, possibly by targeting SGK3-dependent resistance mechanisms. Together, our results provide a strong rationale to investigate further the use of PDPK1 inhibitors in as novel therapeutic strategies for refractory PCa patients.

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AUTHORS’ CONTRIBUTION

IC, COL and AHAR designed the study. GN, FFLC, CWM, LWH, KKC and WML developed the methodology, collected the data and performed the analysis. GN, COL and IC wrote the manuscript. All authors reviewed and approved the manuscript. Geetha Nalairndran: Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal). Azad Hassan Abdul Razack: Conceptualization (equal); Funding acquisition (equal); Project administration (equal); Supervision (equal). Chun-Wai Mai: Investigation (equal); Methodology (equal); Supervision (equal); Validation (equal). Felicia Fei-Lei Chung: Investigation (equal); Methodology (equal); Writing-review & editing (equal). Kok-Keong Chan: Funding acquisition (equal); Project administration (equal); Supervision (equal). Ling-Wei Hii: Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Wei-Meng Lim: Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Ivy Chung: Conceptualization (equal); Funding acquisition (equal); Investigation (equal); Methodology (equal); Project administration (equal); Validation (equal); Writing-original draft (equal); Writing-review & editing (equal). Chee-Onn Leong: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Funding acquisition (equal); Investigation (equal); Methodology (equal); Project administration (equal); Supervision (equal); Validation (equal); Visualization (equal); Writing-original draft (equal); Writing-review & editing (equal).

CONFLICT OF INTEREST

The author(s) declare no competing interests. Where authors are identified as personnel of the International Agency for Research on Cancer/World Health Organization, the authors alone are responsible for the views expressed in this article and they do not necessarily represent the decisions, policy or views of the International Agency for Research on Cancer/World Health Organization.

DATA STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

ORCID

Ivy Chung https://orcid.org/0000-0003-2840-8123
Chee-Onn Leong https://orcid.org/0000-0002-6353-9703

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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