Phosphoinositide Protein Kinase PDPK1 Is a Crucial Cell Signaling Mediator in Multiple Myeloma

Yoshiaki Chinen1, Junya Kuroda1, Yuji Shimura1, Hisao Nagoshi1, Miki Kiyota1, Mio Yamamoto-Sugitani1, Shinsuke Mizutani1, Natsumi Sakamoto1, Masaki R2, Eri Kawata3, Tsutomu Kobayashi1, Yosuke Matsumoto1, Shigeo Horii1, Shinsuke Iida2, and Masafumi Taniwaki1

Abstract

Multiple myeloma is a cytogenetically/molecularly heterogeneous hematologic malignancy that remains mostly incurable, and the identification of a universal and relevant therapeutic target molecule is essential for the further development of therapeutic strategy. Herein, we identified that 3-phosphoinositide–dependent protein kinase 1 (PDPK1), a serine threonine kinase, is expressed and active in all eleven multiple myeloma–derived cell lines examined regardless of the type of cytogenetic abnormality, the mutation state of RAS and FGFR3 genes, or the activation state of ERK and AKT. Our results revealed that PDPK1 is a pivotal regulator of molecules that are essential for myelomagenesis, such as RSK2, AKT, e-MYC, IRF4, or cyclin Ds, and that PDPK1 inhibition caused the growth inhibition and the induction of apoptosis with the activation of BIM and BAD, and augmented the in vitro cytotoxic effects of antilymela agents in myeloma cells. In the clinical setting, PDPK1 was active in myeloma cells of approximately 90% of symptomatic patients at diagnosis, and the smaller population of patients with multiple myeloma exhibiting myeloma cells without active PDPK1 showed a significantly less frequent proportion of the disease stage III by the International Staging System and a significantly more favorable prognosis, including the longer overall survival period and the longer progression-free survival period by bortezomib treatment, than patients with active PDPK1, suggesting that PDPK1 activation accelerates the disease progression and the resistance to treatment in multiple myeloma. Our study demonstrates that PDPK1 is a potent and a universally targetable signaling mediator in multiple myeloma regardless of the types of cytogenetic/molecular profiles. Cancer Res; 74(24); 7418–29. ©2014 AACR.

Introduction

Multiple myeloma is a cytogenetically/symptomatically heterogeneous plasma cell malignancy, and is the second most common among hematologic malignancies, constituting 1% of all cancers (1, 2). The recent advent of novel agents, including immunomodulatory drugs, such as lenalidomide or pomalidomide, and proteasome inhibitors (PI), bortezomib (BTZ) or carfilzomib, has improved the response to therapy and prolonged the survival of patients with multiple myeloma, and has thereby dramatically revolutionized the treatment strategy against multiple myeloma (3–9). However, multiple myeloma remains mostly incurable to date, and the further development of an innovative anti-MM therapy is urgently needed.

The emergence and progression of multiple myeloma are cytogenetic/molecular multistep processes. Initially, a myeloma-initiating cell acquires a primary cytogenetic abnormality involving cyclin Ds (CCND), FGFR3/MMSET, or MAFs, which confers the ability of clonal proliferation and expansion. During the long disease course, myeloma cells progressively acquire various additional cytogenetic, genetic, and/or epigenetic abnormalities that further augment the proliferative phenotype and the resistance to cytotoxic insults, including anticancer agents. In addition to such intracellular oncogenic events, myeloma cells are supported by tumor microenvironment components, including bone marrow (BM) stromal cells (BMSC), osteoclasts and soluble factors, such as IL6, IGF1, or B-cell activating factor belonging to the tumor necrosis factor family (BAFF), in BM. Many of the intrinsic oncogenic cellular events and extrinsic stimuli converge to activate two major signaling pathways, the RAS/RAF/ERK/RSK2 pathway and the PI3K/AKT pathways, which share critical roles in myeloma development and progression (1, 2). In myeloma cells, for instance, the RAS/RAF/ERK/RSK2 pathway has frequently been activated by gene mutations of RAS, BRAF, or FGFR3, and by chromosomal translocation involving FGFR3/MMSET or MAFs (1, 2, 10–12), whereas the PI3K/AKT pathway is active.
in approximately half of patients with multiple myeloma, and AKT activity has been suggested to be mediated by DEP domain-containing mTOR-interacting protein (DEPTOR; refs. 10, 13, 14). Moreover, the abnormal activation of these two pathways is associated with drug resistance and a poor prognosis of multiple myeloma (15–17). Nevertheless, previous studies have demonstrated that a sole inhibition of either the RAS/MAPK/ERK or PI3K/AKT pathways was not sufficiently efficacious to eradicate myeloma cells (10, 16, 18–21). The inhibition of RAS is only effective against myeloma cells with mutated RAS, whereas the inhibition of AKT results in compensative activation of the RAS/RAF/ERK pathway in myeloma cells (16, 22–24). These multifactorial and overlapping molecular mechanisms underlying tumorigenesis serve to make the molecular architecture of myeloma cells highly complex and heterogeneous among patients with multiple myeloma as well as even among subclones in a single patient, and thus, make it even more difficult to identify a universally effective therapeutic target in multiple myeloma.

We have recently identified that the N-terminal kinase domain (NTKD) of RSK2, which is the most downstream signaling mediator of the RAS/ERK pathway in normal tissues, is universally activated thorough phosphorylation in myeloma cells with diverse cytogenetic/molecular abnormalities, regardless of the activation state of ERK and AKT (25). Importantly, the constitutive activation of RSK2-NTKD is responsible for the regulation of critical molecules for myeloma genesis, including c-MYC or CCNDs; however, the underlying mechanism for RSK2-NTKD in myeloma cells has not been identified (25). Because 3-phosphoinositide-dependent protein kinase 1 (PDPK1), a serine/threonine kinase also called PDK1, has been shown to be essential for the activation processes of both RSK2-NTKD and AKT in physiologic condition (26, 27), in this study, we investigated the role of PDPK1 in myeloma genesis and the potential of PDPK1 as a therapeutic target molecule for overcoming the difficulty of treating multiple myeloma due to their interpatient diversity and intraclonal molecular heterogeneity.

Materials and Methods

Cells and reagents

This study used human myeloma cell lines AMO-1, NCI-H929, OPM-2, IP-1 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), PRM8226, and IM9 (ATCCC), which were authenticated by mycoplasma detection, DNA fingerprinting, isozyme detection, and cell vitality detection. KMS-18, KMS-12-BM, KMS-28-PE, KMS-34, and KMS-11 were kind gifts from Dr. T. Ohtsuki (Kawasaki Medical School, Okayama, Japan), and AMU-MM1 was a kind gift from Dr. I. Hanamura (Aichi Medical School, Aichi, Japan). HS-5 is an immortalized human BMSC-derived cell line, which potently secretes G-CSF, GM-CSF, M-CSF, Kit ligand, MIP-1α, IL6, IL8, or IL11 (ATCC; ref. 28). OPM-2, KMS-11, and KMS-18 have been shown to express variants of FGFR3 (K650E, Y373C, and G384D, respectively; ref. 25). Cells were maintained in RPMI-1640 containing 10% FCS, 2 mmol/LL-glutamate, and penicillin/streptomycin. Bortezomib-resistant subclones of OPM-2 and KMS-11 (OPM-2/TZ and KMS-11/TZ, respectively) were developed under continuous exposure to bortezomib (29). Studies using patient samples were approved by the Institutional Review Board of Kyoto Prefectural University of Medicine (Kyoto, Japan; RBMR-G-124-2), and patient-derived samples were collected with informed consent in accordance with the Declaration of Helsinki. BM mononuclear cells were labeled with anti-CD138 MicroBeads and positively isolated with the Mini-MACS separator (Miltenyi Biotec KK). Recombinant human IL6, IGF1, and BAFF were purchased from R&D Systems. Isolated primary myeloma cells were cultured in conventional culture media supplemented with 50 ng/mL IL6. BX-912 and AR-12, PDPK1 inhibitors, were purchased from Symansis NZ Limited. BAY11-7085, an NF-kB inhibitor, was purchased from Calbiochem. Bortezomib, melphalan, and etoposide were provided by the Screening Committee of Anticancer Drugs.

Assays for growth inhibition and apoptosis

Assays for growth inhibition and apoptosis were performed as described in the Supplementary Information.

Drug combination assays

Cells were treated with various concentrations of bortezomib, melphalan, etoposide, or BAY11-7085 alone or in combination with BX-912 for 48 hours. The fractional effect concentrations (i.e., a fractional effect of 0.25 equals a 25% growth inhibitory effect) and the combination index (CI) were calculated with CalcuSyn (Biosoft). This method facilitates the quantification of synergism (CI < 1) and antagonism (CI > 1) at different doses and effect levels. CI calculations were conducted on the assumption that the drug mechanisms were not mutually exclusive.

RNAi

RNAi of PDPK1 was performed by transfecting shRNA plasmid into KMS-18 cells. Briefly, 2.5 × 10⁵ KMS-18 cells were resuspended in 100 µL of transfection buffer and transfected with shRNA plasmid (Qiagen) for PDPK1 or with control shRNA plasmid by means of the CLB-Transfection Kit (Lonza AG) using protocol 9. Two different shRNA plasmids targeting PDPK1 were utilized independently.

Western blot analysis

Western blot analysis was conducted as described elsewhere (25). The primary antibodies used are described in the Supplementary Information.

Assay for NF-κB activity

NF-κB activity was assessed using NF-κB (p65) Transcription Factor Assay Kit according to the manufacturer’s instruction (Cayman Chemical Company).

IHC staining of phospho-PDPK1 in patient-derived BM clot sections and statistical analysis

Approval was obtained from the Institutional Review Board of our institute for the investigation of patient-derived samples and their medical records (RBMR-G-124-2). Formalin-fixed and paraffin-embedded tissues were examined by IHC staining.
with an anti-phosphorylated (p-)PDPK1Ser241 monoclonal Ab. The activity of p-PDPK1 in myeloma cells was evaluated as negative (−), no staining at all; positive (+), moderate staining in myeloma cells; or strong positive (++), strong staining. The p-PDPK1 activity in myeloma cells was independently evaluated by three hematologists who were blinded to the patients’ clinical records. The International Myeloma Working Group criteria were used for the assessment of treatment response, and the disease stage was assessed according to the international staging system (ISS). Progression-free survival (PFS) was estimated from the date of the first assessment of disease progression or the date of death from any cause. OS and PFS were estimated using the Kaplan–Meier method. The confidence interval was 95% for all analyses and P < 0.05 was considered to be statistically significant. The log-rank test was used to compare differences between survival curves.

Analysis of NRAS, KRAS, and BRAF mutations

The cDNA from the myeloma cell lines was amplified by PCR with primers spanning the mutation hotspot codons 12, 13, and 61 for NRAS and KRAS, and codon 600 for BRAF. Analyses of PCR products were conducted as described in the Supplementary Information.

FISH

Cytogenetic abnormalities were examined by means of interphase FISH analysis (30). The probes used are described in the Supplementary Information.

Results

PDPK1 and RSK2-NTKD are constitutively activated through phosphorylation in multiple myeloma–derived cell lines

We first examined the activation state of PDPK1 in association with the activity of ERK, RSK2, and AKT in 11 myeloma-derived cell lines. Phosphorylated (p-)PDPK1Ser241 was used as a surrogate marker for the active confirmation. We simultaneously examined the expression of FGFR3 and DEPTOR, cytogenetic abnormalities that are highly associated with multiple myeloma, and the gene mutation status of NRAS, KRAS, and BRAF in all cell lines examined. As shown in Fig. 1, seven of 11 cell lines exhibited chromosomal translocation t(4;14) involving FGFR3/MMSET, whereas both t(11;14) involving CCND1 and t(14;16) involving c-MAF were identified in one cell line each. Both mutations of NRAS and KRAS were detected in three cell lines each, and their existence was mutually exclusive. None of the cell lines possessed the BRAFV600E mutation. PDPK1Ser241 and RSK2Ser227, NTKD were phosphorylated in all 11 multiple myeloma–derived cell lines examined, whereas ERK, AKT, and RSK2Tyr579, C-terminal kinase domain (CTKD) were activated by phosphorylation in 9, 6, and 3 of 11 multiple myeloma–derived cell lines, respectively (Fig. 1). These results indicated that both PDPK1 and RSK2-NTKD are universally activated through phosphorylation in all 11 multiple myeloma–derived cell lines examined, regardless of the type of cytogenetic abnormalities or the status of upstream signaling molecules, and that myeloma cell lines can be largely subdivided into two types, namely, those that are active for PDPK1, RSK2-NTKD, and AKT and those that are active for PDPK1 and RSK2-NTKD, but inactive for AKT. In addition, all seven cell lines with NRAS, KRAS, or FGFR3 mutations were shown to possess p-ERK, while we found no positive correlation between the expression of DEPTOR and p-AKT status in our series.

PDPK1 inhibition exerts anti-MM effect via G2–M cell-cycle blockade and the induction of apoptosis

Next, we investigated the effects of PDPK1 inhibition on myeloma-derived cell lines using small-molecule inhibitors for PDPK1, BX-912, and AR-12. Although BX-912 is a highly selective inhibitor for PDPK1 (27, 31), AR-12 inhibits not only PDPK1, but also other off target molecules, such as COX-2 (32). Forty-eight–hour exposure of either BX-912 or AR-12 resulted in the time- and dose-dependent growth inhibition in various myeloma cell lines (Supplementary Fig. S1). The IC50 values for the 11 myeloma cell lines were 2.5–128 μmol/L with BX-912, compared with 3.0 to 12.5 μmol/L with AR-12. The growth inhibitory effects of BX-912 and AR-12 were not significantly affected by the p-AKT state of myeloma cells (Fig. 2A and B). To further explore the mechanisms underlying the growth inhibition by PDPK1 inhibition, AMO-1 cells (representative of myeloma cells with active RSK2-NTKD and lacking AKT activity) and NCI-H929 cells (representative of myeloma cells with active RSK2-NTKD and AKT) were subjected to flow-cytometric analyses. As the results, treatment with the PDPK1-specific inhibitor, BX-912 or AR-12, caused G2–M cell-cycle blockade and the induction of apoptosis in both AMO-1 cells and NCI-H929 cells (Fig. 2C and D; Supplementary Fig. S2A and S2B).

Molecular sequelae following PDPK1 inhibition in multiple myeloma cell lines

We next analyzed the molecular sequelae following p-PDPK1 inhibition in AMO-1 and NCI-H929 cells. The blockade of p-PDPK1 by BX-912 caused dephosphorylation of RSK2-NTKD in AMO-1 cells and dephosphorylation of both RSK2-NTKD and AKT in NCI-H929 cells (Fig. 3A). We next examined the effects of PDPK1 inhibition on the downstream target molecules of RSK2 and AKT. As shown in Fig. 3B, inhibition of PDPK1 caused the downregulation of c-MYC, IRF4, and CCNDs, the activation of proapoptotic BH3-only proteins, namely induction of BIML, and BIMI, and dephosphorylation of BAD, and the inhibition of PLK1 through dephosphorylation.

We further validated the effect of PDPK1 inhibition by gene knockdown of PDPK1 on myeloma cells. Two different shRNA vectors were utilized independently to knockdown PDPK1. Regardless of the type of shRNA vector for PDPK1 utilized, transient partial knockdown of PDPK1 expression...
caused the downregulation of c-MYC, IRF4, and CCNDs and the upregulation of BIMEL and BIML in the growth inhibitory stage in KMS-18 cells. As the result, the knockdown of PDPK1 was accompanied by the accumulation of cleaved form of caspase-3 and growth inhibition, indicating that PDPK1 knockdown caused the growth inhibition at least partly via the induction of apoptosis (Fig. 4A and B). In contrast, the transfection of control RNAi vector showed no effect on the expression and/or the activities of PDPK1, as well as the respective downstream molecules, or on the proliferation of KMS-18 cells. These indicate that PDPK1 is a crucial regulator of both RSK2-NTKD and AKT, and, thereby, regulates various molecules essential for disease development, cell cycling, cell proliferation, and survival of multiple myeloma (1, 2, 33, 34).

PDPK1 activity is critical for the viability of patient-derived primary myeloma cells and the long-term outcome of multiple myeloma

CD138-positive primary myeloma cells isolated from 17 BM samples from patients with multiple myeloma were available for the protein preparation for Western blot analyses of PDPK1, RSK2, and AKT, and, 11 of those 17 samples were also available for cytotoxicity assays by BX-912. Seventeen patients included four asymptomatic, so-called smoldering, multiple myeloma (aMM), four symptomatic newly diagnosed multiple myeloma (NDMM), and nine refractory/relapsed multiple myeloma (RRMM; Supplementary Table S1). PDPK1 was active in all 17 primary myeloma cell populations regardless of the disease state, whereas RSK2-NTKD and AKT were active in all NDMM and BRMM and in only two (Pts. 2 and 3) of four aMM (Fig. 5A). In addition, BX-912 treatment resulted in the reduced numbers of viable myeloma cells from 11 patients at concentrations similar to those used for the multiple myeloma–derived cell lines (Fig. 5B). Because untreated primary myeloma cells could survive but did not proliferate significantly in IL6 containing media in our system, our results indicate that the exposure to BX-912 induced cell death in patient-derived primary myeloma cells.

We also retrospectively evaluated the association between PDPK1 activity and treatment outcome of multiple myeloma by IHC staining of p-PDPK1Ser241 in formalin-fixed and paraffin-embedded BM clot specimens from 65 symptomatic NDMM patients that were consecutively diagnosed at our institute from January 2005 to August 2013. The median age of the 65 patients analyzed was 66 years (range, 14–88). The patients’ backgrounds, including gender, M-protein type, and disease stage according to the ISS, are summarized in Table 1. Thirty-six patients were also evaluated for chromosomal abnormalities, including 13q-, t(4;14), t(11;14), t(8;14), 1q21+, NRAS, KRAS, or FGFR3 gene mutations are also shown (26). Normal peripheral lymphocytes were used as the control.
cohorts, the staining intensity of p-PDK1 was similar among myeloma cells (Fig. 5C). Patients with the disease stage III according to ISS were significantly more frequent in the PDK1(+) and/or PDK1(++) cohorts compared with PDK1(−) cohort, while patients’ backgrounds, such as age, gender, or paraprotein type, were not significantly different among the PDK1(−), PDK1(+), and PDK1(++) cohorts (Table 1). Although the overall response rates for BD or HDT/ASCT were not significantly different among the PDK1(−), PDK1(+), and PDK1(++) cohorts, all PDK1(−) patients attained at least a partial response with some kind of therapy. With a median follow-up period of 1,310 days, the median OS period of the smaller population of PDK1(−) cohort was significantly longer than that of the PDK1(++) cohort (2,925 days vs. 1,694 days, P = 0.012), whereas those of the PDK1(+) and PDK1(++) cohorts were not significantly different. In addition, the 8-year OS rate was significantly higher in the PDK1(−) cohort compared with the PDK1(+) plus PDK1(++) cohorts (83% vs. 17%; P = 0.041). In response to BD, the median PFS period of the PDK1(−) cohort was significantly longer than the PDK1(+) or PDK1(++) plus PDK1(++) cohorts (not reached vs. 378 days; P = 0.049 vs. 167 days; P = 0.049). With HDT/ASCT, the median PFS period of the PDK1(−) cohort was significantly longer than that of the PDK1(++) cohort (972 days vs. 305 days; P = 0.046; Fig. 5D). The impact of PDK1 activity on the long-term outcome with Rd was not assessable by statistical analysis, because only 2 patients were treated by Rd in the PDK1(−) cohort.
The clinical implication of targeting p-PDPK1 in the anti-MM treatment

We examined the utility of the targeting p-PDPK1 in the anti-MM therapy. First, we examined the interaction between PDPK1 activity and the stimuli from cell extrinsic tumor microenvironment components, that is, soluble factors IL6 (100 ng/mL), BAFF (200 ng/mL), IGF1 (100 ng/mL), and BMSC (35, 36). As shown in Fig. 6A, the addition of IL6, BAFF, or IGF1 mostly enhanced the phosphorylation states of RSK2 and AKT, but not PDPK1, in both AMO1 cells (RSK2 active, but AKT inactive) and NCI-H929 cells (active RSK2-NTKD and AKT), and dephosphorylation of RSK2Ser227 in AMO-1 cells (active RSK2-NTKD but inactive AKT). PDPK1 inhibition resulted in the reduction of c-MYC, IRF4, and CCNDs, induction of BIM and the activation of BAD through dephosphorylation, and inactivation of PLK1 through dephosphorylation in all three multiple myeloma cell lines examined. Ctl., untreated control.

Figure 3. Molecular sequelae following PDPK1 inhibition by BX-912 in myeloma cells. AMO-1, NCI-H929, and KMS-34 cells were treated with BX-912 at their IC_{50} concentrations (4.8 μmol/L for AMO-1; 12.0 μmol/L for NCI-H929; and 12.0 μmol/L for KMS-34) for 6 to 12 hours (h), respectively. A, PDPK1 inhibition led to dephosphorylation of both RSK2Ser227 and AKTThr308 in NCI-H929 cells (active RSK2-NTKD and AKT), and dephosphorylation of RSK2Ser227 in AMO-1 cells (active RSK2-NTKD but inactive AKT). B, PDPK1 inhibition resulted in the reduction of c-MYC, IRF4, and CCNDs, induction of BIM and the activation of BAD through dephosphorylation, and inactivation of PLK1 through dephosphorylation in all three multiple myeloma cell lines examined. Ctl., untreated control.

Figure 4. Gene knockdown of PDPK1 by RNAi. A, knockdown of PDPK1 caused dephosphorylation of phospho (p)-RSK2Ser227 and p-AKTThr308, downregulation of MYC, IRF4, CCND1, and CCND2, and induction of BIML in KMS-18 cells. Two different shRNA vectors (sh-1 and sh-2) for PDPK1 were examined. Expression levels of target proteins relative to those treated with control RNAi vector were calculated with ImageJ software and are described below each band. B, cell proliferation of KMS-18 cells was inhibited by transfection of sh-1 against PDPK1, but not by the control vector. Cells were stained by Trypan blue, and viable cell numbers were counted under the inverted microscope. Solid lines represent the growth of mock-transfected cells and dotted lines represent that of cells transfected with the sh-1 for PDPK1. Triplicate experiments were carried out for each condition, and cell counts are expressed as the mean ± SD.
inactive) and NCI-H929 cells (both RSK2 and AKT active), while the enhanced phosphorylation of RSK2 or AKT by soluble factors was inhibited by BX-912. These indicate that PDPK1 phosphorylation is independent of soluble factors, while is the prerequisite for the activation of RSK2 and AKT by soluble factors. In addition, BX-912 failed in the complete dephosphorylation of PDPK1 and its downstream kinase(s) under IGF1 stimulation, suggesting that IGF1 may have the stronger potency in enhancing PDPK1-mediated signaling, compared with IL6 or BAFF (Fig. 6A). In contrast, the coculture of

Figure 5. Clinical significance of PDPK1 activity in multiple myeloma. A, Western blot analyses of the phosphorylation state of PDPK1Ser241, RSK2Ser227, and AKTThr308 in CD138-positive patient-derived primary myeloma cells from aMM patients (Pt. 1–4), symptomatic NDMM (Pt. 5–8), and RRMM patients (Pt. 9–17; Supplementary Table S1). Normal peripheral lymphocytes (N.L.) were utilized as a negative control. B, in vitro cytotoxic effect of BX-912 on patient-derived primary myeloma cells. Myeloma cells were exposed to various concentrations of BX-912 in IL-6–containing medium for 48 hours and the relative numbers of viable cells were determined by the modified MTT assay. The IC50 values were 11.3 (Pt. 2), 2.2 (Pt. 3), 8.6 (Pt. 4), 7.9 (Pt. 5), 8.9 (Pt. 6), 6.1 (Pt. 7), 6.5 (Pt. 8), 1.9 (Pt. 10), 3.6 (Pt. 12), 2.6 (Pt. 16), 4.1 (Pt. 17), and 29.3 μmol/L (normal lymphocytes). Results are expressed as the mean of triplicate experiments. C, IHC staining of phospho (p)-PDPK1Ser241 of patient-derived myelomatous BM clot specimens. The top panel, the middle panel, and the bottom panel are representative figures of five patients with p-PDPK1(−), p-PDPK1(+), and p-PDPK1(++) myeloma cells, respectively. Small windows of each figure are the higher-magnification views of the myeloma cells. D, long-term outcomes of patients with multiple myeloma according to p-PDPK1Ser241 status. Two cohorts of p-PDPK1(−; dotted lines) and p-PDPK1(+) plus p-PDPK1(++; solid lines) were compared in the top panels, whereas three cohorts of p-PDPK1(−; larger dotted lines), p-PDPK1(+; solid lines), and p-PDPK1(++; smaller dotted lines) were compared in the bottom panels.
### Table 1. Patient background and treatment outcome in association with PDPK1<sup>Ser241</sup> phosphorylation, as assessed by the IHC staining

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<th></th>
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<th>PDPK1 (-) plus (++)</th>
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<th>PDPK1 (-) plus (++)</th>
<th>P (-) vs. (+)</th>
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<td>CR/VGPR/PR/SD/PD</td>
<td>[50/50/0/0/0 (%)]</td>
<td>[10/3/11/6/0]</td>
<td>[31/10/13/8.9 (8.9)]</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PFS</td>
<td>681</td>
<td>972</td>
<td>567</td>
<td>NS</td>
<td>567</td>
<td>NS</td>
<td>305</td>
<td>0.046</td>
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<tr>
<td>Median, day</td>
<td>83%</td>
<td>59%</td>
<td>NS</td>
<td>64%</td>
<td>NS</td>
<td>33%</td>
<td>NS</td>
<td>33%</td>
</tr>
<tr>
<td>1-y rate (%)</td>
<td>83%</td>
<td>37%</td>
<td>NS</td>
<td>37%</td>
<td>NS</td>
<td>33%</td>
<td>NS</td>
<td>33%</td>
</tr>
</tbody>
</table>

**Abbreviations:** M, male; F, female; Ig, immunoglobulin; BJP, Bence Jones Protein; BD, bortezomib plus dexamethasone; CR, complete response; VGPR, very good partial response; PR, partial response; SD, stable disease; PD, progressive disease; Rd, lenalidomide plus weekly dexamethasone; NA, not available; NS, no significant difference.
myeloma cells with HS-5 cells showed no prominent effects on the phosphorylation status of PDPK1, RSK2, or AKT in our hands (Supplementary Fig. S3).

We next examined the interaction between PDPK1-mediated signaling and NF-κB signaling, because the NF-κB pathway is frequently activated by various kinds of gene mutations, as well as soluble factors in tumor microenvironment, such as BAFF, and, thereby, plays critical roles in promoting cell survival and drug resistance in myeloma cells (1, 2, 17). As shown in Fig. 6B, neither NF-κB inhibitor BAY11-7085 nor bortezomib has inhibitory effect on PDPK1 activity. In addition, although the basal NF-κB activity was much higher in AMO-1 cells compared with that in NCI-H929 cells, BX-912 has no inhibitory effect on NF-κB activity in both cell lines (Fig. 6C). These indicate that PDPK1-mediated signaling and NF-κB signaling promote myeloma cell survival as independent pathways. Given these results, we examined the combinatorial effects of BX-912 and various anticancer drugs, including BAY11-7085 and bortezomib, in the NCI-H929 cells and the AMO-1 cells. The combined use of BX-912 and clinically available agents, such as melphalan, etoposide, bortezomib, or BAY11-7085 were examined in two multiple myeloma derived cell lines, NCI-H929 and AMO-1. Cells were treated with various concentrations of agents for 48 hours. x-axis, fractional effect concentrations; y-axis, CI. CI is expressed as CI ± 1.96 SD. Shaded areas show the synergistic or additive effects of the two agents. In particular, areas below the bidirectional arrows indicate synergism of the two agents.

Figure 6. The utility of PDPK1 inhibition by BX-912 in the anti-MM therapy. A. AMO-1 cells and NCI-H929 cells were cultured with IL6 (100 ng/mL), BAFF (200 ng/mL), or IGF1 (100 ng/mL) in serum-starved (FBS-) media for 48 hours, and were further cultured for 3 hours with (+) or without (−) BX-912. Soluble factors or BX-912 were not added in control sample (c). Intensities of phospho (p)-PDPK1Ser241, p-RSK2Ser227, and p-AKTThr308 relative to those total levels were calculated with ImageJ software and described on individual bands. The activity of each kinase in serum-starved untreated control cells (c) was considered to 1.0. B. AMO-1 cells and NCI-H929 cells were treated by various concentrations of either BAY11-7085 or bortezomib for 3 hours. The activity of p-PDPK1Ser241 was examined by Western blotting. C, AMO-1 cells and NCI-H929 cells were treated by various concentrations of either BAY11-7085 or BX-912 for 3 hours. NF-κB activity was examined by ELISA. D, the combinatory effects of BX-912 and various anticancer agents (melphalan, etoposide, bortezomib, or BAY11-7085) were examined in two multiple myeloma derived cell lines, NCI-H929 and AMO-1. Cells were treated with various concentrations of agents for 48 hours. x-axis, fractional effect concentrations; y-axis, CI. CI is expressed as CI ± 1.96 SD. Shaded areas show the synergistic or additive effects of the two agents. In particular, areas below the bidirectional arrows indicate synergism of the two agents.
OPM-2/BTZ and KMS-11/BTZ cells (29). Although both OPM-2/BTZ and KMS-11/BTZ cells were highly resistant to bortezomib compared with their respective parental cell lines, they exhibited the same sensitivities to BX-912 as their respective parental cells (Supplementary Fig. S4A). Thus, we found no cross-resistance between the bortezomib and PDPK1 inhibitors. We also examined the effect of combinatorial blockade of PDPK1 and NF-kB in AMO-1 cells, NCI-H929 cells, and the bortezomib-resistant cells, using BX-912 and the anti-NF-kB agent, BAY11-7085. This combination therapy exhibited additive effects against all multiple myeloma cell lines examined (Fig. 6D and Supplementary Fig. S4B).

Discussion

The overlapping acquisitions of various cytogenetic/genetic abnormalities as well as the stimulation by the tumor microenvironment are essential for the development and progression of multiple myeloma. Because the types of cell intrinsic cytogenetic/genetic mutations and tumor environmental conditions vary widely among patients with multiple myeloma and even among subclones in a single patient (1, 2), it is unlikely that the therapeutic targeting of individual mutations or cell extrinsic stimulation would be equally effective for eliminating tumor cells of all patients. For the development of a new strategy to treat a broad range of patients with multiple myeloma, it would be ideal to identify a molecule that is responsible for the regulation of a signaling pathway upon which the stimuli by cell intrinsic and extrinsic abnormalities converge. In this study, we demonstrated that PDPK1 is generally activated through phosphorylation in multiple myeloma cell lines, regardless of their types of cytogenetic abnormalities, the existence of Ras or FGFR3 mutations, or the activation state of ERK, RSK2-CTKD, and AKT. In addition, PDPK1 was also found to be active in myeloma cells in approximately 90% of NDMM patients. The phosphorylation of amino acid residue at serine 241 of PDPK1 is essential for the activation of multiple AGC family protein kinases, such as RSK2, AKT, PAK, ILK, or PKC (26, 31, 37). Under physiologic conditions, PDPK1 is essential for B-cell development and survival (38, 39), whereas high PDPK1 activity is required for the proliferation and survival of tumor cells, including putative leukemia-initiating cells, and is also associated with disease progression and metastasis in various solid tumors and acute myeloid leukemias (40–42). However, its pathogenetic role has not been clarified in multiple myeloma.

On the basis of data presented herein, we propose that PDPK1 could be a rational therapeutic target molecule for a broad range of patients with multiple myeloma for the following reasons. First, PDPK1 regulates two pivotal cell signaling molecules, RSK2-NTKD and AKT, and, thereby, controls the activity of molecules that are essential for disease development, proliferation, and survival in myeloma cells. For instance, PDPK1 regulates the activity of PLK1, which may be associated with cell division and the expression of c-MYC (43). Indeed, PDPK1 inhibition caused G2–M cell-cycle arrest in myeloma cells. Furthermore, our data also revealed that PDPK1 regulates the MYC/IRF4 axis, which is essential for myeloma cell survival (44). Presumably, PDPK1 regulates the expression of c-MYC and IRF4 in myeloma cells, at least in part via PLK1, RSK2, and AKT (25, 36, 43, 44). CCNDs, which are critically important for myelomagenesis, are also regulated by PDPK1, presumably through RSK2-NTKD (1, 2, 25, 33), whereas proapoptotic BIM and BAD, which are essential for apoptosis of hematopoietic cells, are negatively regulated by PDPK1 through the activation of ERK/RSK2 with or without AKT activation. Presumably through the comprehensive regulation of those molecules critical for myeloma pathophysiology, PDPK1 inhibition exerted antiproliferative effects via the blockade of cell cycling and the induction of apoptosis in all primary myeloma cells and myeloma-derived cell lines examined, regardless of their molecular features. Second, PDPK1 was generally active in primary myeloma cells from patients with early-stage (aMM) to advanced-stage (RRMM) disease, and in multiple myeloma–derived cell lines, whereas RSK2-NTKD and AKT are also generally active in RRMM and in cell lines, but were less active or inactive in myeloma cells from some aMM and NDMM patients in our series. These findings implicate that PDPK1 activation may be involved in the pathophysiology of multiple myeloma from the early disease phase to the aggressive phase. Third, our IHC analysis revealed that small numbers of patients with inactive PDPK1 had significantly more favorable prognoses compared with the larger population of PDPK1-active patients. Because the response rates to the anti-MM therapies were not significantly different between the PDPK1(−) and the PDPK1(+) plus PDPK1(+) cohorts, the shorter PFS and OS of the PDPK1-active cohorts suggest that PDPK1 promotes the earlier acquisition of resistance to therapy and the expansion of residual tumor cells. It would be anticipated that inhibition of PDPK1 would improve the long-term outcome of the major population of patients with multiple myeloma with active PDPK1 similar to that of the PDPK1-inactive cohort. Because the numbers of patients with multiple myeloma analyzed were limited in this study, the clinical data presented here should be taken with caution. However, considering the significant prognostic impacts of PDPK1 activity in other cancers (40–42), the clinical significance of PDPK1 activity in multiple myeloma also needs to be addressed in the larger cohorts in future. Finally, PDPK1 inhibition exhibited additive and/or synergistic antimyeloma effects when combined with several clinically available drugs. In addition, the cytotoxic effect of PDPK1 inhibition was not hampered by the acquired resistance to bortezomib. Because the prognoses of patients with multiple myeloma who have acquired resistance toward novel agents, especially bortezomib, continue to be dismal, our results suggest that PDPK1 inhibition is useful for overcoming resistance to bortezomib are encouraging. It is also interesting that the inhibition of PDPK1 exerts an additive cytotoxic effect with the inhibition of NF-kB on myeloma cells. Because nearly 50% of multiple myeloma cell lines and most primary patient-derived primary myeloma cells have been shown to possess high levels of NF-kB activity due to both cell intrinsic and extrinsic mechanisms, the combination of inhibitors for NF-kB and PDPK1 may be an attractive future therapeutic strategy for multiple myeloma.
Although PDPK1 is most likely to be an attractive therapeutic target for multiple myeloma, it remains unknown whether or not PDPK1 inhibition is safe in the clinical setting, such as hyperglycemia (45–47). Our study showed that normal lymphocytes were much less sensitive to RX-912 compared with myeloma cells, and PDPK1 inhibition led to no life-threatening deleterious effect in several clinical trials for human and preclinical studies with animals (40, 43, 47). For instance, previous study has shown that PDPK1 hypomorphic mice expressing only 10% of the normal level of PDPK1 were viable, fertile, and exhibited no obvious harmful phenotype (48). Nevertheless, cautions for adverse events should be needed when PDPK1 inhibitor is used for human. Although rare, unforeseeable severe adverse events, such as cardiac events, have been occasionally reported with various molecular targeted agents, when utilized for human patients. It is expected to develop more powerful and selective inhibitors for PDPK1 that can induce anti-MM effects at much lower concentrations without profound toxicities. In addition, the utility of PDPK1 activity as a biomarker for predicting prognosis may be of value, because 10% of all patients with multiple myeloma with inactive PDPK1 can be isolated as having the favorable prognostic “PDPK1-inactive variant” myeloma. Our study also provides a new research topic in multiple myeloma. PDPK1 potently activates RSK2-NTKD and AKT. However, although PDPK1 and RSK2-NTKD are generally active in myeloma-derived cell lines, AKT was only active in limited cell lines. This suggests that although RSK2-NTKD activity is largely regulated by PDPK1, some unknown mechanisms are also involved in AKT activity in myeloma cells. Finally, the underlying cell intrinsic mechanism for PDPK1 activation in myeloma cells remains to be verified in this study. Recent studies have indicated that the deregulation of miR-375 (miR375) associates with the deregulated expression and activity of PDPK1 in various cancers (49). Moreover, our preliminary data coincide with the previous finding that reported the decreased expression of miR375 in a large majority of patients with multiple myeloma regardless of their cytogenetic features (50). We are currently investigating the functional role of miR375 deregulation for PDPK1 activation in myeloma cells. In conclusion, PDPK1 is generally phosphorylated in multiple myeloma, which is a highly molecularly heterogeneous hematologic disorder that continues to be incurable. Inactivation of PDPK1 is a promising and attractive therapeutic target for multiple myeloma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: Y. Chinen, J. Kuroda, M. Kiyota, Y. Matsumoto
Development of methodology: Y. Chinen, J. Kuroda
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Chinen, J. Kuroda, Y. Shimura, H. Nagoshi, N. Sakamoto, M. Ri, E. Kawata
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Chinen, J. Kuroda, Y. Shimura, H. Nagoshi, M. Yamamoto-Sugitani, N. Sakamoto, T. Kobayashi
Writing, review, and/or revision of the manuscript: Y. Chinen, J. Kuroda, H. Nagoshi, S. Horíke, S. Iida
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Chinen, J. Kuroda, Y. Shimura, N. Sakamoto, S. Horíke, S. Iida
Study supervision: Y. Chinen, S. Mizutani, M. Tanikawa

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Phosphoinositide Protein Kinase PDPK1 Is a Crucial Cell Signaling Mediator in Multiple Myeloma

Yoshiaki Chinen, Junya Kuroda, Yuji Shimura, et al.


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