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Protein Kinase D 1 Predicts Poor Treatment Response and Unfavorable Survival of Bortezomib-Based Treatment, and Its Knockdown Enhances Drug Sensitivity to Bortezomib in Multiple Myeloma

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Abstract

Objective: The present study aimed to explore the correlation of protein kinase D 1 with prognosis in bortezomib-treated multiple myeloma patients and further investigate the effect of protein kinase D 1 knockdown on drug sensitivity to bortezomib in multiple myeloma cells. **Methods:** Totally, 104 *de novo* symptomatic multiple myeloma patients treated with bortezomib-based regimens and 30 healthy controls were recruited. Bone marrow mononuclear cells–derived plasma cells were collected from multiple myeloma patients before initial treatment and from healthy controls on the bone marrow donation, respectively, then protein kinase D 1 protein/messenger RNA expressions were detected by Western blot and reverse transcription quantitative polymerase chain reaction, respectively. The effect of protein kinase D 1 knockdown on drug sensitivity to bortezomib was detected by transfecting protein kinase D 1 knockdown plasmid and control plasmid into RPMI8226 and U266 cells. **Results:** Protein kinase D 1 protein/messenger RNA expressions were both upregulated in multiple myeloma patients compared with healthy controls and presented good value in differentiating multiple myeloma patients from healthy controls. Furthermore, protein kinase D 1 protein/messenger RNA expressions were both associated with high International Staging System stage and *t* (4; 14). Furthermore, both complete response rate and overall response rate were reduced in protein kinase D 1 high patients compared with protein kinase D 1 low patients; similarly, progression-free survival and overall survival were both decreased in protein kinase D 1 high patients compared with protein kinase D 1 low patients. In addition, in RPMI8226 and U266 multiple myeloma cells, protein kinase D 1 knockdown increased drug sensitivity to bortezomib. **Conclusion:** Protein kinase D 1 has the potential to predict poor treatment response and unfavorable survival of bortezomib-based treatment in multiple myeloma patients, and its knockdown enhanced drug sensitivity to bortezomib in multiple myeloma cells.

Keywords

protein kinase D 1, multiple myeloma, drug sensitivity, prognosis, bortezomib

Abbreviations

AUC, area under the curve; BD, bortezomib/dexamethasone; BAD, Bortezomib/doxorubicin/dexamethasone; BCD, Bortezomib/cyclophosphamide/dexamethasone; BM, bone marrow; BMMCs, bone marrow mononuclear cells; BTD, Bortezomib/thalidomide/

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dexamethasone; CI, confidence interval; CR, complete response; EMT, epidermal-to-mesenchymal transition; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HC, healthy control; ISS, International Staging System; IMWG, International Myeloma Working Group; IQR, interquartile range; IC_{50}, U266 cells by 50%; JNK, c-Jun N-terminal kinase; MM, Multiple myeloma; M $\pm$ SD, mean $\pm$ standard deviation; mRNA, messenger RNA; NF-κB, nuclear factor kappa B; ORR, overall response rate; OS, overall survival; PFS, Progression-free survival; PKD1, Protein kinase D 1; PR, Partial response; qPCR, quantitative polymerase chain reaction; RPMI, Roswell Park Memorial Institute; VGPR, Very good partial response

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Introduction

Multiple myeloma (MM), the malignancy of plasma cells, is characterized by clonal proliferation of plasma cells accumulating in the bone marrow (BM), which leads to bone destruction and marrow failure.[1] The epidemiology reports that MM accounts for 1.8% of all cancer types and represents as one of the most common hematologic malignancies globally.[1,2] The progression of MM is associated with the presence of genetic mutations, chromosome abnormalities, and epigenetic alterations, which contribute to a more aggressive MM.[3,4] Due to the development of various treatment approaches and therapeutic agents, such as bortezomib, the clinical outcomes of patients with MM have achieved a great improvement and the treatment responses become durable.[5-7] Bortezomib, the first-line proteasome inhibitor, selectively inhibits the ubiquitin proteasome pathway and degrades various intracellular proteins involved in the MM progression, which is antimyeloma in MM treatment.[8,9] Although bortezomib is reported to have great efficacy against MM, approximately 20% patients with MM still do not respond to bortezomib over time or suffer from expectable disease relapse.[8,10] Therefore, it is important to enhance the understanding of MM pathology and to identify novel biomarkers for predicting treatment response to bortezomib in MM treatment.

Protein kinase D 1 (PKD1), a member of protein kinase D family of serine/threonine, resides in diverse subcellular locations, including cytosol, Golgi apparatus, nucleus, and mitochondria.[11] Activation of PKD1 is implicated in various cellular functions, such as Golgi generation, plasma membrane–directed transport, and cell immune responses, as well as involved in the physiological activities associated with malignant progression, such as cell proliferation, and epidermal-to-mesenchymal transition (EMT).[12,13] Recent studies have revealed that PKD1 is correlated with somatic mutations, which further cause various phenomenon for oncogenesis, and its biological role has been demonstrated in the progression of several solid tumors via modulating several signaling pathways, including nuclear factor kappa B (NF-κB) signaling, mitogen-activated protein Kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling, c-Jun N-terminal kinase (JNK) signaling.[14-16] These signaling pathways have been reported to play critical roles in the pathophysiology of MM.[10,17,18] Activation of NF-κB signaling contributes to the interaction of myeloma cells with the BM microenvironment, and MEK/ERK-signaling is associated with RAS signaling cascade, resulting in mutation-driven MM cases.[10,18] As for JNK signaling, it is shown to be involved in the MM cellular process, including migration, proliferation, apoptosis, and so on.[17] In addition, prior evidence shows that the bortezomib resistance is associated with the activation of NF-κB and inactivation of JNK signaling pathways, meanwhile, PKD1 has a strong regulatory effect on NF-κB signaling as well as JNK signaling pathways. Therefore, we hypothesized that PKD1 might be implicated in the pathology and the generation of bortezomib resistance in MM.[11,19,20] However, there is still no evidence available. We performed this clinical study to explore the correlation of PKD1 with treatment response and survival in patients with MM who treated with bortezomib-based regimen and further conducted cellular experiments to investigate the effect of PKD1 knockdown on drug sensitivity to bortezomib in MM cells.

Materials and Methods

Participants

A total of 104 *de novo* symptomatic patients with MM who were treated with bortezomib-based regimens in our hospital were consecutively enrolled. And the period of the enrollment ranged from January 2016 to December 2019. The inclusion criteria were (1) newly diagnosed as symptomatic MM according to International Myeloma Working Group (IMWG) diagnosis criteria for MM,[21] (2) aged between 18 and 80 years, (3) scheduled to receive bortezomib-based regimens, and (4) no active infectious diseases. The exclusion criteria were (1) relapsed MM or mixed MM; (2) complicated with other hematologic malignancies or solid tumors; (3) primary or secondary plasma cell leukemia; (4) history of chemotherapy, radiotherapy, or stem cell transplantation; and (5) females who were pregnant or lactating. Besides, 30 healthy participants who underwent BM donation in our hospital were enrolled as healthy controls (HCs). This study was approved by the institutional review board of our hospital. All participants or their guardians provided the written informed consents before enrollment.

Data Collection

The demographics, immunoglobulin subtype, organ impairment, Durie-Salmon stage, International Staging System (ISS) stage, biochemical indexes level, and key cytogenetics of

patients with MM were collected after enrollment. Organ impairment including bone lesion and renal impairment were assessed according to the IMWG diagnosis criteria for MM.[21] Durie-Salmon stage of patients with MM was evaluated referring to the criteria of Durie-Salmon based on hemoglobin level, serum calcium level, bone X-ray result, and low M-component production rate.[22] The ISS stage of patients with MM was evaluated referring to the criteria of ISS, based on serum β-2 microglobulin level and serum albumin level.[22,23]

Sample Collection

For patients with MM, the BM samples were collected before initiation of treatment, and for HCs, the BM samples were collected when undergoing BM donation. After collection, the BM samples were processed by gradient density centrifugation for separating the bone marrow mononuclear cells (BMMCs). Then, CD138-positive plasma cells were purified from BMMCs using CD138-coated magnetic beads (Miltenyi Biotec).

Protein Kinase D 1 Protein Detection

The expression of PKD1 protein in plasma cells from patients with MM and HCs was detected by Western blot assay. Briefly, protein in the plasma cells was extracted using RIPA Buffer (Sigma-Aldrich) and quantified with the use of Bicinchoninic Acid Kit for Protein Determination (Sigma-Aldrich). Then, equal amount of protein was separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (Invitrogen) before being transferred to a polyvinylidene difluoride membrane (Whatman GmbH). The membrane was incubated with an anti-PKC mu/PKD antibody (1:1000, Abcam) overnight at 4 °C. Next day, the membrane was incubated with goat anti-Rabbit IgG H&L (horse radish peroxidase; 1:3000, Abcam) antibody for 1.5 hours at room temperature. Finally, the membrane was visualized by Amersham ECL detection system (GE Healthcare), and the image was obtained on Gel Imager (Thermo Scientific).

Protein Kinase D 1 mRNA Detection

The expression of PKD1 messenger RNA (mRNA) in plasma cells from patients with MM and HCs was detected by reverse transcription–quantitative polymerase chain reaction. Total RNA was extracted from plasma cells using TRIzol Reagent (Invitrogen) and then reversely transcribed using ReverTra Ace quantitative polymerase chain reaction (qPCR) RT Master Mix (Toyobo). Subsequently, qPCR was performed using SYBR Green Realtime PCR Master Mix (Toyobo) to quantify PKD1 expression. In addition, PKD1 expression was calculated using $2^{-\Delta\Delta Ct}$ method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference. The primers of PKD1 and GAPDH used in this study were as follows: PKD1 forward primer: 5′-GCCAGAGCGCATAACGAA-3′, reverse primer: 5′-TACCCACCACTCACCTCC-3′; GAPDH forward primer: 5′-TGACCACAGTCCATGCCATCAC-3′, reverse primer: 5′-GCCTGCTTCACCACCTTCTTGA-3′.

Treatment and Assessment

All patients with MM received induction therapy with regimens based on proteasome inhibitor (bortezomib), which included: bortezomib/dexamethasone (BD), bortezomib/doxorubicin/dexamethasone (BAD), bortezomib/cyclophosphamide/dexamethasone (BCD) or bortezomib/thalidomide/dexamethasone (BTD). After 2-cycle treatment, clinical response to induction therapy was assessed according to national comprehensive cancer network (NCCN) clinical practice guidelines in Oncology: Multiple Myeloma (2015.V4). And complete response (CR) was defined as negative immunofixation on the serum and urine and disappearance of any soft tissue plasmacytomas and $\leq 5\%$ plasma cells in BM. Very good partial response (VGPR) was defined as serum and urine M-protein detectable by immunofixation but not on electrophoresis or 90% or greater reduction in serum M-protein plus urine M-protein level <100 mg per 24 hours. Partial response (PR) was defined as 50% reduction of serum M-protein and reduction in 24 hours urinary M-protein by 90% or to <200 mg per 24 hours. Besides, overall response rate (ORR) was defined as the proportion of patients who achieved CR, VGPR, or PR.

Follow-Up

Regular follow-up was carried out and the last follow-up date was December 31, 2019. Progression-free survival (PFS) was calculated from the date of initiation of treatment to the date of disease progression, disease relapse, or death. Overall survival (OS) was calculated from the date of initiation of treatment to the date of death. As for the patients not known whether the disease had progressed or whether they had died at the last follow-up date, they were censored on the date of last visit or the date they were last known to be alive.

Cell Culture

Human MM cell lines RPMI8226 and U266 were purchased from the American Type Culture Collection. RPMI8226 and U266 cells were cultured in 90% Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco) with 10% fetal bovine serum (Gibco) at the condition of 37 °C with 95% air and 5% CO_2.

Transfection and Drug Sensitivity

The PKD1 knockdown plasmid and the control knockdown plasmid were constructed using pGPH1 vector (Invitrogen). Then the plasmids were transfected into RPMI8226 and U266 MM cells using HilyMax (Dojindo). The cells transfected with PKD1 knockdown plasmid were defined as PKD1(−) cells, and the cells transfected with control knockdown plasmid were defined as control(−) cells. After transfection for 24 hours, both PKD1(−) cells and control(−) cells were plated in 96 multi-well plates with a concentration of 5000 cells/well. Then, the PKD1(−) cells and control(−) cells

were incubated with different concentration (0, 1, 2, 4, 8, 16, and 32 nM) of bortezomib (Velcade) for another 48 hours. After incubation with bortezomib for 48 hours, the viability of PKD1(−) cells and control(−) cells was determined by Cell Counting Kit-8 (Dojindo Molecular Technologies), according to manufacturer's manual. And the relative cell viability was calculated referring to the viability of 0 nM bortezomib treated PKD1(−) cells and control(−) cells, respectively. Finally, the bortezomib concentration required to inhibit growth of RPMI8226 and U266 cells by 50% (IC_{50}) value was estimated by Probit regression analysis.

Statistical Analysis

Statistical analysis was performed using SPSS 24.0 software (IBM), and the figure was plotted using GraphPad Prism 7.00 software (GraphPad Software). Data were expressed as mean ± standard deviation, median (interquartile range, IQR), and count (percentage). According to the median value of PKD1 protein expression in plasma cells extracted from patients with MM, all patients with MM were classified as PKD1 protein high expression group and PKD1 protein low expression group. And according to the median value of PKD1 mRNA expression in plasma cells extracted from patients with MM, all patients with MM were classified as PKD1 mRNA high expression group and PKD1 mRNA low expression group as well. Comparison between 2 groups was determined by Wilcoxon rank-sum test or Student *t* test. Correlation was determined by χ^2 test or Spearman rank correlation test. The ability of PKD1 protein/mRNA in discriminating patients with MM from HCs was assessed by receiver operating characteristic curve and area under the curve (AUC) with 95% CI. Kaplan-Meier curve was plotted to illustrate PFS and OS, and the difference in PFS and OS between 2 groups was determined by Log-rank test. *P* value <.05 was considered as significant.

Results

Clinical Characteristics

The mean age of patients with MM was 54.5 ± 7.8 years (Table 1). There were 43 females and 61 males included. Regarding immunoglobulin subtype, there were 57 (54.8%) patients with IgG, 28 (26.9%) patients with IgA, and 19 (18.3%) patients with other immunoglobulin subtypes. The number of patients at Durie-Salmon stages II and III was 11 (10.6%) and 93 (89.4%), respectively. The number of patients at ISS stages I, II, and III was 25 (24.0%), 34 (32.7%), and 45 (43.3%), respectively. As for key cytogenetics, there were 11 (10.6%) patients with *t*(4; 14), 8 (7.7%) patients with *t*(11; 14), 6 (5.8%) patients with *t* (14; 16) and 5 (4.8%) patients with Del (17p). In terms of treatment regimens received, 41 (39.5%) patients received BAD, 30 (28.8%) patients received BCD, 23 (22.1%) patients received BTD, and 10 (9.6%) patients received BD. More detailed information of clinical characteristics was shown in Table 1.

Table 1. Characteristics of Patients With MM.

Items	MM patients (N = 104)
Demographics	
Age (years), M ± SD	54.5 ± 7.8
Gender (female/male), no.	43/61
Immunoglobulin subtype, no. (%)	
IgG	57 (54.8)
IgA	28 (26.9)
Others	19 (18.3)
Organ impairment, no. (%)	
Bone lesion	74 (71.2)
Renal impairment	36 (34.6)
Durie-Salmon stage, no. (%)	
II	11 (10.6)
III	93 (89.4)
ISS stage, no. (%)	
I	25 (24.0)
II	34 (32.7)
III	45 (43.3)
Biochemical indexes	
Hb (g/L), M ± SD	99.3 ± 24.6
Calcium (mg/dL), M ± SD	9.7 ± 2.1
Scr (mg/dL), M ± SD	1.8 ± 0.6
ALB (g/L), median (IQR)	33.5 (28.3-37.0)
β2-MG (mg/L), median (IQR)	5.0 (2.5-7.5)
LDH (U/L), median (IQR)	210.0 (182.2-241.4)
Key cytogenetics, no. (%)	
t (4; 14)	11 (10.6)
t (11;14)	8 (7.7)
t (14; 16)	6 (5.8)
Del (17p)	5 (4.8)
Induction therapy	
BAD	41 (39.5)
BCD	30 (28.8)
BTD	23 (22.1)
BD	10 (9.6)

Abbreviations: ALB, albumin; BAD, bortezomib/doxorubicin/dexamethasone; BCD, bortezomib/cyclophosphamide/dexamethasone; BTD, bortezomib/thalidomide/dexamethasone; BD, bortezomib/dexamethasone; Hb, hemoglobin; IgA, immunoglobulin A; IgG, immunoglobulin G; IQR, interquartile range; ISS, International Staging System; LDH, lactate dehydrogenase; MM, multiple myeloma; M ± SD, mean ± standard deviation; Scr, serum creatinine; β2-MG, beta-2-microglobulin.

Protein Kinase D 1 Expression in Patients With MM and HCs

Protein Kinase D 1 protein expression was increased in patients with MM (0.691 [0.458-0.991]) compared with HCs (0.303 [0.138-0.632] ; $P < .001$; Figure 1A and B). Protein kinase D 1 mRNA expression was also elevated in patients with MM (2.391 [1.803-3.213]) compared with HCs (1.005 [0.593-1.653] ; $P < .001$; Figure 1C). Besides, PKD1 protein expression (AUC: 0.901, 95% CI: 0.714-0.905) and PKD1 mRNA expression (AUC: 0.837, 95% CI: 0.754-0.920) were both of good value in differentiating patients with MM from HCs (Figure 1D).

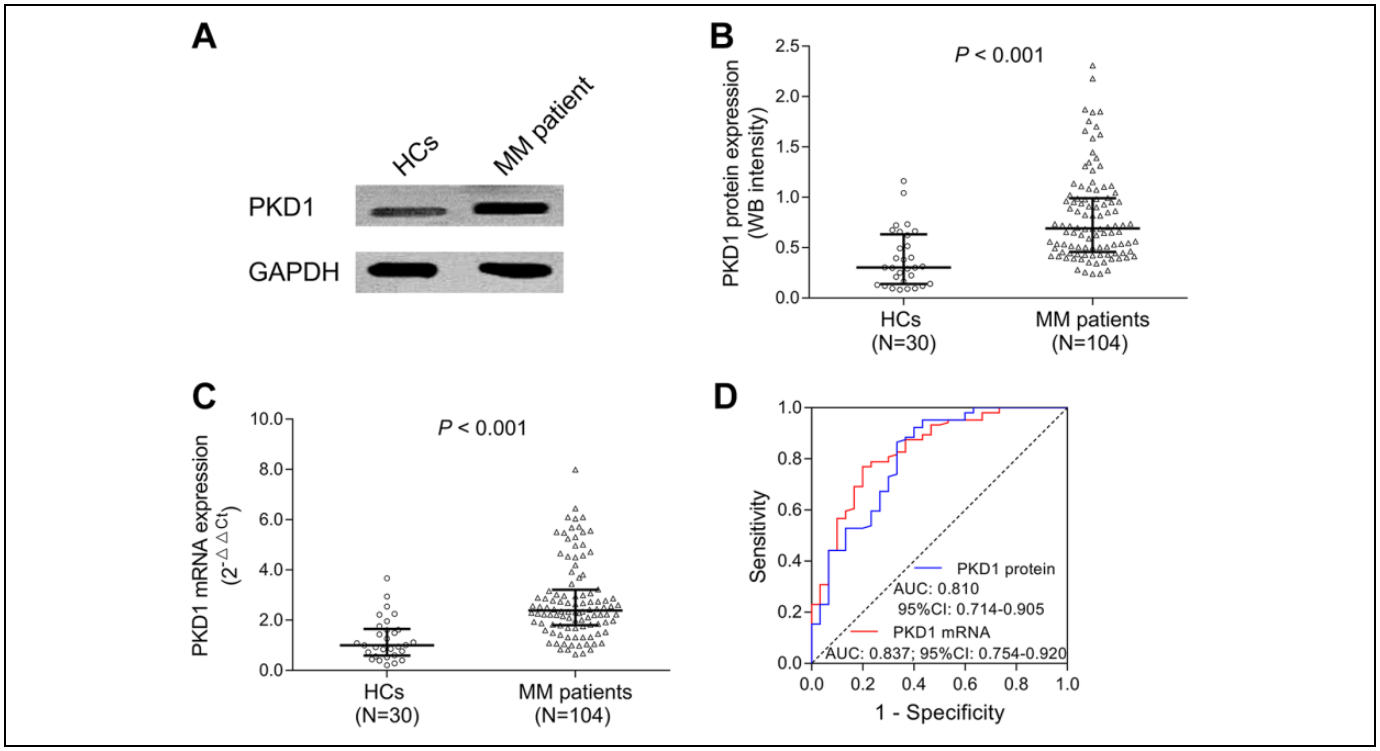

Figure 1. PKD1 was upregulated in patients with MM compared with HCs. Comparison of PKD1 protein (A, B) and mRNA (C) expression between patients with MM and HCs. The ability of PKD1 protein/mRNA in discriminating patients with MM from HCs (D). AUC indicates area under the curve; HC, healthy controls; MM, multiple myeloma; mRNA, messenger RNA; PKD1, protein kinase D 1; ROC, receiver operating characteristic; WB, Western blot.

Correlation of PKD1 With Disease Stage and Key Cytogenetics in Patients With MM

According to the median value of PKD1 protein (median value: 0.691, IQR: 0.458-0.991)/mRNA (median value: 2.391, IQR: 1.803-3.213) expression in patients with MM, all patients with MM were classified as PKD1 protein/mRNA high expression group and PKD1 protein/mRNA low expression group, respectively. Protein kinase D 1 protein expression was positively associated with ISS stage ($P = .006$) and $t(4; 14)$ ($P = .026$), while there was no correlation of PKD1 protein expression with immunoglobulin subtype ($P = .676$), Durie-Salmon stage ($P = .753$), $t(11; 14)$ ($P = .141$), $t(14; 16)$ ($P = .400$) or Del (17p) ($P = .647$; Table 2). Furthermore, PKD1 mRNA expression was positively associated with ISS stage ($P = .017$) and $t(4; 14)$ ($P = .026$), while there was no correlation of PKD1 mRNA expression with immunoglobulin subtype ($P = .729$), Durie-Salmon stage ($P = .344$), $t(11;14)$ ($P = .462$), $t(14; 16)$ ($P = .093$), or Del (17p) ($P = .647$), either.

Correlation of PKD1 With Treatment Response to Bortezomib in Patients With MM

Complete response ($P = .024$) and ORR ($P = .007$) were both reduced, but VGPR was similar ($P = .126$) in patients with PKD1 protein high expression compared with those with PKD1 protein low expression, while there was no difference of VGPR between (Table 3). Similarly, ORR ($P = .028$) and VGPR ($P = .001$) were both decreased in patients with PKD1 mRNA high expression compared with those with PKD1 mRNA low expression; however, there was no difference of CR between patients with PKD1 mRNA high expression and those with PKD1 mRNA low expression ($P = .065$).

Correlation of PKD1 With PFS and OS in Patients With MM

Progression-free survival ($P = .036$; Figure 2A) and OS ($P = .029$; Figure 2B) were reduced in patients with PKD1 protein high expression compared with patients with PKD1 protein low expression. Progression-free survival ($P = .023$; Figure 2C) and OS ($P = .008$; Figure 2D) were also decreased in patients with PKD1 mRNA high expression compared with patients with PKD1 mRNA low expression.

Effect of PKD1 Knockdown on Drug Sensitivity to Bortezomib in MM Cells

In RPMI8226 cells, relative cell viability was decreased in 2 nM ($P = .008$), 4 nM ($P = .023$) bortezomib-treated PKD1(−) cells compared with control(−) cells, while it was similar between 0 nM ($P = .980$), 1 nM ($P = .171$), 8 nM ($P = .582$), 16 nM ($P = .640$), and 32 nM ($P = .953$) bortezomib-treated PKD1(−) cells and control(−) cells (Figure 3A);

Table 2. Correlation of PKD1 Protein/mRNA Expression With Disease Stage and Key Cytogenetics.[a]

	PKD1 protein expression			PKD1 mRNA expression		
Items	Low (n = 52)	High (n = 52)	*P* value	Low (n = 52)	High (n = 52)	*P* value
Immunoglobulin subtype, no. (%)			.676			.729
IgG	30 (57.7)	27 (51.9)		30 (57.7)	27 (51.9)	
IgA	12 (23.1)	16 (30.8)		14 (26.9)	14 (26.9)	
Others	10 (19.2)	9 (17.3)		8 (15.4)	11 (21.2)	
Durie-Salmon stage, no. (%)			.753			.344
II	6 (11.5)	5 (9.6)		7 (13.5)	4 (7.7)	
III	46 (88.5)	47 (90.4)		45 (86.5)	48 (92.3)	
ISS stage, no. (%)			.006			.017
I	17 (32.7)	8 (15.4)		18 (34.6)	7 (13.5)	
II	19 (36.5)	15 (28.8)		16 (30.8)	18 (34.6)	
III	16 (30.8)	29 (55.8)		18 (34.6)	27 (51.9)	
t(4; 14), no. (%)			.026			.026
No	50 (96.2)	43 (82.7)		50 (96.2)	43 (82.7)	
Yes	2 (3.8)	9 (17.3)		2 (3.8)	9 (17.3)	
t(11;14)			.141			.462
No	46 (85.5)	50 (96.2)		47 (90.4)	49 (94.2)	
Yes	6 (11.5)	2 (3.8)		5 (9.6)	3 (5.8)	
t(14; 16), no. (%)			.400			.093
No	50 (96.2)	48 (92.3)		51 (98.1)	47 (90.4)	
Yes	2 (3.8)	4 (7.7)		1 (1.1)	5 (9.6)	
Del (17p), no. (%)			.647			.647
No	50 (96.2)	49 (94.2)		50 (96.2)	49 (94.2)	
Yes	2 (3.8)	3 (5.8)		2 (3.8)	3 (5.8)	

Abbreviations: IgA, immunoglobulin A; IgG, immunoglobulin G; ISS, International Staging System; PKD1, protein kinase D1.
[a]Correlation was determined by χ^2 test or Spearman rank correlation test.

Table 3. Correlation of PKD1 Protein/mRNA Expression With Treatment Response.[a]

	PKD1 protein expression			PKD1 mRNA expression		
Items	Low (n = 52)	High (n = 52)	*P* value	Low (n = 52)	High (n = 52)	*P* value
CR, no. (%)			.024			.065
No	28 (53.8)	39 (75.0)		29 (55.8)	38 (73.1)	
Yes	24 (46.2)	13 (25.0)		23 (44.2)	14 (26.9)	
VGPR, no. (%)			.126			.001
No	34 (65.4)	41 (78.8)		30 (57.7)	45 (86.5)	
Yes	18 (34.6)	11 (21.2)		22 (42.3)	7 (13.5)	
ORR, no. (%)			.007			.028
No	5 (9.6)	16 (30.8)		6 (11.5)	15 (28.8)	
Yes	47 (90.4)	36 (69.2)		46 (88.5)	37 (71.2)	

Abbreviations: CR, complete response; ORR, overall response rate; PKD1, protein kinase D1; VGPR, very good partial response.
[a]Correlation was determined by χ^2 test.

meanwhile, IC_{50} value of bortezomib was decreased in PKD1(−) cells compared with control(−) cells ($P = .021$; Figure 3B). In U266 cells, relative cell viability was decreased in 2 nM ($P = .016$), 4 nM ($P = .018$) bortezomib-treated PKD1(−) cells compared with control(−) cells, while it was similar in 0 nM ($P = .651$), 1 nM ($P = .075$), 8 nM ($P = .523$), 16 nM ($P = .793$), and 32 nM ($P = .914$) bortezomib-treated PKD1(−) cells and control(−) cells (Figure 3C); besides, IC_{50} value of bortezomib was decreased in PKD1(−) cells compared with control(−) cells ($P = .003$; Figure 3D). These data suggested that PKD1 knockdown increased drug sensitivity to bortezomib in MM cells.

Discussion

In the present study, we found that (1) PKD1 was upregulated in patients with MM compared with HCs and presented good value in differentiating patients with MM from HCs. (2) PKD1 was positively associated with ISS stage and *t*(4; 14) in patients with MM. (3) PKD1 was negatively associated with treatment response and survival with regard to bortezomib-based treatment in patients with MM. (4) In cellular experiments, PKD1 knockdown increased drug sensitivity to bortezomib in MM cells.

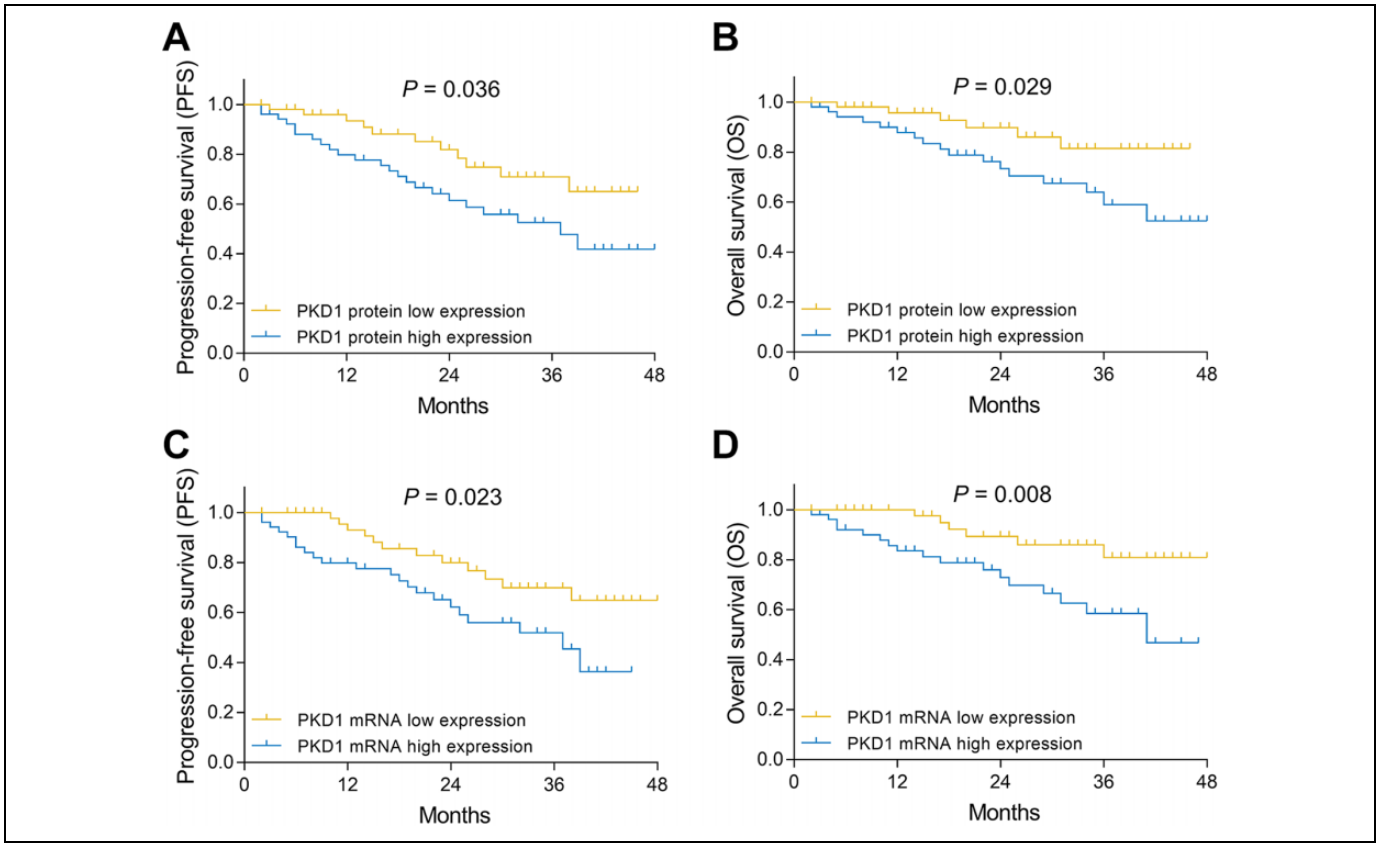

Figure 2. PKD1 was associated with survival profiles in patients with MM. Comparison of PFS (A) and OS (B) between PKD1 protein high patients and PKD1 protein low patients. Comparison of PFS (C) and OS (D) between PKD1 mRNA high patients and PKD1 mRNA low patients. MM indicates multiple myeloma; OS, overall survival; PFS, progression-free survival; PKD1, protein kinase D 1.

Protein kinase D 1 is one isoform of PKD, which serves as an important regulator in various biological processes including protein trafficking, oxidative stress response, and immune response.[13] Besides, as a signaling regulator protein, the involvement of PKD1 in malignant signaling pathway has recently attracted increasing attention.[11,13,24,25] Mechanically, PKD1 activates the downstream vascular endothelial growth factor–induced ERK signaling pathway and modulates the MEK/ERK pathway, promoting cell proliferation via phosphorylation in skin cancer.[26,27] Also, PKD1 modulates NF-κB signaling pathway, which regulates transcription factor snail-induced EMT, tumor initiation, and metastasis in breast cancer.[28] In addition, the MEK/ERK pathway cascade is associated with RAS/RAF mutations, further driving mutation-inducing MM, and the activation of NF-κB is critical in supporting MM cell growth as well as in the formation of drug resistance.[19,29] Therefore, we speculated that PKD1 might be upregulated and associated with clinical characteristics in patients with MM, while the related evidence is lacking. First, we found that PKD1 mRNA and protein expressions were both increased in patients with MM compared with HCs and presented good value in distinguishing patients with MM from HCs. Furthermore, PKD1 was positively associated with ISS stage and $t(4; 14)$ in patients with MM. The possible reasons might include the following: (1) Previous evidence showed that PKD1 upregulation linked to the P53 mutation and the biallelic events in tumor suppressor TP53, which led to abnormal cell-cycle arrest, apoptosis, and malignancy initiation in MM. Therefore, PKD1 level was elevated in patients with MM compared with HCs.[30] (2) In addition, PKD1 might lead to a set of chromosomal abnormalities including immunoglobulin heavy chain translocations (eg, t (4; 14)), dysregulated oncogenes (eg, CCND1) via activating NF-κB signaling and stimulating RAS-RAF mutations, which further led to the progression of MM; hence, patients with MM with upregulation of PKD1 might have worse disease condition.[13,31]

Bortezomib is one of the main backbone agents of antimyeloma treatment, and previous studies reveal that bortezomib not only selective and reversible inhibits proteasome but also induces MM cell death through suppressing NF-κB signaling, which exerted its antineoplastic action in MM treatment.[8,20] However, some patients with MM still suffer from poor treatment response to bortezomib, which leads to unfavorable prognosis. Therefore, it is important to investigate the biomarkers for predicting treatment response and survival profiles with regard to bortezomib-based treatment in patients with MM. In addition, according to the prior evidence, PKD1 has regulatory effect on NF-κB and JNK signaling, and drug resistance to bortezomib is associated with stimulation of NF-κB signaling but inactivation of JNK signaling.[11,19,20] Moreover, PKD1

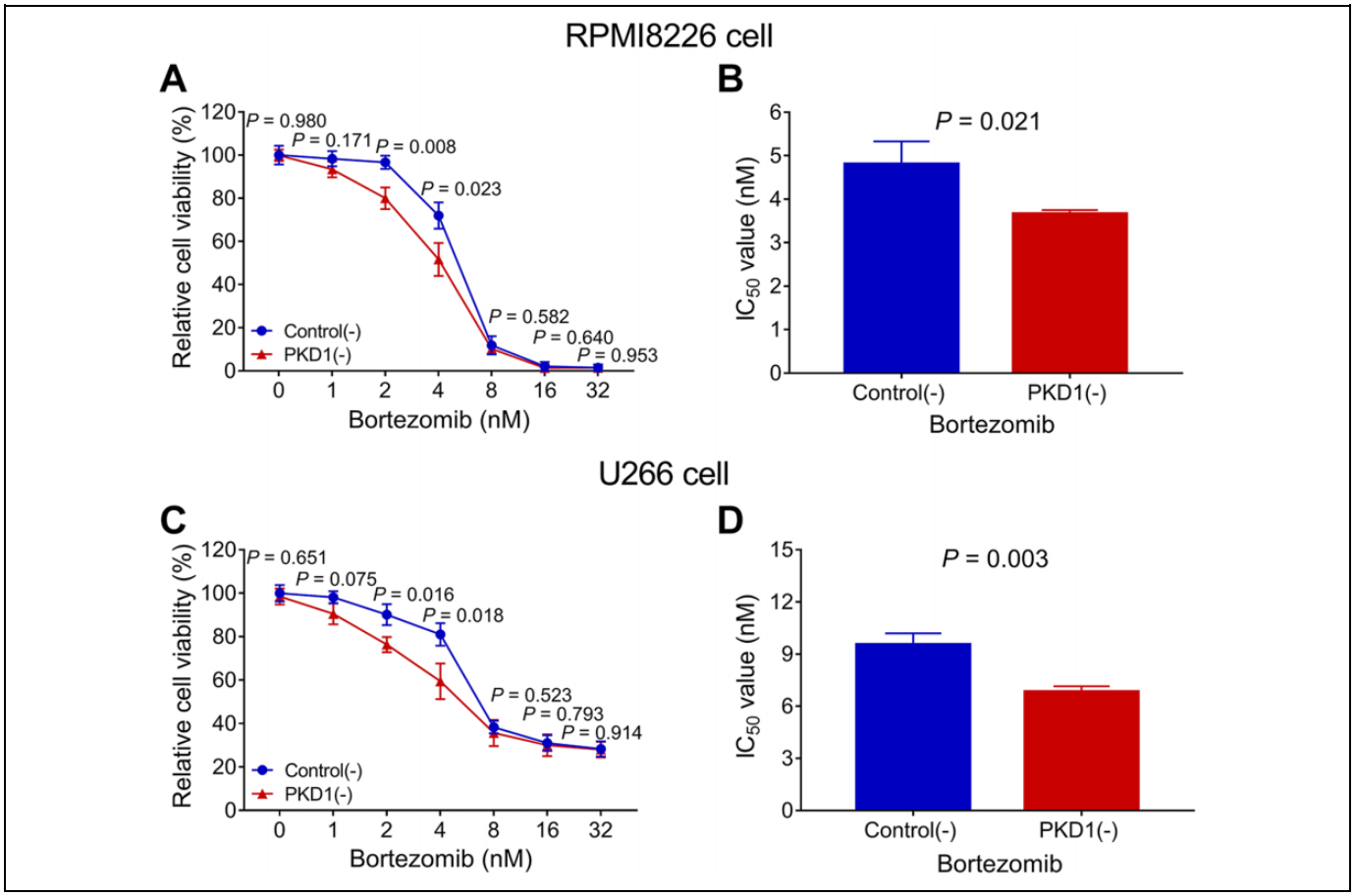

Figure 3. PKD1 knockdown increased drug sensitivity to bortezomib in MM cells. Comparison of relative cell viability between 0, 1, 2, 4, 8, 16, and 32 nM bortezomib-treated PKD1(−) and control(−) of RPMI8226 cells (A). Comparison of IC_{50} value of bortezomib between PKD1(−) and control(−) of RPMI8226 cells (B). Comparison of relative cell viability between 0, 1, 2, 4, 8, 16, and 32 nM bortezomib-treated PKD1(−) and control(−) of U266 cells (C). Comparison of IC_{50} value of bortezomib between PKD1(−) and control(−) of U266 cells (D). Control(−) cells, the cells transfected with control knockdown plasmid; IC_{50}, drug concentration required to inhibit growth cells by 50%. MM indicates multiple myeloma; PKD1, protein kinase D 1; PKD1(−) cells, the cells transfected with PKD1 knockdown plasmid.

activates MEK/ERK signaling cascade, which is associated with RAS/RAF mutations, which is involved in the development of drug resistance in MM treatment. According to these aforementioned evidence, we speculated that PKD1 might be correlated with prognosis in patients with MM treated with bortezomib-based regimens and found that PKD1 was negatively associated with treatment response and survival with regard to bortezomib-based treatment in patients with MM. The possible reasons might include that (1) PKD1 might decrease drug sensitivity to bortezomib in MM cells, thus patients with MM with PKD1 high expression had poor treatment response to bortezomib. This was further explored in our following cellular experiments. (2) According to the previous finding, PKD1 was associated with the presence of *t*(4; 14) and advanced ISS stage in patients with MM, which were considered to be high-risk factor for poor prognosis; therefore, patients with MM with higher PKD1 expression might display poor prognosis.[32]

The above results suggested that PKD1 was involved in the generation of drug resistance to bortezomib in patients with MM, and furthermore, we conducted cellular experiments to investigate whether PKD1 knockdown had influence on drug sensitivity to bortezomib. We observed that PKD1 knockdown enhanced drug sensitivity to bortezomib in MM cells. The possible reasons might include (1) PKD1 might facilitate carcinogenesis and decrease drug sensitivity to bortezomib via activating NF-κB and inactivating JNK cascade. Therefore, PKD1 knockdown increased drug sensitivity to bortezomib in MM cells.[19,20] (2) According to the abovementioned results, PKD1 was associated with genetic mutations (such as *t* (4; 14)), which might further contribute to the formation of drug resistance, thereby, PKD1 knockdown might increase drug sensitivity to bortezomib in MM cells.[33]

However, there still existed some limitations in our study including (1) our study was a single-center study, while a further larger sample from multiple centers were needed for validating the results; (2) since the present study excluded the patients with relapsed MM or mixed MM, the potential prognostic role of PKD1 was needed further investigation in these patients; and (3) the detailed mechanism of PKD1 knockdown

in inducing drug sensitivity to bortezomib was not included in the present study, which needed further exploration.

In summary, PKD1 has the potential to predict poor treatment response and survival in patients with MM treated with bortezomib-based regimens, and its knockdown enhanced drug sensitivity to bortezomib in MM cells, suggesting its clinical significance as a prognostic biomarker to bortezomib therapy in MM management.

Authors' Note

X.L. and Y.Y. contributed equally to this work. The present study was approved by the Wuhan First Hospital medical ethic committee with the approval number "2015-09." All participants or their guardians provided the written informed consents before enrollment.

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