Supporting Information

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SI Text

Cell Lines, Patients, and Tissues Sample. LNCaP, PC-3 and DU145 cells were obtained from the American Type Culture Collection. PrEC cells were obtained from Clonetics. All cell lines were maintained with suitable medium (F-12 supplemented with 10% FCS for LNCaP and PC-3, MEM supplemented with 10% FCS for DU145, PrEGM for PrEC) under 5% CO_2.

Paired human untreated primary prostate cancer tissues and normal [or benign prostatic hypertrophy (BPH)] ($n = 29$) tissues from same patients were obtained during radical prostatectomy at Yokohama City University Hospital and its affiliates. The sampling and usage of all prostate tissues in this study were approved by the ethical committee of Yokohama City University Graduate School of Medicine and performed only after obtaining informed consent from each patient. Patients received neither hormonal nor radiation therapy pre- or postoperatively. All prostate cancer and normal/BPH tissues were obtained from specimens by conventional microdissection. All tissues were stored at -80 °C until each experiment. Clinical features are listed at Table S1. Pathological diagnosis was performed according to the Japanese General Rules for Clinical and pathological Studies on Prostate Cancer (1). Whole frozen tissues were used for RNA extraction.

RNA Extraction and Real-Time Quantitative PCR (qPCR). Total RNA from cell lines, prostate tissues and xenografts were extracted using ISOGEN (NipponGene) according to the manufacturer's instructions. After cDNA had been synthesized with random hexomers and MMLV (Moloney Murine Leukemia Virus), qPCR was performed with an iCycler and SYBR Green Supermix (Bio-Rad). Primers for aPKCλ/ι and IL-6 were determined by PrimerExpress software (Applied Biosystems). β-actin was used as an internal control. Primer sequences were as follows; aPKCλ/ι 5′-TTT GCC ACA GGA ACC AGT GA-3′ and 5′-TTT GCC ACT TTC CCT GGT GT-3′, IL-6 5′-ACC AGG CAA GTC TCC TCA TTG A-3′ and 5′-GCC CTG AGA AAG GAG ACA TGT AAC-3′, β-actin 5′- TGA GCG CGG CTA CAG CTT-3′ 5′- TCC TTA ATG TCA CGC ACG ATT T-3′. Reaction conditions were 3 min at 95 °C for preheating, then 30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C for 40 cycles. All specific quantities were divided by the quantity of β-actin. DU145 cDNA was used for standard curve preparation. All PCR products were confirmed using the melt curve method for specific amplification.

Immunohistochemistry. Tissue microarrays with a 3-mm diameter were prepared from formalin-fixes and paraffin-embedded tissue blocks using the manual tissue microarray technique (Azumaya Corp.). Briefly, 4-μm thick paraffin sections were deparaffinized, rehydrated in ethanol, and autoclaved (121 °C, 15 min) in 10 mM citrate buffer (pH 6.0) for antigen retrieval. The sections were then immersed in 0.3% hydrogen peroxide at room temperature for 30 min to quench the intrinsic peroxidase activity and further incubated with the anti-aPKCι antibody (1:500 dilution) at 4 °C overnight. Labeled antigens were visualized with a Histo Fine kit (Nichirei), followed by a DAB plus reaction (Dako Cytomation). Finally, the sections were counterstained with hematoxylin. Low intensity (0 and + 1) and high intensity ($+2 \leq$) were given based on the predominant pattern in each case.

Cytokine Membrane Array. Cytokines in the conditioned medium were detected using Human Cytokine Array III (Ray Biotech) according to the manufacturer's instructions. Following the seeding of 5×10^5 DU-C or DU-P cells onto six-well plates and incubation for 24 h, the medium was changed to phenol red-free RPMI1640 with 0.1% BSA and incubation was continued for another 48 h. Then, the medium was changed to fresh phenol red-free RPMI1640 with 0.1% BSA. After another 24 h incubation, the conditioned media were collected and used in the experiment.

ELISA (ELISA) for IL-6 Secretion. IL-6 secretion in the collected medium was measured using a human IL-6 ELISA kit according to the manufacturer's instructions (R&D Systems). Briefly, DU-C or DU-P cells were seeded onto six-well plates at 5×10^5 cells/well and the culture was continued for another 24 h. Next, the medium was changed to phenol red-free RPMI1640 with 0.1% BSA and the culture was continued for another 48 h. Media were replaced with fresh phenol red-free RPMI1640 with 0.1% BSA, and then collected after another 24-h incubation.

Western Blot. Cells were washed in ice-cold PBS (PBS) three times and then dissolved in lysis buffer (20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 100 mM sodium fluoride, 200 mM sodium orthovanadate, 1 mM EGTA, 2 mM PMSF, 1 μg/mL leupeptin, and 3 μg/mL aprotinin). After centrifugation and quantification of the supernatants using a Bio-Rad protein assay (Bio-Rad), equal volume proteins of total cell lysate were electrophoresed by SDS/PAGE and transferred to Immobilon-P membranes (Millipore) electrophoretically. After the transferred membranes had been blocked with blocking reagent (NOF Corp.), they were incubated at room temperature for 1 h with a specific antibody in Tris buffered saline containing Tween 20 (TBST: 150 mM NaCl, 20 mM Tris, and 0.05% Tween 20). The membranes were washed with TBST three times for 10 min before incubation with the secondary antibody. After the membranes had been treated with the horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h and washed three times for 10 min, specific signals were detected using an ECL Kit (GE Healthcare) and X-ray film (Kodak).

Luciferase Assay. ≈1.2 kb of the IL-6 5′-flanking region was generated using PCR from genomic DNA extracted from DU145 cells, and cloned into the pGL4.0 [*luc*2] vector (pGL4-IL6p) (Promega). Wild type aPKCλ/ι was obtained as described in previous reports (2, 3). phRL-SV40 was used as the internal control for the luciferase assay (Promega).

To examine the influence of wild type aPKCλ/ι, 1×10^5 DU145 cells were seeded onto 24-well plates and transfected with a combination of pGL4, pGL4-IL6p, phRL-SV40, empty and wild type aPKCλ/ι expression vectors using Transfast reagents (Promega). After a 48 h incubation, luciferase activity was measured using the dual-luciferase reporter assay system (Promega) and a luminometer, TD-20/20 (Turner Design).

For IL-6 promoter activation in DU-C and DU-P cells, 1×10^5 DU-C or DU-P cells were seeded onto 24-well plates followed by pGL4-IL6p and phRL-SV40 transfection. After a 12-h incubation, transfected cells were lysed and luciferase activity was measured

Electrophoretic Mobility Shift Assay (EMSA). Nuclear proteins of DU-C or DU-P were extracted using NE-PER (Pierce Biotech-

nology Inc.). After the nuclear protein concentration had been determined using a Bio-Rad protein assay (Bio-Rad), 2 μg protein were used for the reaction. NFκB or AP-1 oligo were purchased from Promega. All reactions including labeling of each oligo with ^{32}P were carried out using gel shift assay systems (Promega) according to the manufacturer's instructions. Reacted samples were separated in 6% PAGE and visualized by autoradiography on X-Ray film.

Statistical Analysis. All statistical analyses were performed using SPSS for windows (SPSS Inc.). Data were analyzed by the paired or unpaired *t* test, ANOVA followed by Bonferroni test, as appropriate. All statistical analyses were two-sided. Results of Western blot and RT-PCR were quantitated by densitometry and analyzed statistical significances. To assess relationships between aPKCλ/ι expression and clinical factors, the median values of aPKCλ/ι mRNA expression, PSA and patient age served as cut-off values. The associations of aPKCλ/ι mRNA expression with PSA failure time were analyzed using the Kaplan-Meier method and the log rank test. The Cox proportional hazards model was used for univariate and multivariate analyses of PSA failure. A $P < 0.05$ was considered statistically significant in all analyses. Correlation between aPKCλ/ι mRNA expression and IL-6 mRNA expression was analyzed by Spearman rank test.

1. Japanese Urological Association and the Japanese Society of Pathology (2001) in *General Rules for Clinical and Pathological Studies on Prostate Cancer* (Kanehara Shuppan Co. Ltd, Tokyo), 3rd Ed.
2. Akimoto K, et al. (1996) EGF or PDGF receptors activate atypical PKCλ through phosphatidylinositol 3-kinase. *EMBO J* 15:788–798.
3. Akimoto K, et al. (1998) Atypical protein kinase Cλ binds and regulates p70 S6 kinase. *Biochem J* 335:417–424.

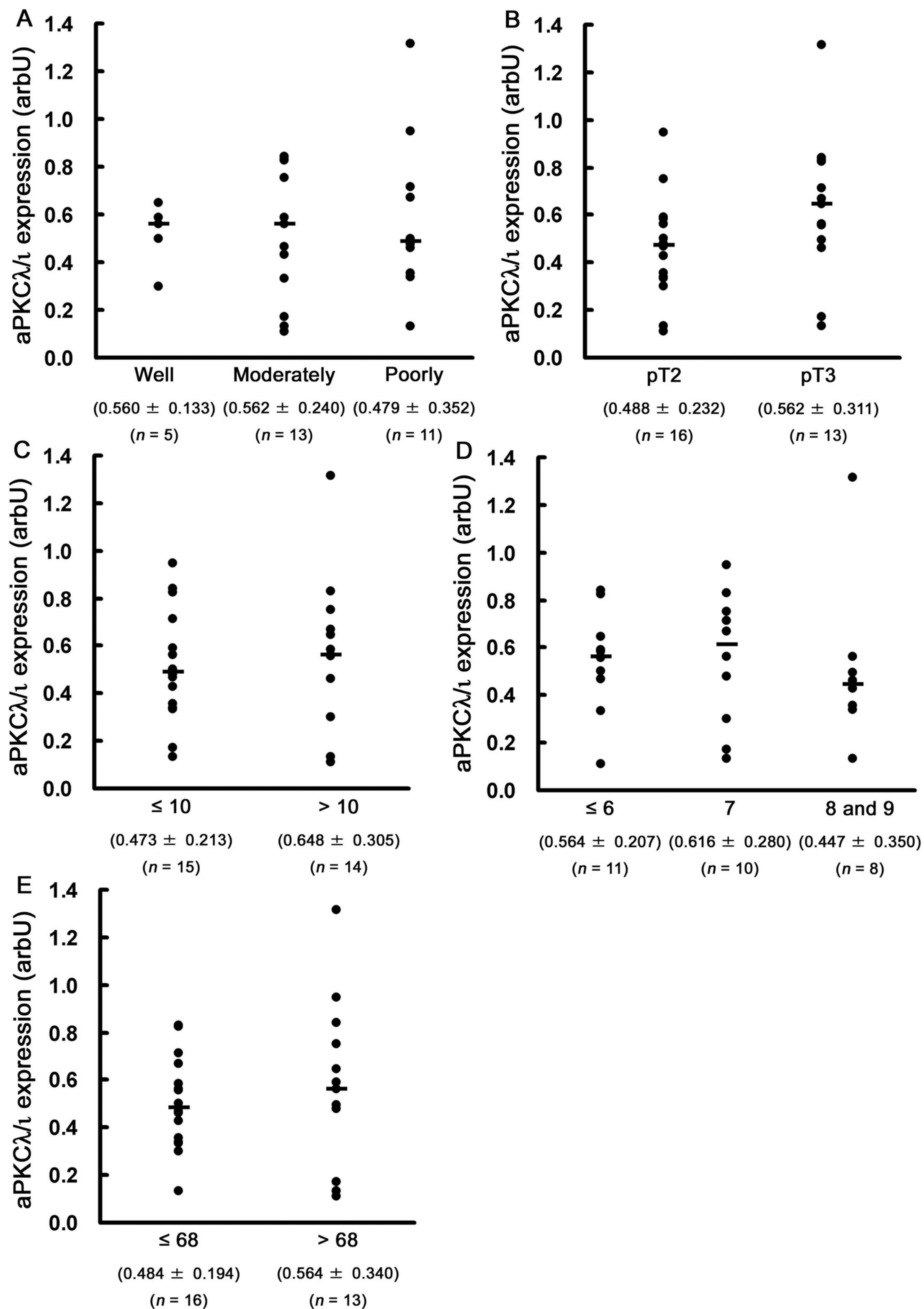

Fig. S1. Relationships between aPKCλ/ι expression and various clinicopathological features in prostate cancer tissues. Differences in aPKCλ/ι expression between (*A*) Tumor histology based on degree of differentiation (well; $n = 5$, moderately; $n = 13$ and poorly; $n = 11$, $P = 0.968$ by ANOVA test), (*B*) Tumor stages (pT2; $n = 16$ and pT3; $n = 13$, $P = 0.531$ by unpaired *t* test), (*C*) PSA secretion (PSA secretion was divided into two groups based on the median value (≤ 10 ng/mL: $n = 15$, and >10 ng/mL: $n = 14$), $P = 0.112$ by unpaired *t* test), (*D*) Gleason score (Gleason score ≤6; $n = 11$, Gleason score 7; $n = 10$, and Gleason scores 8 and 9; $n = 8$, $P = 0.368$ by ANOVA test) and (*E*) Patient age (specimens were divided into two groups based on the median patient age (≤ 68: $n = 16$, and >68: $n = 13$), $P = 0.428$ by unpaired *t* test). arbU: arbitrary units. Values indicate medians ± SD.

Fig. S2. Kaplan-Meier and log rank analysis of clinical features and PSA failure time. Correlation between clinical features and PSA failure time was analyzed. (*A*) Tumor histology based on degree of differentiation (well; $n = 5$, moderately; $n = 13$ and poorly; $n = 11$, $P = 0.646$), (*B*) Tumor stages (pT2; $n = 16$ and pT3; $n = 13$, $P = 0.334$), (*C*) PSA secretion (PSA secretion was divided into two groups based on the median value ($\leq$ 10 ng/mL: $n = 15$, and $>$10 ng/mL: $n = 14$), $P = 0.789$), (*D*) Gleason score (Gleason score $\leq$6; $n = 12$, Gleason score 7; $n = 9$, and Gleason score 8 and 9; $n = 8$, $P = 0.723$) and (*E*) Patient age (specimens were divided into two groups based on the median patient age ($\leq$ 68: $n = 16$, and $>$68: $n = 13$), $P = 0.935$).

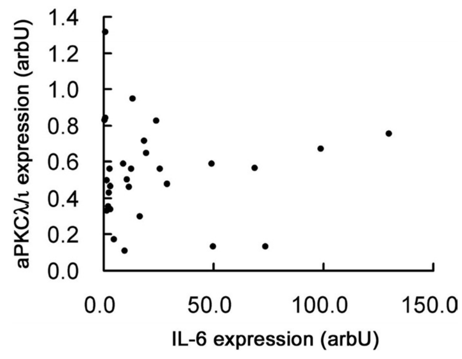

Fig. S3. Correlation between IL-6 mRNA and aPKCλ/ι mRNA expression in prostate cancer tissues. IL-6 mRNA and aPKCλ/ι mRNA expression by qPCR was analyzed (n = 29, P = 0.943 by Spearman rank test). arbU: arbitrary units.

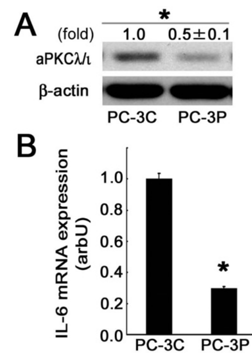

Fig. S4. PC-3 transfected aPKCλ/ι and decreasing of IL-6 mRNA expression. (*A*) PC-3 cells transfected with siRNA for aPKCλ/ι expression vector (PC-3P cells) and empty vector (PC-3C cells) were confirmed by Western blot. β-actin was used as an internal control. Values indicate means ± SD from at least three independent experiments (set as 1.0 in PC-3C). *, $P < 0.001$ by unpaired *t* test. (*B*) Expressions of IL-6 mRNA in PC-3C and PC-3P cells. IL-6 mRNA expression was investigated by qPCR ($n = 3$ in each group). Bars represent means ± SD from at least three independent experiments. *, $P = 0.014$ by unpaired *t* test. arbU: arbitrary units.

Table S1. Clinicopathological features of the samples used in qPCR analysis

No.	Age	Histology*	Gleason score	Pathological stage	PSA (ng/ml)	Follow up (Days)	PSA failure (Days)
1	74	Well	6	pT3a	20.9	995	-
2	68	Well	6	pT3a	14.2	3028	-
3	54	Well	6	pT2b	4.1	1181	156
4	71	Well	6	pT2b	6.6	1544	767
5	62	Well	7	pT3a	4.8	4579	2704
6	69	Moderately	7	pT2b	19.2	976	-
7	71	Moderately	6	pT2a	19.9	1023	-
8	75	Moderately	6	pT2a	6.1	1262	-
9	64	Moderately	6	pT2a	4.6	1682	-
10	75	Moderately	7	pT3a	8.8	2116	-
11	67	Moderately	8	pT2a	6.2	2249	-
12	63	Moderately	7	pT3b	22.2	1061	389
13	68	Moderately	6	pT2a	12.7	1090	0
14	63	Moderately	8	pT3a	21.2	1786	1315
15	52	Moderately	6	pT3a	7.0	1071	1516
16	70	Moderately	6	pT3a	6.4	1362	417
17	63	Moderately	6	pT2b	9.8	1520	0
18	70	Moderately	7	pT3a	9.9	675	178
19	68	Poorly	9	pT2a	8.6	981	-
20	59	Poorly	7	pT2b	57.6	1340	-
21	69	Poorly	9	pT2a	6.5	1409	-
22	72	Poorly	8	pT2b	13.3	1456	-
23	67	Poorly	9	pT2b	7.3	1544	-
24	73	Poorly	7	pT2a	6.6	1321	-
25	68	Poorly	7	pT3a	17.1	2426	-
26	73	Poorly	7	pT2b	12.3	1066	996
27	64	Poorly	8	pT3a	12.3	1584	1570
28	71	Poorly	9	pT3a	13.7	1982	153
29	65	Poorly	7	pT3a	35.7	2369	420

*Well: Well-differentiated adenocarcinoma, Moderately; Moderately-differentiated adenocarcinoma, Poorly: Poorly-differentiated adenocarcinoma.

Table S2. aPKCλ/ι expression and Gleason score

		Normal	Gleason score					*n*
			6	7	8	9	10	
aPKCλ/ι expression level	+0 and + 1	2	4	3	3	1	0	13
	+2<	1	16	6	5	1	1	30
	n	3	20	9	8	2	1	43

Data was analyzed by χ^2 test, $P = 0.560$.