Cancer Cell, Volume *34*

Supplemental Information

Targeting PKCδ as a Therapeutic Strategy against Heterogeneous Mechanisms of EGFR Inhibitor Resistance in EGFR-Mutant Lung Cancer

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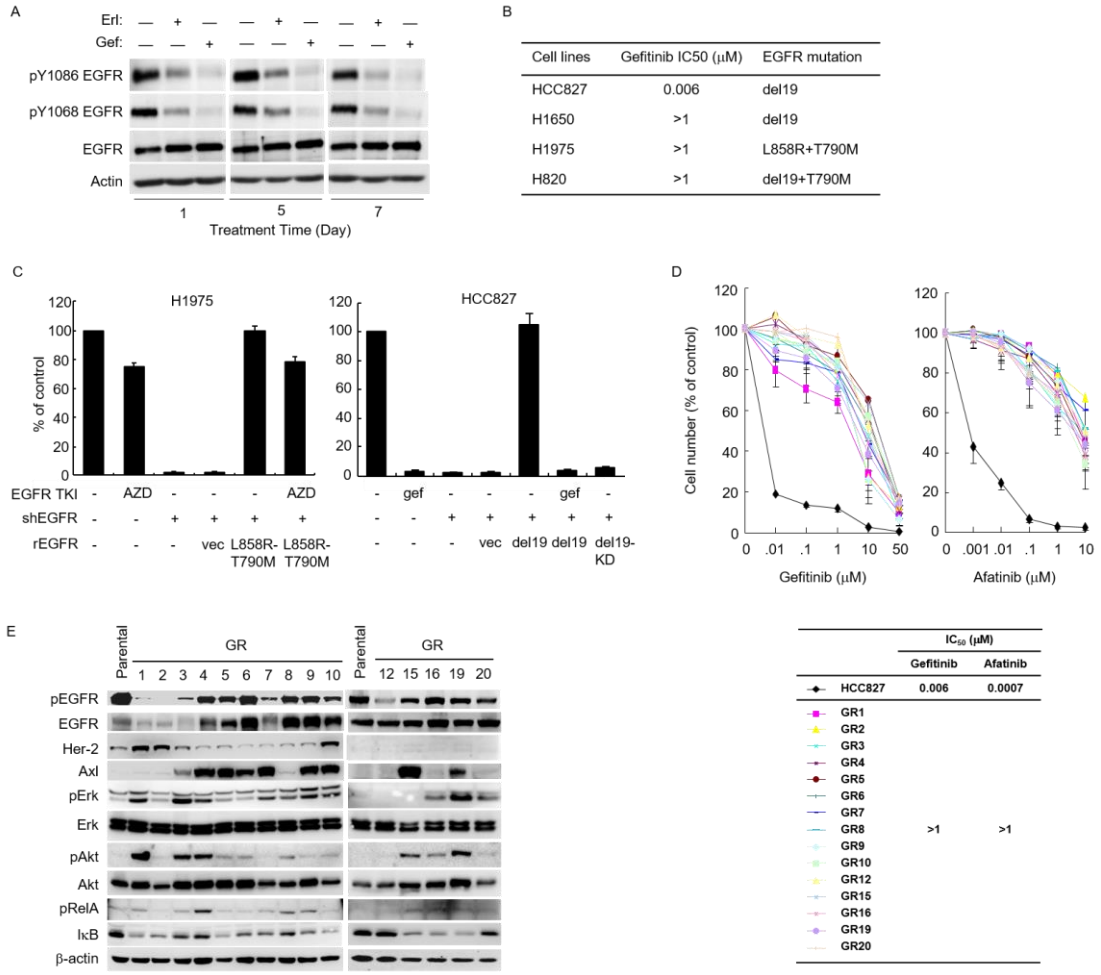

Figure S1, related to Figure 1. (A) Western blot showing Y1068 and Y1086 phosphorylation of EGFR in H1650 cells treated with gefitinib (gef, 1 μM) or erlotinib (erl, 0.1 μM) for 1, 5, and 7 days. (B) IC_{50} of gefitinib in HCC827 sensitive and H1650, H1975, and H820 resistant cells**.** (C) Re-expression of endogenous EGFR reversed EGFR depletion–induced cell death. H1975 and HCC827 cells were infected with EGFR shRNA and re-expressed shRNA-resistant EGFR (rEGFR) variants, L858R+T790M (H1975) and del19 (HCC827), in presence or absence of 1 μM gef and 0.1 μM AZD9291 (AZD), respectively. The cells were counted after treatments for 7 days. (D) Cells were treated with gefitinib and afatinib for 3 days. Cells were counted and expressed as percent of control cells. IC_{50} of gefitinib and afatinib in HCC827 parental and 15 GR cells were calculated and showed in bottom. (E) Parental and GR cell lysates were subjected to Western blots analysis with the indicated antibodies. Antibodies used correspond to previously reported features of known TKI resistance.

Data in (C) and (D) represent mean ± SD (n = 3).

Table S1, related to Figure 2. Twenty-seven potential mediators in the TKI-insensitive pathways identified by antibody array.

Modifier	shCtrl (A)	shCtrl+TKI (A+)	shEGFR (B)	EDR (C)	ratio of (C) / (B)
B-RAF (Ab-446)*	1.00	0.97	0.04	1.20	30.51
CaMK2 (Phospho-Thr305)	1.00	0.86	0.02	1.03	49.32
CaMK4 (Phospho-Thr196/200)	1.00	1.17	0.06	0.95	16.63
Catenin beta (CTNNB) (Phospho-Tyr489)*	1.00	0.91	0.10	1.15	11.58
Catenin delta-1 (Ab-228)	1.00	1.14	0.06	1.13	18.41
CD5 (Ab-453)	1.00	1.15	0.14	1.11	8.10
c-Jun (Ab-73)	1.00	1.20	0.05	1.27	26.78
CK2-b (Phospho-Ser209)	1.00	0.85	0.13	0.63	4.93
c-met (Ab-1003)*	1.00	0.95	0.08	2.05	26.95
cofilin (Ab-3)	1.00	1.13	0.12	1.06	9.00
Cytokeratin 8 (Ab-431)	1.00	1.16	0.08	0.55	7.12
Ephrin B (Ab-330)	1.00	0.99	0.07	1.88	28.43
Estrogen Receptor-alpha (Ab-106)	1.00	0.81	0.03	0.93	29.80
FAK (Ab-576)*	1.00	0.85	0.11	0.91	8.00
FKHR (Phospho-Ser319)*	1.00	0.96	0.09	1.79	19.37
HDAC5 (Ab-498)	1.00	1.04	0.05	0.98	21.25
LAT (Ab-191)	1.00	0.84	0.09	0.61	6.65
MAP3K7/TAK1 (Ab-439)	1.00	1.13	0.05	0.83	16.26
NFkB-p105/p50 (Ab-337)*	1.00	0.92	0.03	0.72	24.17
PAK1 (Ab-212)	1.00	1.10	0.08	0.76	9.03
PKC beta/PKCB (Phospho-Ser661)	1.00	0.90	0.12	0.96	8.32
PKC delta (Phospho-Thr505)	1.00	0.99	0.14	0.68	4.74
PLCg2 (Ab-1217)	1.00	0.88	0.12	0.86	7.29
Raf1 (Ab-338)*	1.00	0.91	0.14	1.39	9.91
Rb (Ab-795)	1.00	1.08	0.10	1.07	10.53
SRF (Phospho-Ser77)	1.00	0.99	0.11	2.22	19.65
VASP (Ab-238)	1.00	1.13	0.06	0.64	11.51

*Candidates that have previously been implicated with resistance to EGFR inhibitors.

Table S2, related to Figure 2. Top 32 affected pathways by Ingenuity Pathway Analysis based on the 27 potential mediators identified.

Ingenuity Canonical Pathways	-log (p-value)	Molecules
Molecular Mechanisms of Cancer	1.69E01	Raf1,NF-kB-p105/p50,B-Raf,FAK,RB,PAK1,c-Jun,FKHR,PKC delta,MAP3K7,Catenin beta,PKC beta,catenin delta,CaMK2
B Cell Receptor Signaling	1.55E01	FAK,Raf1,CaMK4,c-Jun,FKHR,Cofilin,PLCg2,MAP3K7,NF-kB-p105/p50,CaMK2,PKC beta
HGF Signaling	1.38E01	FAK,c-Met,Raf1,PAK1,c-Jun,PKC delta,PLCg2,MAP3K7,PKC beta
Chemokine Signaling	1.34E01	FAK,Raf1,CaMK4,c-Jun,Cofilin,PLCg2,CaMK2,PKC beta
LPS-stimulated MAPK Signaling	1.31E01	Raf1,PAK1,c-Jun,PKC delta,SRF,MAP3K7,NF-kB-p105/p50,PKC beta
GNRH Signaling	1.3E01	FAK,Raf1,PAK1,c-Jun,PKC delta,MAP3K7,NF-kB-p105/p50,CaMK2,PKC beta
Protein Kinase A Signaling	1.18E01	FAK,B-Raf,Raf1,CaMK4,PKC delta,PLCg2,NF-kB-p105/p50,Catenin beta,VASP,CaMK2,PKC beta
Renin-Angiotensin Signaling	1.17E01	FAK,Raf1,PAK1,c-Jun,PKC delta,PLCg2,NF-kB-p105/p50,PKC beta
ERK/MAPK Signaling	1.15E01	FAK,B-Raf,Raf1,PAK1,PKC delta,PLCg2,SRF,ER alpha,PKC beta
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	1.14E01	Raf1,CaMK4,c-Jun,PKC delta,PLCg2,MAP3K7,NF-kB-p105/p50,Catenin beta,CaMK2,PKC beta
Melatonin Signaling	1.13E01	B-Raf,Raf1,CaMK4,PKC delta,PLCg2,CaMK2,PKC beta
ErbB Signaling	1.06E01	Raf1,PAK1,c-Jun,FKHR,PKC delta,PLCg2,PKC beta
Glioma Signaling	1.03E01	Raf1,RB,CaMK4,PKC delta,PLCg2,CaMK2,PKC beta
Role of NFAT in Cardiac Hypertrophy	9.98E00	Raf1,CaMK4,PKC delta,PLCg2,MAP3K7,HDAC5,CaMK2,PKC beta
Corticotropin Releasing Hormone Signaling	9.86E00	B-Raf,Raf1,CaMK4,c-Jun,PKC delta,PLCg2,PKC beta
IL-8 Signaling	9.84E00	FAK,B-Raf,Raf1,c-Jun,PKC delta,NF-kB-p105/p50,VASP,PKC beta
Thrombin Signaling	9.77E00	FAK,Raf1,CaMK4,PKC delta,PLCg2,NF-kB-p105/p50,CaMK2,PKC beta
Axonal Guidance Signaling	9.67E00	FAK,c-Met,Raf1,Ephrin B,PAK1,Cofilin,PKC delta,PLCg2,VASP,PKC beta
PI3K Signaling in B Lymphocytes	9.43E00	Raf1,CaMK4,c-Jun,PLCg2,NF-kB-p105/p50,CaMK2,PKC beta
Erythropoietin Signaling	9.31E00	Raf1,c-Jun,PKC delta,PLCg2,NF-kB-p105/p50,PKC beta
IL-3 Signaling	9.16E00	Raf1,PAK1,c-Jun,FKHR,PKC delta,PKC beta
Phospholipase C Signaling	9.04E00	Raf1,CaMK4,PKC delta,PLCg2,LAT,NF-kB-p105/p50,HDAC5,PKC beta
PDGF Signaling	8.94E00	Raf1,c-Jun,PLCg2,SRF,CK2-b,PKC beta
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	8.91E00	Raf1,CaMK4,c-Jun,PLCg2,LAT,NF-kB-p105/p50
Xenobiotic Metabolism Signaling	8.69E00	Raf1,CaMK4,PKC delta,MAP3K7,NF-kB-p105/p50,HDAC5,CaMK2,PKC beta
NF-κB Signaling	8.46E00	B-Raf,Raf1,PLCg2,MAP3K7,CK2-b,NF-kB-p105/p50,PKC beta
UVC-Induced MAPK Signaling	8.45E00	B-Raf,Raf1,c-Jun,PKC delta,PKC beta
IGF-1 Signaling	8.33E00	FAK,Raf1,c-Jun,FKHR,SRF,CK2-b
Cholecystokinin/Gastrin-mediated Signaling	8.22E00	FAK,Raf1,c-Jun,PKC delta,SRF,PKC beta
Rac Signaling	8.17E00	FAK,Raf1,PAK1,c-Jun,Cofilin,NF-kB-p105/p50
Leukocyte Extravasation Signaling	8.06E00	FAK,PKC delta,PLCg2,Catenin beta,VASP,catenin delta,PKC beta
Natural Killer Cell Signaling	8.05E00	Raf1,PAK1,PKC delta,PLCg2,LAT,PKC beta

Table S3, related to Figure 2. Synthetic lethal screen of gefitinib with inhibitors targeting potential mediators or their affected pathways in H1650 cells.

Target	Inhibitor name	Clinical status	Dose range	Synergy with gefitinib (CI)
β-catenin nuclear complex	ICG-001		1-15 μM	>1.1
	PNU-74654		1-15 μM	>1.1
	Resveratrol (non-selective)		1-50 μM	>1.1
β-catenin	Aspirin (non-selective, NSAID)	FDA approved	0.5-8 μM	0.5-0.9
	Sulindac (non-selective, NSAID)	FDA approved	1-20 μM	0.5-0.9
CaMKII	KN-62		0.1-10 μM	0.5-0.9
FZD-Dvl	Dvl-PDZ (3289-8625)		0.5-25 μM	0.9-1.1
	NSC668036		0.5-50 μM	>1.1
IκB kinase	BMS-345541		1-30 μM	0.4-1.1
JNK	SP600125		1-20 μM	0.9-1.1
MEK	Trametinib	FDA approved	2.5-20 nM	0.4-0.5
NFAT	NFAT inhibitor		1-20 μM	>1.1
PKCβ	Enzastaurin**	Phase 3	0.5-20 μM	1.0-1.4
PKC-pan	**Go6983****		1-40 μM	***0.2**-0.8
	Sotrastaurin (AEB071) **	**Phase 2**	0.5-20 μM	***0.1**-0.8
PLC	U-73122 (Stroidamine)		1-5 μM	*0.3-0.7
PI-PLC	Edelfosine		1.5-30 μM	>1.1
PLD1, PLD2	FIPI		0.1-10 μM	0.5-0.9
PLD	VU0359595		0.1-10 μM	0.5-0.9
Wnt[#]	IWP2 (Porcupine inactivator)		0.05-10 μM	0.5-0.9
	IWP4 (Porcupine inactivator)		0.5-50 μM	0.5-0.9
	LGK-974 (Porcupine inhibitor)		0.01-1 μM	>1.1

Non-steroidal anti-inflammatory drug (NSAID); combination index (CI).

*Strong synergy (CI value ≤ 0.3); shown in red.

**Go6983 inhibited PKCα, β, γ, δ, ζ, and μ isoforms, and sotrastaurin inhibited α, β, δ, ε, η, and θ. Enzastaurin (so-call PKCβ inhibitor) inhibited PKCα and β.

[#]Mediators involved in canonical and non-canonical Wnt signaling although the Wnt receptor was not identified (Table S2). PKC inhibitors are shown in green and blue.

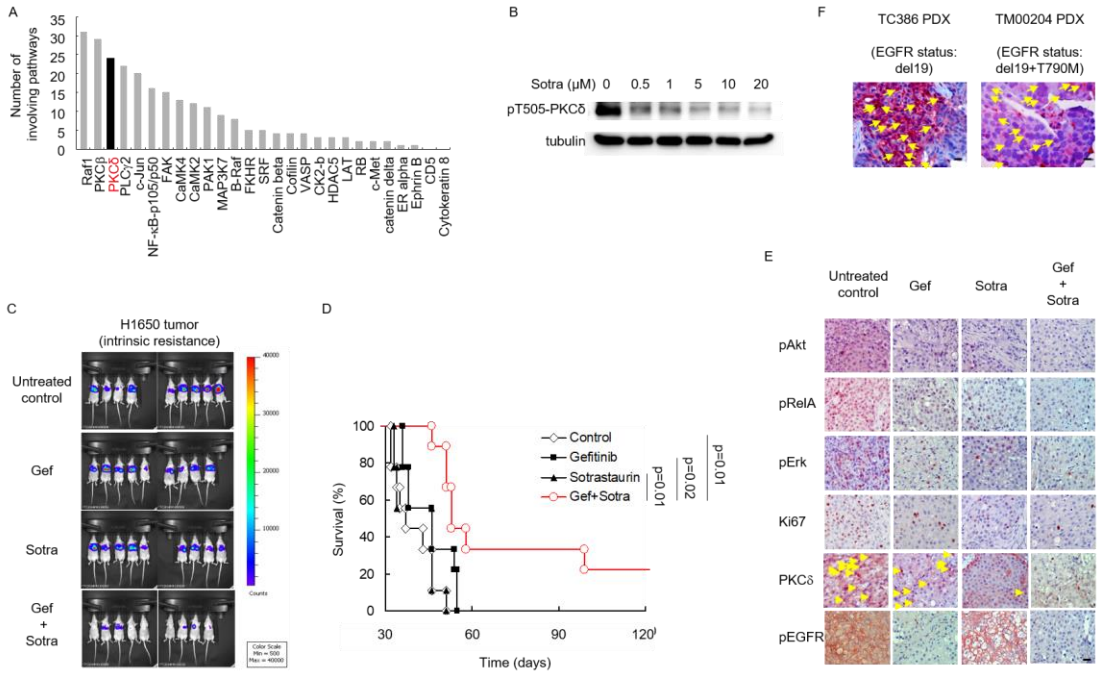

Figure S2, related to Figure 2. (A) The numbers of canonical pathways involving the 27 candidates identified in Figure 2B. Ingenuity pathway analysis identified a total 32 canonical pathways involving the 27 candidates (Table S2). The canonical pathways involving each individual candidate were counted. (B**)** The effects of sotrastaurin (sotra) treatment on T505 phosphorylation of PKCδ in H1650 cells. Western blot showing T505 phosphorylation of PKCδ in H1650 cells treated with sotra for 24 hr. (C) Images of mice with H1650 tumors at day 14. (D) Mice survival in combination group compared to control, gef, and sotra alone groups. Data represent mean ± SEM (n = 9). $p < 0.05$ statistically significant by Student's t test. (E) Related to Figure 2G. Representative IHC images of pAkt, pRelA, pErk, Ki67, PKCδ, and pEGFR in H1650 xenografts from mice treated as indicated. Yellow arrows denote representative nuclear PKCδ-positive cells. Bar, 50 μm. (F) Representative IHC images of nPKCδ positive PDX tumors. Yellow arrow denotes representative nuclear PKCδ-positive cells. Bar, 10 μm.

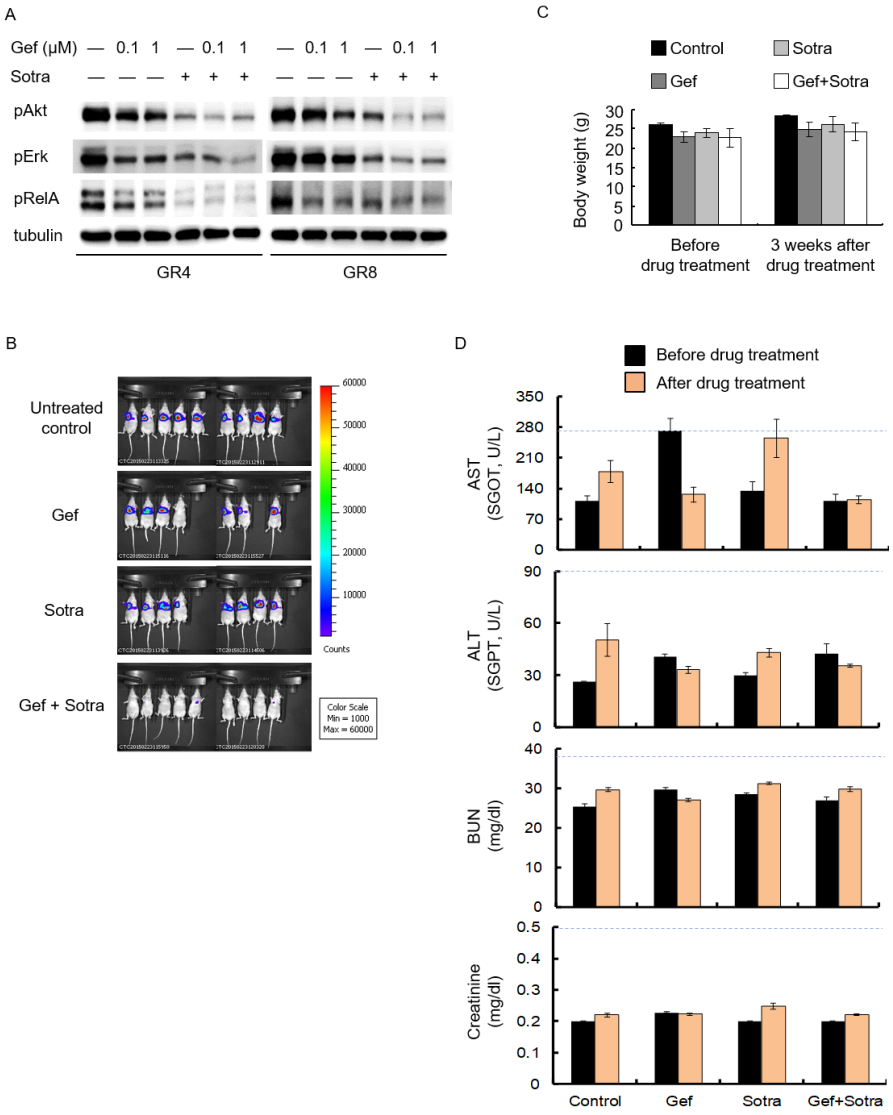

Figure S3, related to Figure 3. (A) Western blot showing pAkt, pErk, pRelA in GR cells treated with gef, 10 μM sotra, and the combination for 24 hr. (B) Images of mice with GR6 tumors at day 28. (C, D) Body weight changes (C) as well as indicators for liver and kidney functions (D) in each treatment group before and after drug treatment for 3 weeks. The normal range of aspartate aminotransaminase (AST), alanine aminotransaminase (ALT), blood urea nitrogen (BUN), and creatinine are 63–253 U/L, 35–90 U/L, 17–38 mg/dl, and 0.3–0.5 mg/dl, respectively.

Data in (C) and (D) represent mean ± SD (n = 3).

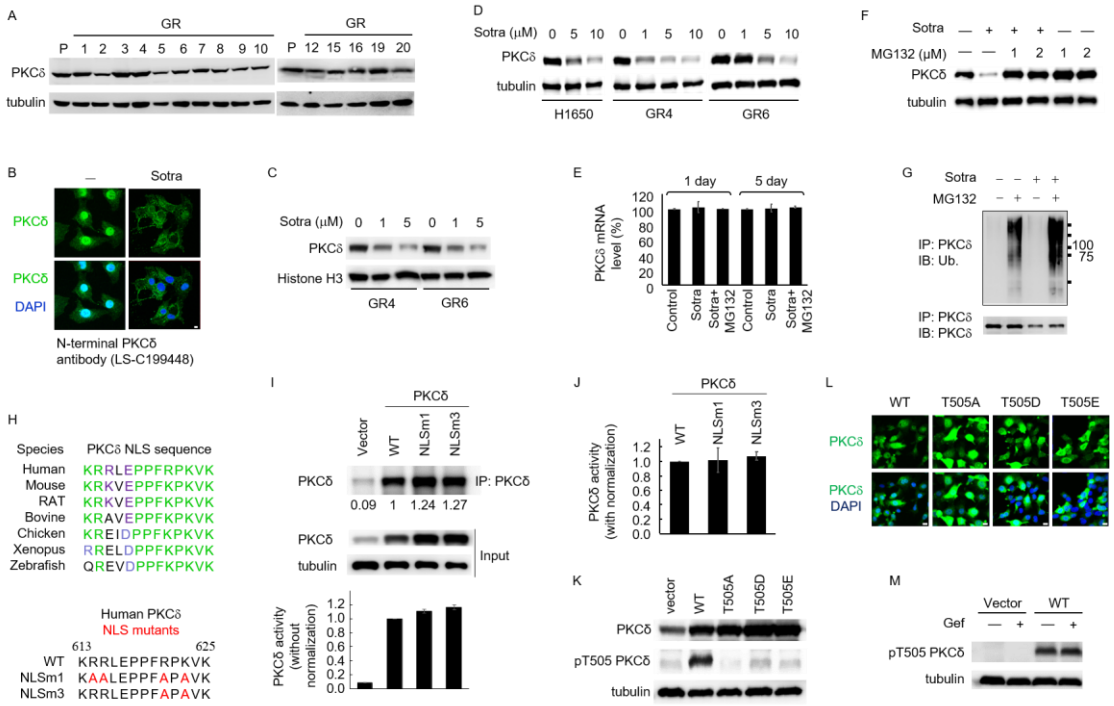

Figure S4, related to Figure 4. (A) Western blots showing total PKCδ expression in HCC827 parental (P) and GR cells. (B) Confocal microscopy analysis of PKCδ localization in H1650 cells treated with 10 μM sotra for 24 hr using the LS-C199448 antibody that recognizes the N-terminal region of PKCδ. Bar, 10 μm. (C) Western blots showing PKCδ expression in nuclear extracts of GR4 and GR6 cells treated with sotra for 24 hr. (D) Western blots showing total PKCδ expression in whole cell extracts of H1650, GR4, and GR6 cells treated with sotra for 24 hr. (E) Real-time PCR analysis of PKCδ messenger RNA levels in H1650 cells treated with 10 μM sotra followed by the treatment with or without proteasome inhibitor MG132 (2 μM) for 24 hr. (F, G) Western blots showing PKCδ protein (F) and ubiquitination (G) levels in H1650 cells treated with 10 μM sotra followed by the treatment with or without MG132 for 24 hr. (H) Top, sequence alignment of PKCδ nuclear localization signal (NLS) domain across species. Bottom, NLS of human wide-type (WT) PKCδ and NLS mutants. Red letters indicate the mutated amino acid residues within the NLS. (I, J) Analysis of PKCδ kinase activity in HCC827 cells expressing endogenous PKCδ (Vector), exogenous WT PKCδ, PKCδ mutant NLSm1, or PKCδ mutant NLSm3. (I) PKCδ protein expression levels (top) and the raw PKCδ activities (bottom) normalized to PKCδ activity by protein expression level (J). (K) Western blot analysis of total PKCδ and pT505 PKCδ expression in HCC827 cells expressing vector, WT, T505A, T505D, or T505E mutant PKCδ. (L) Confocal microscopy analysis of PKCδ localization in HCC827 expressing WT, T505A, T505D, or T505E mutant PKCδ. Bar, 20 μm. (M) Western blot analysis of pT505 PKCδ expression in ectopic WT PKCδ expressing HCC827 cells treated with 0.1 μM gefitinib for 24 hr.

Data in (E), (I), and (J) represent mean ± SD (n = 3).

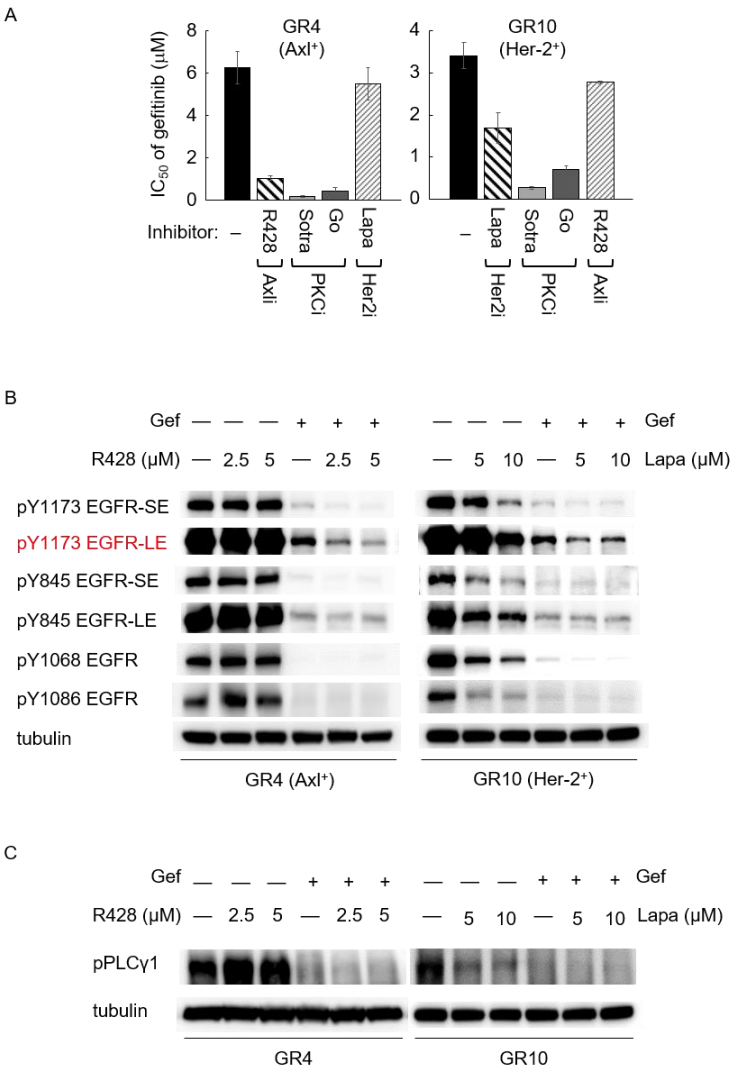

Figure S5, related to Figure 5. (A) The median inhibitory concentrations (IC_{50}) of gefitinib in Axl-positive GR4 and Her-2-positive GR10 cells measured after 10 days of treatment of R428 (Axli, 2.5 μM) and Lapatinib (Lapa, Her2i, 5 μM) as well as sotra and Go6983 (Go), (PKCi, 10 μM) in GR4 and GR10 cells with gefitinib. Data represent mean ± SD (n = 3). (B) Western blot showing phosphorylation of EGFR Y1173, Y845, Y1068, and Y1086 in GR4 and GR10 cells after 24 hr treatment of 0.1 μM gefitinib with R428 and Lapa, respectively. SE, short exposure; LE, long exposure. (C) Western blot showing phosphorylation of PLCγ1 in cells treated as in (B).

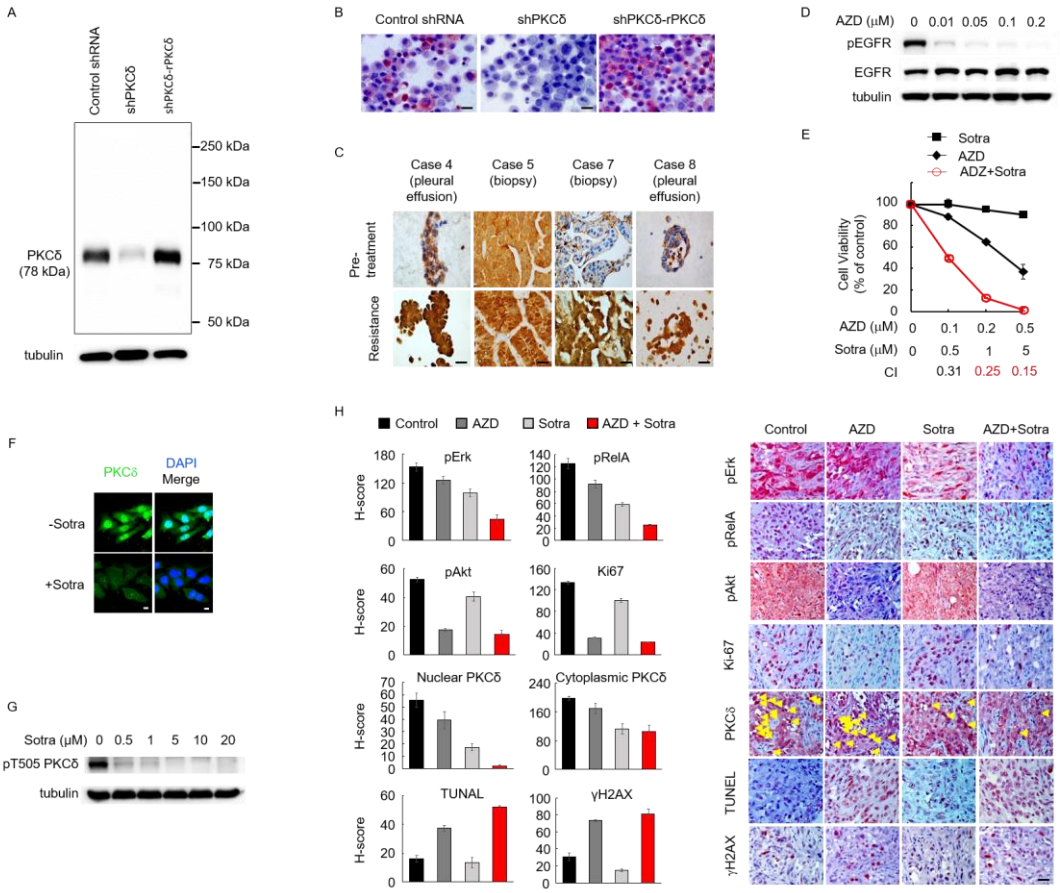

Figure S6, related to Figure 6. (A and B) Western blot (A) and IHC staining (B) in H1650 cells showing the specificity of PKCδ antibody (abcam ab182126) used for human IHC staining. The samples were H1650 control shRNA, PKCδ-depleted (shPKCδ), and re-expressing shRNA-resistant PKCδ (rPKCδ) cells from left to right in that order. PKCδ-depleted samples were used as negative control. Bar, 30 μm. (C) Representative images of PKCδ by IHC staining in paired pretreatment and resistance specimens of cases 4, 5, 7, and 8 in Table 1. Bar, 50 μm. (D) H1975 cells were treated with AZD9291 for 24 h. The cell extracts were subjected to Western blotting. (E) Synergistic effects of sotra with AZD9291 (AZD) in H1975 cells. Cells were treated with AZD and/or sotra for 15 days. Cell viability was assayed by crystal violet staining and the value of combination index (CI) calculated as in Figure 2E. (F) Confocal microscopy analysis showing PKCδ localization in H1975 cells treated with 10 μM sotra for 24 hr. Bar, 10 μm. (G) H1975 cells were treated with sotra for 24 h. The cell extracts were subjected to Western blotting. (H) Left, the H-score of Erk, RelA, and Akt phosphorylation, proliferation marker Ki67, nuclear and cytoplasmic PKCδ, apoptosis (TUNEL), and γH2AX in H1975-derived xenograft tumors from mice treated as indicated. Right, representative IHC images of pErk, pRelA, pAkt, Ki67, PKCδ, TUNEL, and γH2AX in H1975 xenografts from mice treated as indicated for 5 days. Yellow arrows denote representative nuclear PKCδ-positive cells. Bar, 50 μm.

Data in (E) and (H) represent mean ± SD (n = 3).

Table S4. related to Figure 6. Objective response and progression-free survival of 19 patients with high nPKCδ tumors.

ID	smoking	EGFR alteration	TKI	Objective response	PFS (m)
1	no	19DEL	Gef	SD	9.2
2	no	19DEL	Erl	PR	4.9
3	no	L858R	Gef	PD	1.6
4	no	L858R	Gef	PD	2.7
5	no	19DEL	Gef	SD	6.1
6	no	19DEL	Gef	SD	13.0
7	no	19DEL	Gef	SD	8.4
8	no	G719A	Gef	SD	7.9
9	no	V769_D770inASV	Erl/Gef	PD	1.9
10	no	19DEL	Gef	PR	15.9
11	no	L858R	Gef	PR	3.2
12	no	19DEL	Gef	NA	2.1
13	no	19DEL	Erl	PD	1.5
14	no	T790M, L858R	Gef	PD	0.7
15	no	S768I, G719A	Erl	SD	6.8
16	no	19DEL	Gef	SD	6
17	yes	19DEL	Gef	PD	0.5
18	no	L858R	Gef	PR	3.9
19	no	19DEL	Gef/Erl	PD	3

Gef, gefitinib; Erl, erlotinib; SD, stable disease; PR, partial response;
PD, progression disease; NA, data not available.