Towards personalized therapy in pediatric acute lymphoblastic leukemia: RAS mutations and prednisolone resistance

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Supplemental Data

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Materials and Methods

Processing of patients' leukemic cells

Bone marrow samples were collected from children with newly diagnosed ALL after written consent as approved by the institutional review board. Mononuclear cells were isolated by lymphoprep density gradient centrifugation, as previously described [7]. Only leukemic samples with ≥ 90% leukemic blasts were used in the present study. If applicable, enrichment of leukemic blasts was achieved with immunomagnetic beads. Each patient was examined for the following genomic lesions, i.e. hyperdiploid (>50 chromosomes), *ETV6-RUNX1*$^{+}$, *TCF3-PBX1*$^{+}$, *MLL*-rearrangement, *BCR-ABL1*$^{+}$ and *BCR-ABL1*$^{+}$-like by means of FISH, PCR and by utilizing the 110-probeset classifier [8]. Patients negative for aforementioned genomic aberrations or signature were named B-other. Cells were cultured in RPMI Dutch modification (Gibco) supplemented with 0·1% insulin-transferrin-sodium selenite (Sigma), 0·4 mM glutamine (Invitrogen), 0.25 µg/ml gentamycine (Gibco), 100 IU/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 0.125 µg/ml fungizone (Gibco) and 20% fetal calf serum (Integro) at 37°C in humidified air containing 5% CO_2.

Reverse Phase Protein Array

Proteins were isolated from 1)unexposed primary BCP-ALL cells obtained at initial diagnosis 2)normal mononuclear cells obtained from non-leukemic pediatric bone marrow samples and 3)primary BCP-ALL cells that were exposed for 48h to 0µg/ml, 1µg/ml or 250µg/ml prednisolone. Proteins were isolated with protein lysis buffer and protein concentration was quantified by means of the BCA assay (Pierce). Hereafter, lysates were spotted twice in triplicate on glass-backed nitrocellulose-coated array slides by the facility of Dr. E. F. Petricoin, George Mason University,Manassas,USA. Slides were subsequently stained with indicated antibodies, incubated with a biotinylated secondary antibody and scanned using the NovaRay scanner. The MicroVigene Software was used to calculate protein levels relative to the total amount of protein per sample. Antibodies used were: phospho-STAT6(Y641 (Cell signaling (CS)#9361), phospho-MET(Y1234-1235)(CS#3126), RAS (Millipore #05-516), phospho RAS-GRF1(S916)(CS #3321), phospho-ARAF(S299)(CS #4431), phospho-BRAF(S455)(CS#2696), phospho-CRAF(S338)(CS#9427), phospho-MEK1/2(S217-221) (CS#9121), phospho-AKT(S473)(CS #9271), phospho-NFκB(S536)(CS#3031), phospho-p38MAPK(T180-Y182)(CS#9211), phospho-SAPK-JNK(T183-Y185)(CS#9251), phospho-JAK2(Y1007)(CS#3771), phospho-TYK2(Y1054/55)(CS#9321), phospho-STAT5(Y694)(CS#9351), phospho-P70S6K(T389)(CS#9208), phospho-CREB(S133)(CS#9191) and phospho-PLCgamma2(Y759)(CS#3874).

Western Blot

Proteins were isolated from primary patients' cells treated for 4 days with the indicated inhibitor. There were only enough leukemic cells of patient D for extensive western blotting studies. Protein samples were loaded on pre-cast gels and transferred to nitrocellulose membranes (Bio-Rad). Blots were blocked and probed with the following antibodies; phospho-MEK1/2(S217-221)(CS#9121), phospho-ERK1/2(Thr202/Tyr204)(CS#9101), phospho-AKT(S473)(CS#9271), phospho-BRAF(S455)(CS#2696), and β-Actin (Abcam, ab6276). Hereafter, protein levels were quantified using the Odyssey 3.0 application software (Li-COR).

Ion Torrent deep sequencing

DNA was extracted from leukemic blasts (≥ 90% purity) by means of Trizol isolation according to the manufacturer's protocol (Invitrogen). DNA concentration was determined by the Quantit picogreen method (Invitrogen). Deep sequencing was performed on the Ion PGM using the Ion AmpliSeq Library Kit 2·0, the Ion AmpliSeq Cancer Panel Primer Pool and Ion Xpress Barcode adapters 1-32 (Life Technologies). The multiplexed PCR covered several hotspot mutations in BRAF, NRAS, HRAS, KRAS, PTPN11, FLT3 and cMET, as was reported in the Cosmic database (Supplemental Table 1). A maximum of 16 indexed samples were pooled in equimolar fashion and sequenced on an Ion Torrent 318B chip using the 200bp sequencing chemistry according to manufacturer's protocol. Sequences were analyzed using the Torrent_Suite 3.4.2 software (variant caller v3.4.51874). Variants were annotated using an in-house developed pipeline using the Ensembl databases (www.ensembl.org).

Materials and Methods

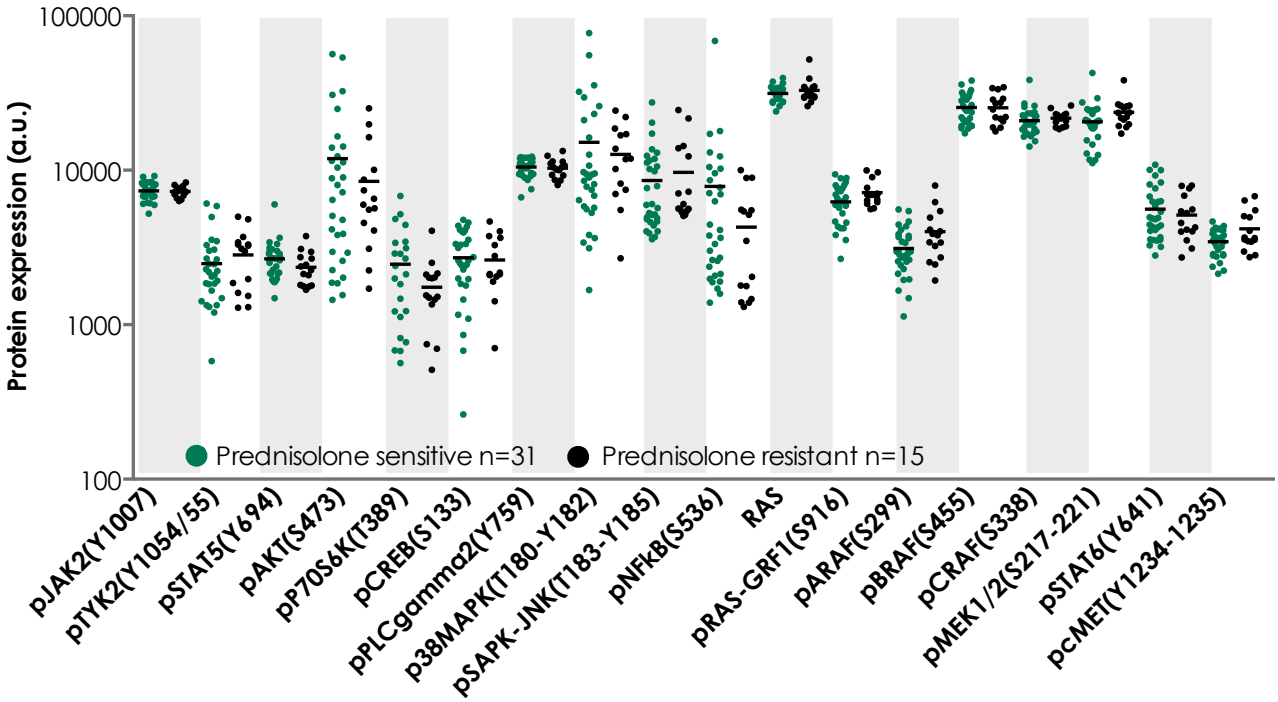
MTT assay

Cytotoxicity of prednisolone (Bufa Pharmaceutical Products) in primary patients' cells (as indicated in Table 1) was determined by the *in vitro* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) drug-resistance assay after 4-days of exposure, as previously described [7]. Optical density values were measured on the Versamax (Molecular Devices) at λ=562 nm and λ=720nm. *In vitro* prednisolone sensitivity was defined by a concentration of prednisolone lethal to 50% of the cells (LC_{50}) below 0·1 μg/mL and prednisolone resistance was defined by a LC_{50} value above 150 μg/mL as shown previously to be predictive for clinical outcome in pediatric ALL [7,9]. Cytotoxicity to Trametinib, Sorafenib, Crizotinib (Selleckchem) and AS1517499 (Axon Medchem) in primary patients' cells was determined by the MTT assay after 4-days of exposure. These inhibitors were dissolved in 100% DMSO and were tested in a serial dilution ranging between 0.0002 and 20 μM. Cytotoxicity of these inhibitors together with prednisolone was determined after 4 days by the MTT assay. Prednisolone dose-response curves were corrected for loss of cell viability caused by the inhibitors and the solvent itself. Data was only used when >50% of DMSO control patient cells survived compared to input.

Statistical Analysis

Prednisolone-induced changes in protein expression were analyzed with a Kruskal-Wallis test. A T-test was used to compare data obtained in resistant and sensitive patients, and to test the prednisolone sensitizing effects of inhibitors on cell viability compared to vehicle control. The dose-response curves of prednisolone in combination with an inhibitor was analyzed by two-way ANOVA, testing the interaction between inhibitor*prednisolone. A p-value below 0·05 was considered statistically significant.

Supplemental Figure 1.

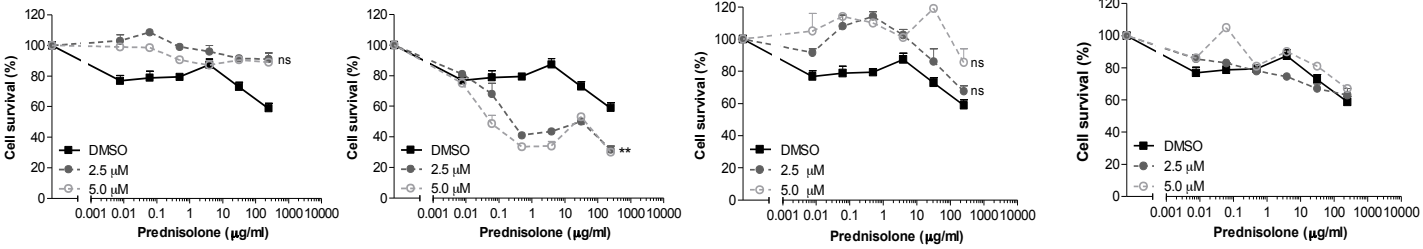


Supplemental Figure 1. Basal protein expression of 18 tyrosine-kinase pathway proteins are not different in prednisolone-resistant patients compared to sensitive patients.
(A) Protein (phosphorylation) levels of 18 proteins were analyzed by means of reverse phase protein array (relative to total protein) of 31 *in vitro* prednisolone sensitive and 15 prednisolone resistant unexposed samples taken from BCP-ALL patients' at initial diagnosis. P-value was not significant for all proteins.

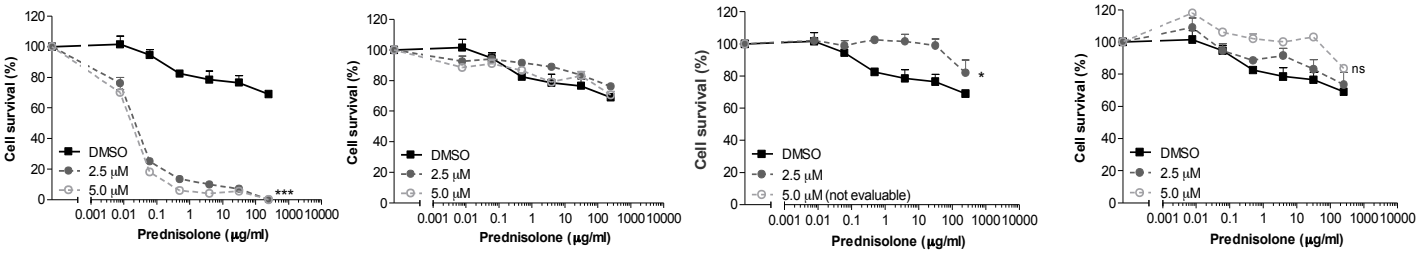
Supplemental Figure 2.

Trametinib – MEK1/2 | Sorafenib - BRAF | AS1517499 – STAT6 | Crizotinib – cMET

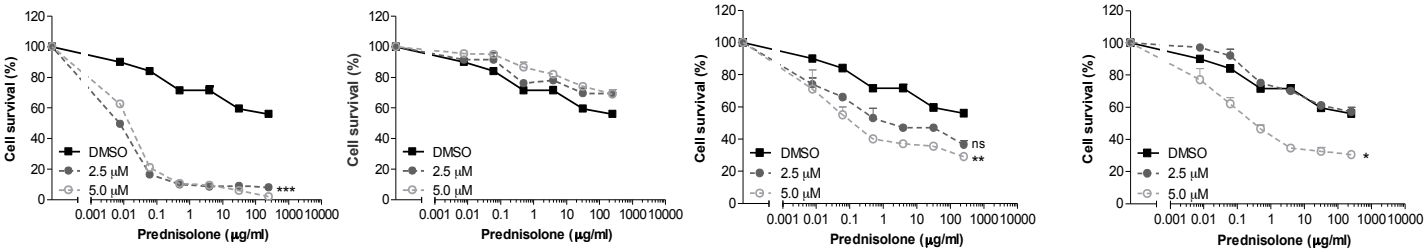
Patient A KRAS/NRAS mt



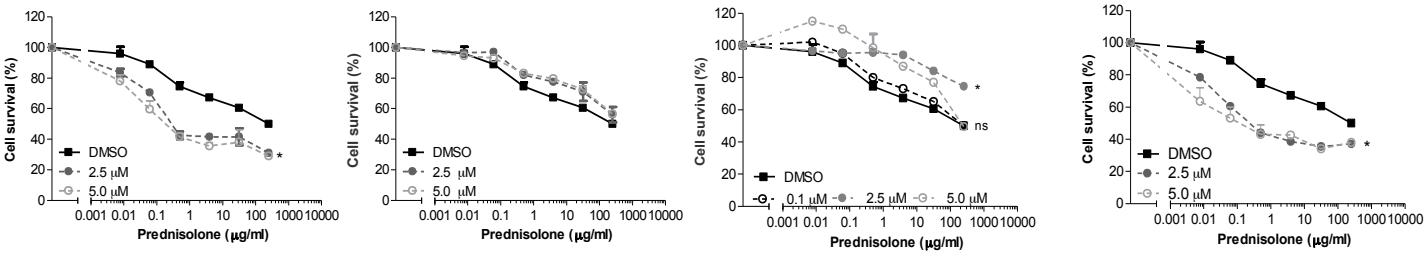
Patient B KRAS mt



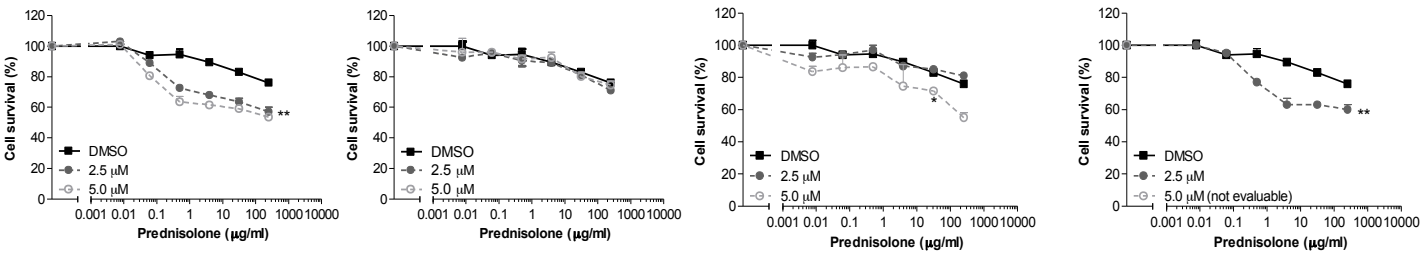
Patient C NRAS/KRAS mt



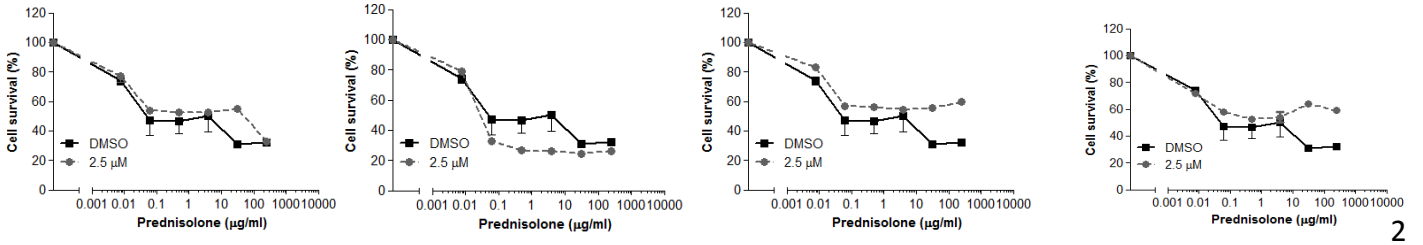
Patient D RAS-WT



Patient E RAS-WT



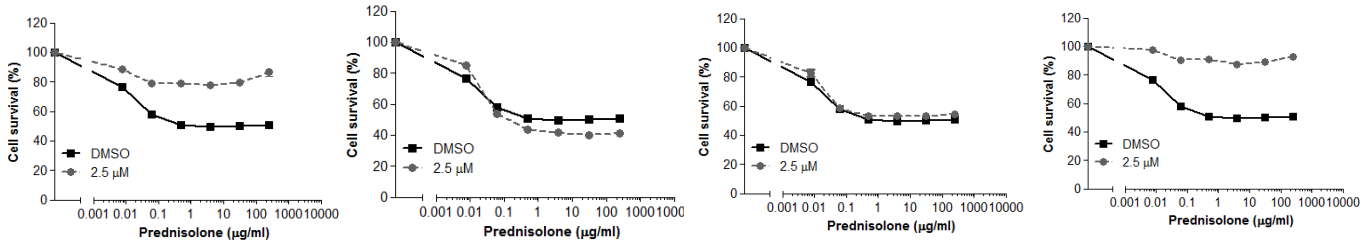
Patient F RAS-WT



Supplemental Figure 2.

Trametinib – MEK1/2 | Sorafenib - BRAF | AS1517499 – STAT6 | Crizotinib – cMET

Patient G KRAS/NRAS wt



Supplemental Figure 2. ***Trametinib (MEK inhibitor) and Sorafenib (BRAF inhibitor) restored prednisolone sensitivity in RAS-mutant patients*** Dose-response curves of 7 pediatric BCP-ALL patients' cell samples exposed to prednisolone together with 2.5 or 5.0 µM of inhibitor or vehicle (DMSO). Data are presented as mean plus SEM of a duplicate experiment (repeated measurement two-way ANOVA, *p<0.05, **p<0.01, ***p<0.001). To facilitate assessment of cellular sensitization to prednisolone by the inhibitors, cell survival was corrected for the cell death induced by the inhibitor. Patients A, B and C have RAS-mutations, patients D,E,F and G are RAS-wildtype (see also Table 1).

Supplemental Table 1a.

Patient Number	Pred exposure Protein Study	Inhibitor Study	Genetic Subtype	LC50 Prednisolone µg/ml	Category Prednisolone	Treatment Protocol	Risk-group	Non-response	Relapse	Death	EFS	Mutated Gene	Genomic Variant	Protein Variant	Coverage Depth	Heterozygous Frequency	% Mutated cells
1			BO	>250	Resistant	COALL03	HR	0	0	0	4.54	NRAS	T/C	Q61R	1163	13.59	27.18
												NRAS	C/T	G13D	1534	2.48	4.96
												FLT3	A/C	D835E	2042	12.10	24.2
												PTPN11	G/A	E69K	1913	6.53	13.06
2			BO	>250	Resistant	COALL03	HR-S	0	0	0	3.75	NRAS	C/A	G13V	1570	43.89	87.78
												NRAS	C/T	G12D	1562	5.12	10.24
3			BO	>250	Resistant	COALL03	HR-S	0	0	0	6.34	KRAS	C/T	G12D	1073	45.29	90.58
4		X Patient A	BO	>250	Resistant	ALL10	MR	0	0	0	4.48	KRAS	C/T	G13D	613	13.7	27.4
												NRAS	C/T	G12D	1109	6.85	13.7
												NRAS	T/C	Q61R	1012	2.47	4.94
5	X		BO	>250	Resistant	COALL03	HR-S	0	0	0	5.05	NRAS	C/T	G13D	2033	58.31	116.62
6			BAL	>250	Resistant	COALL97	HR-S	0	0	0	12.36	KRAS	C/T	G12D	666	35.14	70.28
7		X Patient B	ER	>250	Resistant	COALL03	LR-I	0	0	0	7.45	KRAS	C/G	G12R	1010	51.58	103.16
8	X	X Patient C	ER	154	Resistant	COALL03	LR-I	0	0	0	4.79	NRAS	C/A	G12V	1523	35.00	70.00
												KRAS	C/T	G12S	616	7.95	15.9
9			BAL	0.06	Sensitive	COALL03	HR-S	0	0	0	5.83	KRAS	C/T	G12S	624	27.56	55.12
10	X		ER	0.04	Sensitive	COALL03	HR-S	0	0	0	5.48	KRAS	C/A	G12V	1096	29.56	59.12
												NRAS	A/C	Y64D	1231	10.07	20.14
11			ER	0.06	Sensitive	COALL03	LR-S	0	0	0	5.48	KRAS	C/T	G12S	532	12.41	24.82
												NRAS	C/T	G12S	1306	2.83	5.66
12			BAL	>250	Resistant	ALL10	MR	0	0	0	4.08	wildtype					
13			BAL	195	Resistant	COALL03	HR-S	0	0	0	8.84	wildtype					
14		X Patient D	ER	>250	Resistant	ALL9	/	0	0	0	/	wildtype					
15			ER	>250	Resistant	COALL03	LR-S	0	0	0	5.99	wildtype					
16	X	X Patient E	ER	>250	Resistant	ALL10	MR	0	0	0	4.78	wildtype					
17	X	X Patient G	BO	0.07	Sensitive	COALL03	HR-S	0	0	0	4.84	wildtype					
18			BO	0.05	Sensitive	COALL03	LR-R	0	0	0	3.87	wildtype					
19			BO	0.01	Sensitive	ALL10	MR	0	0	0	2.86	wildtype					
20			BO	0.04	Sensitive	COALL03	HR-S	0	1	0	2.23	wildtype					
21			BAL	0.03	Sensitive	COALL03	HR-S	0	0	0	4.00	wildtype					
22			BAL	0.04	Sensitive	COALL03	HR-S	0	0	0	4.64	wildtype					
23	X		BAL	0.05	Sensitive	COALL03	HR-S	0	1	0	1.08	wildtype					
24			ER	0.01	Sensitive	COALL03	LR-R	0	0	0	5.96	wildtype					
25		X Patient F	ER	0.05	Sensitive	COALL03	LR-R	0	0	0	4.93	wildtype					
26			ER	0.01	Sensitive	ALL10	SR	0	0	0	4.33	wildtype					

Subtype: BO=B-other (negative for hyperdiploidy, hypodiploidy, ETV6-RUNX1$^+$, BCR-ABL1$^+$, BCR-ABL1-Like, TCF3-PBX1$^+$, MLL rearranged), ER=ETV6-RUNX1$^+$, BAL=BCR-ABL1-like ; LC50 prednisolone (µg/ml): Prednisolone concentration (µg/ml) that killed 50% of leukemic cells ; Category Prednisolone: sensitive ≤0.1 µg/ml, resistant ≥150 µg/ml [7] ; Riskgroup: HR=high risk, MR=medium risk, LR=low risk, S=standard protocol, I=Intensified protocol, R=Reduced protocol ; EFS=Event-free survival (years) ; Non-response, relapse, death: 0=no event, 1=event ; Coverage depth: Number of reads.

Supplemental Table 1b.

RES	RES	RES	RES	RES	RES	RES	RES	SENS	SENS	SENS		
B-Other	B-Other	B-Other	B-Other	B-Other	BCR-ABL like	ETV6-RUNX1	ETV6-RUNX1	BCR-ABL like	ETV6-RUNX1	ETV6-RUNX1		
	10%		14%								NRAS	G12D
							70%				NRAS	G12V
										6%	NRAS	G12S
5%				100%							NRAS	G13D
	88%										NRAS	G13V
27%			5%								NRAS	Q61R
									20%		NRAS	Y64D
		91%			70%						KRAS	G12D
						103%					KRAS	G12R
							16%	55%		25%	KRAS	G12S
									59%		KRAS	G12V
			27%								KRAS	G13D
24%											FLT3	D835E
13%											PTPN11	E69K

Supplemental Table 2.

Gene	Cosmic hotspot codons examined
BRAF	444, 464, 466, 469, 471, 581, 587, 592, 594, 595, 596, 597, 599, 600, 601 and 605
NRAS	12, 13, 18, 61 and 64
HRAS	12, 13 and 61
KRAS	12,13,19,22,59,61 and 146
PTPN11	60, 61, 69, 72, 73,76, 502 and 503
FLT3	451,572, 592,597, 599, 601, 602, 603, 834, 835, 836 and 842
cMET	168, 375, 1010,1112, 1248,1253 and 1268

Supplemental Table 3.

In vitro prednisolone response	RAS-mutations			Total
	B-other	*BCR-ABL1*-like	*ETV6-RUNX1+*	
Sensitive	0% (0/4 patients)	25% (1/4 patients)	40% (2/5 patients)	23% (3/13 patients)
Resistant	100% (5/5 patients)	33% (1/3 patients)	40% (2/5 patients)	62% (8/13 patients)
Total	56% (5/9 patients)	29% (2/7patients)	40% (4/10 patients)	42% (11/26 patients)

Supplemental Table 4.

Inhibitor	Main Target	FDA approval
Trametinib	MEK1-2	FDA approved for melanoma
Sorafenib	BRAF	FDA approved for renal cell carcinoma and hepatocellular carcinoma
AS1517499	STAT6	No clinical trial data
Crizotinib	cMET	FDA approved for non-small lung carcinoma