SUPPLEMENTARY APPENDIX FOR:

Selective RET Kinase Inhibition for Patients with *RET*-Altered Cancers

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SUPPLEMENTARY METHODS

In vitro studies

Cell lines and assays

LC-2/ad (*CCDC6-RET* NSCLC) cells were obtained from Sigma-Aldrich. TT and SW620 cells were obtained from ATCC. MZ-CRC-1 (*RET M918T* MTC) and TPC1 (*CCDC6-RET* thyroid cancer) cells were obtained from Dr. James Fagin, Memorial Sloan Kettering Cancer Center. *RET* gene alteration-negative human cancer cell lines were selected from the Eurofins OncoPanel collection of cell lines (Eurofins). NIH 3T3 and HEK-293 cells were obtained by Array BioPharma. Eurofins cell lines were authenticated by short-tandem repeat (STR) analysis (Genetica DNA Laboratories, Inc., last July 2015). LC-2/ad, TT, MZ-CRC-1 and TPC1 cells were authenticated by confirmation of the presence of each *RET* alteration (e.g. *CCDC6-RET, RET C634W, RET M918T* or *CCDC6-RET,* respectively) by the Oncomine Focus Assay NGS assay (Thermo Fisher Scientific, Inc) within 12 months of experiments. Cell lines were tested regularly for Mycoplasma (MycoAlert™, Lonza, Inc. or STAT-Myco, Idexx, BioResearch, Inc.). Frozen stocks prepared after ~1-2 passages were thawed ~3-6 days (~2-3 passages) before use.

Generation of HEK-293 engineered cell lines and assessment of target activity

HEK-293 cells stable expressing doxycycline-inducible mutant versions of RET (KIF5B-RET -/+ V804M, RET M918T), wild-type KDR/VEGFR2 or wild-type FGFR1 were generated using standard transfection methods.

For assessment of cellular target activity, cells were harvested, counted and added to flat-bottom 96-well assay plates at 4-5X10^4 cells/well in100 µL/well of DMEM growth medium containing 10% FBS and 1 µg/mL doxycycline, and allowed to attach for 24 hours at 37°C, 5% CO_2. Cells were treated for one hour with each inhibitor, each prepared as a 1:3 dilution series starting at a maximum of 16.7 or 1.67 µM (RET), 5 µM (KDR/VEGFR2, FGFR1) or 10 µM (Aurora) and a constant DMSO concentration of 0.5%. Control wells contained either 0.5% DMSO alone (no inhibition control) or 1 µM LOXO-292 (complete inhibition control). The levels of phosphorylated-RET were determined by In Cell Western assay (LI-COR) using antibodies to phosphorylated-RET (Tyrosine 1062, Santa Cruz Biotechnology) and GAPDH (Millipore). Plates

were analyzed by reading optical density at 700/800 nM using an infrared scanner (Aerius), and the phosphorylated-RET signal for each well was normalized to the GAPDH signal. The levels of phosphorylated KDR/VEGFR2 were determined after 5-minute treatment with human VEGF by sandwich immunoassay using antibodies to total (capture) and phosphorylated KDR/VEGFR2 (detection). Plates were analyzed using an electrochemiluminescent detection instrument (Meso Scale Discovery). The levels of phosphorylated-FGFR1 were determined after 5-minute treatment with human acidic FGF by ELISA assay (R & D Systems), using total FGFR1 (capture) and phosphorylated tyrosine (detection) antibodies. For assessment of Aurora kinase activity, the levels of phosphorylated Histone H3 were determined by In-Cell Western (LI-COR) using antibodies to phosphorylated Histone-H3 (Serine 10, Cell Signaling Technology) and total ERK (Santa Cruz Biotechnology), as for phosphorylated RET. hERG activity in individual HEK-293 cells engineered to express cloned hERG and treated with LOXO-292 at a concentration range of 0-10 μM was determined by patch clamp analysis (Charles River Laboratories). For each target, IC_{50} values were calculated by 4-parameter logistic regression. hERG IC_{50} values for cabozantinib and vandetinib were previously published [1, 2].

In vivo studies

Mouse efficacy

All animals were obtained at 6-8 weeks of age, housed in groups of 5 and allowed a one-week acclimation period before cancer cell injection. Food, water, temperature and humidity were prepared per Pharmacology Testing Facility performance standards which are in accordance with the 1996 Guide for the Care and Use of Laboratory Animals and AAALAC-International.

The KIF5B-RET and KIF5B-RET V804M NIH-3T3 tumor cell lines (5 x 10e6 cells), TT cells (1 x 10e7 cells), or minced tumor fragments derived from prior xenografts of LC-2/a cells, KIF5B-RET PDX (Champions Oncology, CTG-0838), CCDC6-RET PDX (Crown Bioscience, CRL-2518) and CCDC6-RET-V804M PDX (Crown Bioscience, CRL-2545), were injected subcutaneously into the right flank of female nu/nu NCr mice (SCID-beige for LC-2/a). Tumors could grow to ~ 100 - 200 mm3, and animals were randomized by tumor size into dosing groups of 7-12 animals. Animals were dosed by oral gavage with vehicle, LOXO-292 at doses of 3, 10, 30 mg/kg and 50 mg/kg (KIF5B-RET PDX) twice daily, cabozantinib at 60 mg/kg (Scid-beige 40

mg/kg) daily or ponatinib at 20-25mg/kg daily. Body weight and tumor size were monitored after cell implantation and at regular intervals during dosing. Tumor diameters were measured with digital calipers, and the tumor volume in mm^3 was calculated by the formula: Volume = $((width)^2 \times length)/2$.

For the intracranial tumor model, suspensions of the CCDC6-RET PDX (4 x 10e5 cells) were injected orthotopically into the brain. Treatment was initiated 7 days after injection. Animals were monitored at regular intervals and sacrificed for morbid condition. Survival analysis was performed using the Kaplan-Meyer method.

PK-PD analysis

Steady-state minimum and maximum concentrations (C_{min}, C_{max}) for LOXO-292 were determined from plasma samples collected predose and at defined intervals after dosing on days 1 and 8 of the starting dose and each subsequent dose escalation. LOXO-292 concentration was analyzed using validated LC/MS-MS, and noncompartmental pharmacokinetic parameters were determined. C_{min}/C_{max} values for vandetanib, cabozantinib and alectinib were obtained from published sources [1-3]. Human plasma and brain protein binding were determined by incubating each inhibitor in 100% human plasma or mouse brain homogenate at 1μM final concentration for 4.5-6 hours, followed by precipitation of proteins and determination of free inhibitor concentration in the supernatant using LC-MS/MS. Estimated CNS penetration for alectinib was obtained from published sources [4]. IC_{50} values for each agent/RET target pair were determined using the HEK-293 cell assays, and were corrected for human plasma protein binding and estimated brain protein binding (LOXO-292) or CNS penetration (alectinib). The percent RET target inhibition at the C_{min}/C_{max} for each agent was determined with the following formula:

$$\% \text{ target inhibition at each dose} = \frac{[\text{agent}]}{([\text{agent}] + \text{corrected } IC_{50})}$$

Tumor mutational analysis

All tumor molecular profiling was performed at the treating institutions or by Foundation Medicine (tumor tissue) or Guardant (plasma cell-free tumor DNA) in CLIA-approved laboratories (Fig. S7).

REFERENCES

1. Research CfDEa. NDA 22-405 Clinical Pharmacology Review – Vandetanib 2010.
2. Research CfDEa. NDA 203756 Clinical Pharmacology Review - Cabozantinib 2012.
3. Alecensa Product Information - EMEA/H/C/004161-II/0001. 2017.
4. Kodama T, Hasegawa M, Takanashi K et al. Antitumor activity of the selective ALK inhibitor alectinib in models of intracranial metastases. Cancer Chemother Pharmacol 2014; 74: 1023-1028.

A

5637	D283 Med	LC-2/ad	SH-4
647-V	DBTRG-05MG	M0-91	SHP-77
A101D	Detroit 562	MCF7	SJRH30
A172	DK-MG	MDA MB 231	SK-BR-3
AN3 CA	DMS114	MDA MB 453	SK-LMS-1
AU565	DMS53	MDA MB 468	SK-MEL-28
BFTC-905	DU145	MG-63	SK-N-DZ
BHT-101	EFM-19	Mia PaCa-2	SK-N-FI
BT20	FaDu	MV-4-11	SNB-19
BT474	HEL-92-1-7	MZ-CRC-1	SNU-16
BT-549	HMCB	NCI-H292	SU-DHL-10
BxPC-3	Hs 578T	NCIH441	SW1353
C32	HT1376	NCI-H520	SW579
Cal 27	HT-29	NCI-H661	T24
CAL-62	J82	OE19	T47D
CAMA-1	JeKo-1	REC-1	TCCSUP
CaOV3	K562	RT112 84	TF-1
CGTH-W-1	KATO III	SaOS2	TPC1
CHL-1	KG-1	SCaBER	TT
CML-T1	KM12-Luc	SCC-25	U-138MG
COR-L105	KPL-1	SCC-4	UM-UC-3
CUTO-3	L-428	SCC-9	

B

	LOXO-292			Cabozantinib			Vandetanib		
RET Alteration	**IC_{50} (nM)**	**n**	**Fold vs. LOXO-292**	**IC_{50} (nM)**	**n**	**Fold vs. LOXO-292**	**IC_{50} (nM)**	**n**	**Fold vs. LOXO-292**
KIF5B-RET	4 ± 2	55	1	75 ± 27	4	19	935 ± 679	4	234

Figure S1. (A) Cell lines analyzed for Figure 1A left. *RET*-altered cell lines are indicated in red. (B) 50% inhibitory concentrations for LOXO-292, cabozantinib and vandetanib in the cellular phospho-KIF5B-RET assay. Values are mean ± standard deviation. Abbreviations: IC_{50}-50% inhibitory concentration; nM-nanomolar; n-number of replicates.

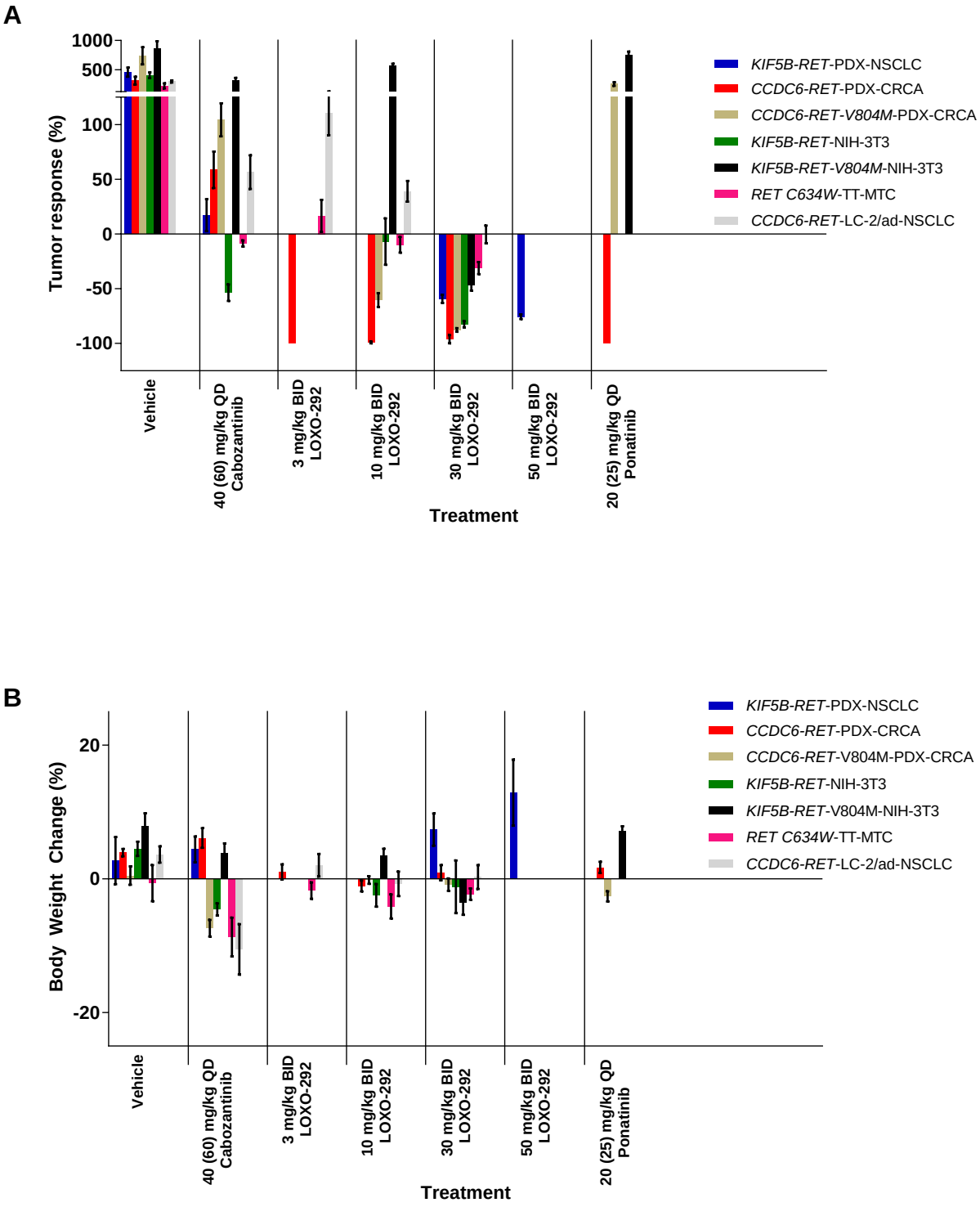


Figure S2. (A) Tumor response and (B) body weight change for individual doses tested in mouse subcutaneous tumor models in Figure 1B. Abbreviations: mg-milligrams; kg-kilograms; QD-once daily; BID-twice daily; PDX-patient-derived xenograft; NSCLC-non-small cell lung cancer; CRCA-colorectal cancer; MTC-medullary thyroid cancer.

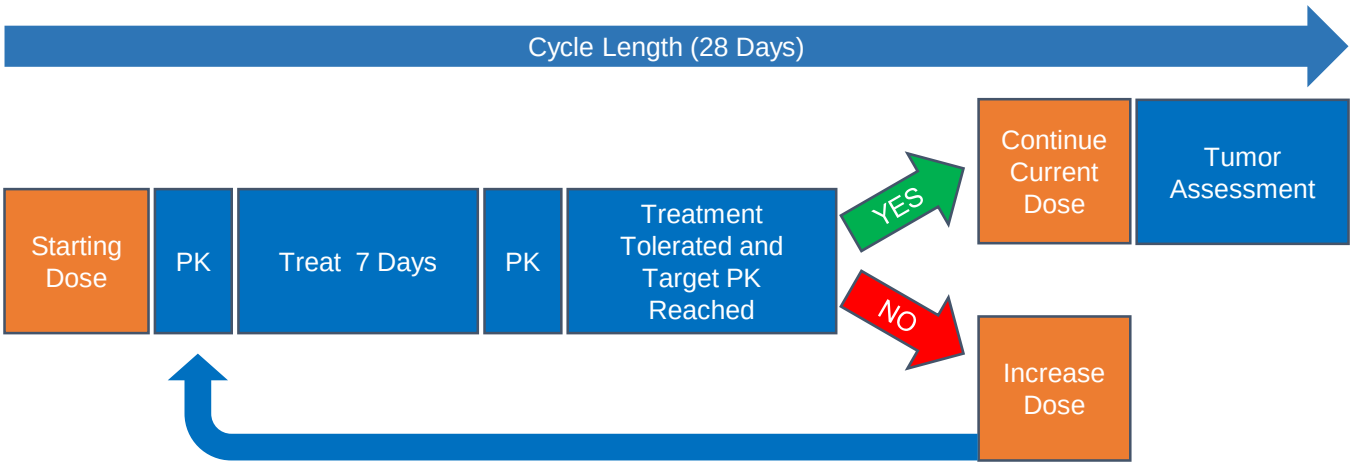


Figure S3. Single patient protocol study schema. Abbreviations: PK-pharmacokinetics.

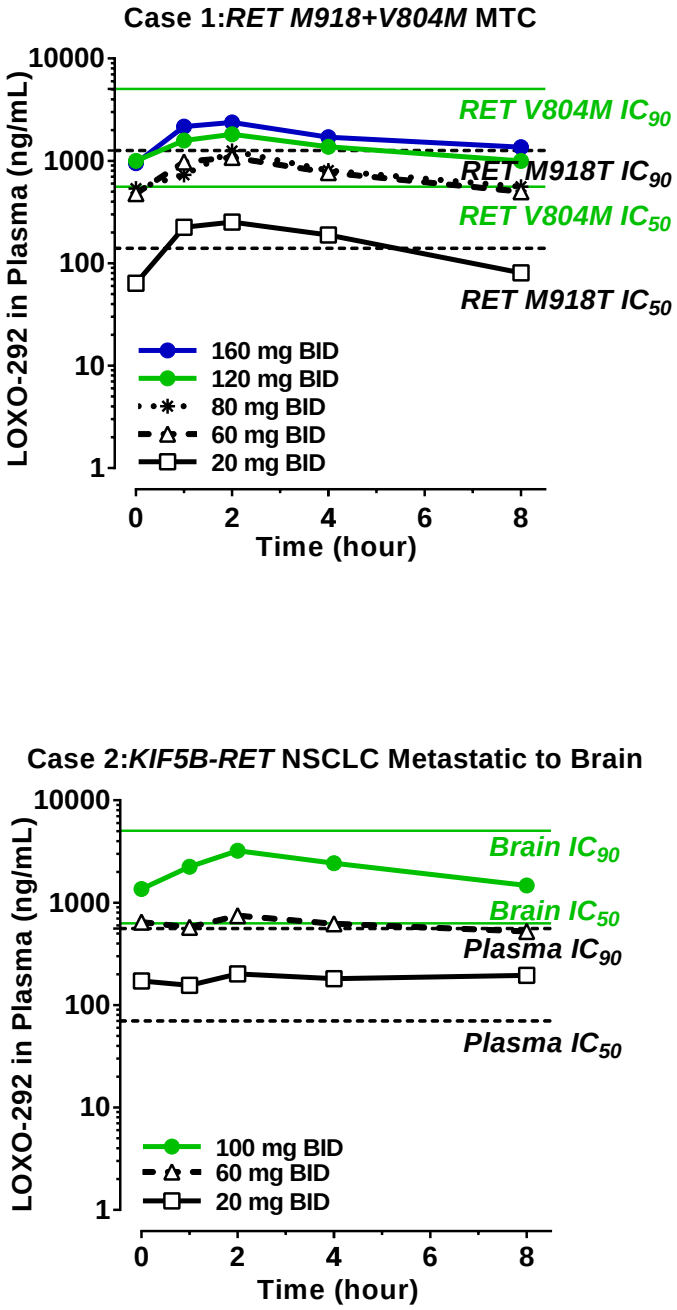
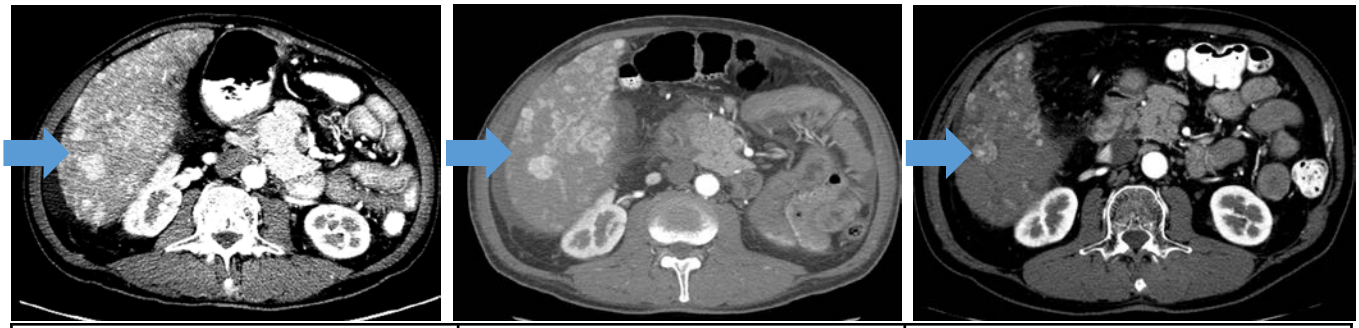
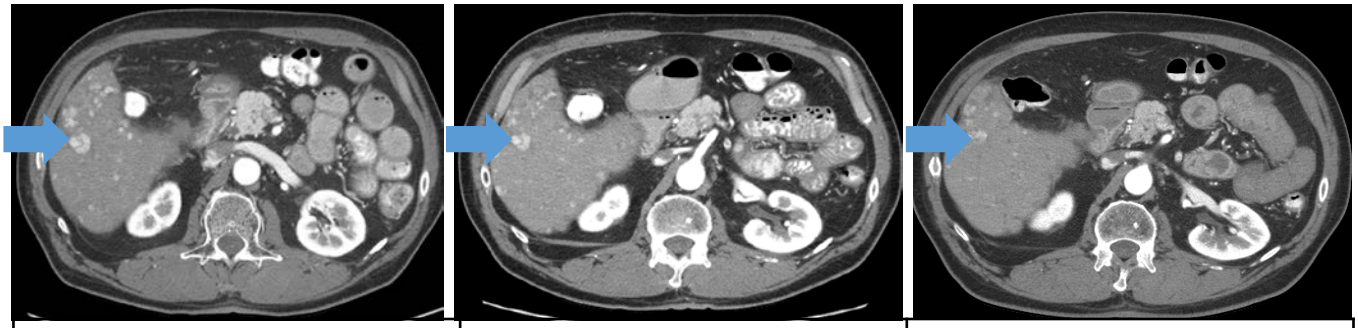


Figure S4. Real-time pharmacokinetic analysis for the two patients. Abbreviations: ng-nanograms; mL-milliliters; MTC-medullary thyroid cancer; NSCLC-non-small cell lung cancer; IC_{50}-half-maximal inhibitory concentration; IC_{90}-90% maximal inhibitory concentration; BID-twice daily.



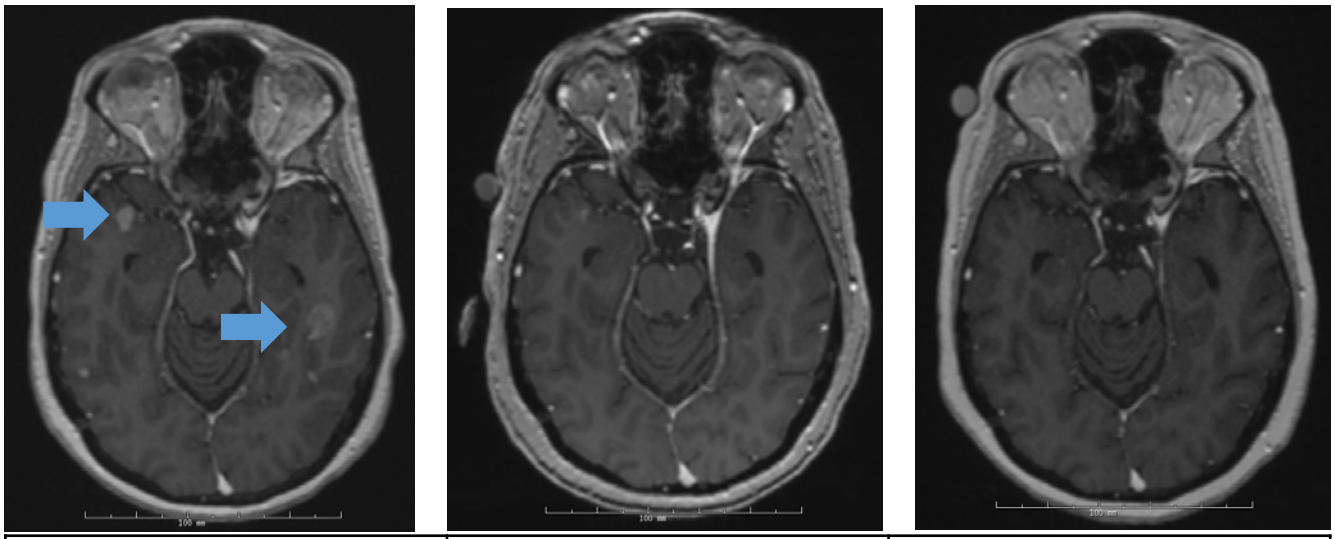
Baseline	1.2 Mo.	2.6 Mo.
↓ Target Lesions (RECIST 1.1)	-12%	-21%



4.2 Mo.	5.2 Mo.	6.9 Mo.
-31% (PR)	-42% (cPR)	-54% (cPR)

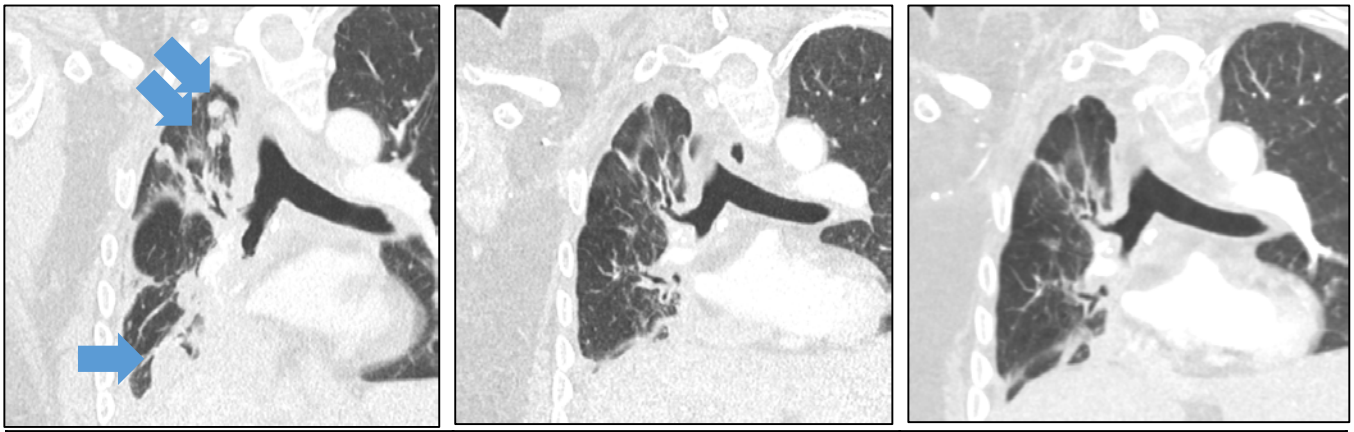
Figure S5. Axial images of the liver for Case 1 (*RET M918T* + *RET V804M* MTC). Note enhancing, infiltrative, non-discrete pattern of liver involvement, and significant decrease with time of LOXO-292 treatment. Abbreviations: Mo.-month; PR-partial response; cPR-confirmed partial response.

A



Baseline	2 Mo.	5 Mo.
↓ Target Lesions (RANO-BM)	-89% (PR)	-100% (cPR)

B



Baseline	2 Mo.	5 Mo.
↓ Target Lesions (RECIST 1.1)	-57% (PR)	-67% (cPR)

Figure S6. (A) Axial images of the brain for Case 2 (*KIF5B-RET* NSCLC) indicating a second target lesion in the right temporal lobe together with the left temporal lobe target lesion shown in Figure 3C. (B) Coronal images of the lungs for Case 2. Blue arrows denote target lesions followed over time. Abbreviations: Mo.-month; PR-partial response; cPR-confirmed partial response.

Case 1 Cancer Mutation - 50 genes (MDACC)					
Date of Collection	Date of Report	Specimen Site	Gene	Mutation	Notes
10/8/2009	7/30/2015	Thyroid Tumor	RET	*M918T*	

Case 1 FoundationOne					
Date of Collection	Date of Report	Specimen Site	Gene	Mutation	Notes
10/8/2009	12/17/2014	Thyroid Tumor	*RET*	M918T	
			SF3B1	K700E	
			ARID1B	G319del	VUS
			ASXL1	G704W	VUS
			EPHA3	S377N	VUS
			EZH2	amplification	VUS
			HRAS	P169fs*4	VUS
			KEL	amplification	VUS
			KMT2C	amplification	VUS
			MLL2	P4175Q	VUS
			PALB2	S689W	VUS
			PIK3R2	R65Q	VUS

Abbreviations: VUS--Variant of Unknown Significance

Case 1 Guardant360						
Date of Collection	Date of Report	Specimen Site	*RET M918T*	*RET V804M*	*RET V804L*	*RET Y806C*
5/23/2017	6/2/2017	Plasma	12.50%	2.20%	ND	ND
6/14/2017	6/23/2017		19.00%	1.30%	0.40%	ND
7/18/2017	7/28/2017		9.10%	1.10%	ND	ND
8/16/2017	8/29/2017		2.50%	0.70%	ND	0.30%
11/28/2017	12/6/2017		1.00%	0.50%	ND	ND
12/19/2017	1/4/2017		0.60%	0.60%	ND	ND

Date of Collection	Date of Report	Specimen Site	*BRAF AMP*	*AR R618Q*	*FGFR1 R58W*	*RB1 L670fs*	*EGFR Exon 20 deletion*
5/23/2017	6/2/2017	Plasma	2.19 (+)	ND	0.10%	ND	ND
6/14/2017	6/23/2017		2.50 (+)	ND	0.10%	0.20%	ND
7/18/2017	7/28/2017		2.24 (+)	0.20%	ND	ND	ND
8/16/2017	8/29/2017		ND	ND	0.09%	ND	ND
11/28/2017	12/6/2017		ND	ND	0.20%	ND	ND
12/19/2017	1/4/2017		ND	ND	ND	ND	0.03%

Case 2 FoundationOne					
Date of Collection	Date of Report	Specimen Site	Gene	Mutation	Notes
6/16/2016	8/5/2016	Lung Tumor	*RET*	KIF5B-RET fusion	
			MLL3	R1906*	
			SPTA1	R891*	subclonal
			CSF1R	R83G	VUS
			EZH2	E649K	VUS
			HNF1A	S345C	VUS
			IRS2	R693_A694InsA	VUS
			MAP2K4	G9R	VUS
			SETD2	N1628K	VUS
			TET2	R1926C	VUS