Targeting Anaplastic Lymphoma Kinase (ALK) in Rhabdomyosarcoma (RMS) with the second-generation ALK inhibitor Ceritinib

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Electronic Supplementary Material

Supplementary Table 1. Clinical trials with ALK inhibitors in solid tumors (including RMS) as of March 2017

#[a]	*Tumor type*	*ALK status*	*RMS (Y/N)*[b]	*Age*	*Inhibitor*	*Target*	*Phase*	*Status*[a]	*Preliminary data*[c]
NCT01524926	**Solid tumors**	**ALK and/or MET alterations**	**Y**	**>1 y**	**crizotinib**	**ALK, MET, ROS1**	**II**	**Recruiting**	Inclusion of metastatic ARMS
NCT02034981	**Hematological and solid tumors**	**ALK, MET or ROS1 alterations**	**Y**	**>1y**	**crizotinib**	**ALK, MET, ROS1**	**II**	**Recruiting**	Ten RMS included, no amplification or translocation of ALK, no treatment response reported
NCT01742286	**Pediatric malignancies**	**ALK alteration or ALK$^+$ IHC (for RMS)**	**Y**	**1-17y**	**ceritinib**	**ALK, IGF1R, IR**	**I**	**Recruiting**	Six RMS included, no treatment response reported
NCT02465528	All except ALK-positive lung cancer	ALK positive	N	>1y	ceritinib	ALK, IGF1R, IR	II	Recruiting	
NCT02186821	Hematological and solid tumors	ALK or ROS alterations	N	>18y	ceritinib	ALK, IGF1R, IR	II	Recruiting	
NCT02321501	Locally advanced/metastatic solid tumors	N.S.	N	>18y	ceritinib + everolimus	ALK, IGF1R, ROS1 + mTOR	I	Recruiting	
NCT02227940	Gemcitabine-based treated solid tumors	N.S.	N	>18y	ceritinib + chemo	ALK, IGF1R, IR	I	Recruiting	
NCT00939770[1]	Relapse or refractory solid tumors and ALCL	ALK fusion, mutation or amplification (>4-fold)	N	1-21	crizotinib	ALK, MET, ROS1	I/II	Active	Three RMS included in dose-escalating phase, not eligible for treatment response analysis due to lack ALK alterations.
NCT02693535	Multiple, incl. advanced solid tumors	ALK, ROS1, MET mutations	N	>18y	crizotinib	ALK, MET, ROS1	II	Recruiting	
NCT02465060	Solid tumor or lymphoma	ALK translocation	N	>18y	crizotinib	ALK, MET, ROS1	II	Recruiting	
NCT01606878	Relapsed or refractory solid tumors or ALCL	N.S.	N	1-21	crizotinib + chemo	ALK, MET, ROS1	I	Recruiting	
NCT01999972	Solid tumor	N.S.	N	>18y	crizotinib + axitinib	ALK, MET, ROS1 + multi kinase	I	Recruiting	
NCT02650401	Solid tumors, CNS tumors and NB	N.S.	N	2-22y	entrectinib	TrkA/B/C, ROS1, ALK	I	Recruiting	
NCT02568267	Solid tumors incl. sarcomas	NTRK1/2/3, ROS1 or ALK gene rearrangement	N	>18y	entrectinib	TrkA/B/C, ROS1, ALK	II	Recruiting	
NCT02097810[2, 3]	Locally advanced or metastatic solid tumors	ALK, ROS1, NTRK1/2/3 alterations	N	>18y	RXDX-101	ALK, ROS1, TrkA/B/C	I	Recruiting	
NCT01625234	Advanced solid tumors	N.S.	N	>18y	X-396	ALK	I	Recruiting	
NCT02048488	Advanced solid tumors and lymphomas	ALK or TRK-positive status	N	>18y	TSR-011	ALK, TRK	I/II	Active	
NCT01401504	Relapse or refractory solid tumor	N.S.	N	>20y	ASP3026	ALK, ROS1, ACK	I	Completed	
NCT01284192[4]	Advanced solid tumors and BCL	ALK or ROS alterations	N	>18y	ASP3026	ALK, ROS1, ACK	I	Completed	

[a] according to Clinicaltrials.gov (as of 29-3-2017), RMS: rhabdomyosarcoma, [b] mentioning of inclusion RMS patients, [c]up to April 2017, Y: inclusion RMS patients, N: not mentioned, y: years of age, N.S: not specified, ARMS: alveolar rhabdomyosarcoma, ALCL: anaplastic large cell lymphoma, CNS: central nervous system, NB: neuroblastoma, BCL: B-cell lymphoma, ALK: anaplastic lymphoma kinase, IGF1R: insulin-like growth factor 1 receptor, IR: insulin receptor

2. Supplementary materials and methods

2.6 Western Blot

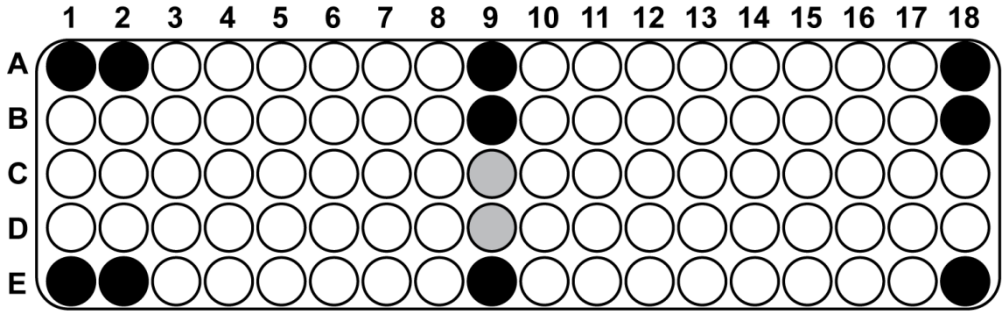
Monoclonal rabbit anti-phosphorylated (p) AKT (Ser473) (1:1000, cat.#4060S), anti-pERK (Thr202/Tyr204) (1:500, cat.#4376S), anti-pS6 (S6 ribosomal protein) (Ser235/236) (1:1000, cat.#2211S), anti-pALK (Tyr1507) (1:1000, cat,#14678S), anti-pIGF1R (Tyr1135/1136) (1:500, cat.#3024S), anti-pSrc (Tyr416) (1:1000, cat.#2101S), anti-ALK (1:1000, cat.#3633S), anti-IGF1R (1:500, cat.#3027S) and anti-poly ADP ribose polymerase (PARP, 1:2000; cat.#9542) were purchased from Cell Signaling Technology (Danvers, MA, USA). Loading control monoclonal mouse anti-α-tubulin (1:1000, cat.#A11126) or anti-GAPDH (1:10000, cat.#Ab8245) were purchased from Thermo Scientific (Breda, The Netherlands) or Abcam (Cambridge, UK), respectively.

2.9 Immunohistochemistry (IHC)

IHC of tumor xenografts was performed to evaluate general tumor characteristics (vascularization, CD34; proliferation, Ki67; and apoptosis, Caspase-3) as previously described [5]. Effects on the PI3K/Akt and MEK/ERK pathways (pAkt and pERK1/2) were assessed with antibodies directed against pAkt (Ser473) and pERK (Thr202/Tyr204). Tumor sections (4μm) were deparaffinized in xylol and rehydrated in a graded ethanol into water series. Antigen retrieval was performed by heating the slides for 10 min at 100ºC while in 10mM citrate buffer, pH 6. Endogenous peroxidase activity was blocked in 3% H_2O_2 for 10 min at RT. Non-specific binding was blocked with 20% normal goat serum in TBS for 30 min. Sections were incubated overnight at 4ºC with the anti-pAkt (1:50, cat.#4060S) or anti-pERK (1:100, cat.#4376S) antibody (Cell Signaling Technology, Danvers, MA, USA), followed by a 30 min incubation at RT with goat-anti-rabbit biotinylated secondary antibody (1:200, Vector laboratories, Peterborough, UK). Lastly, avidin-biotin-enzyme complex (1:100, Vector Laboratories) was added for 30 min at RT followed by 7 min incubation with 3,3'-diaminobenzidine (Bright-DAB) for visualization of antigen expression. Slides were counterstained with hematoxylin, dehydrated, and mounted followed by expression analysis.

Ki67, CD34 and Caspase-3 slides were digitally analyzed with the KS400-Axiophot light microscope (Carl Zeiss, Jena, Germany) and expression was quantitatively analyzed by KS400 software (Carl Zeiss). Random selection of fifteen (CD34; Caspase-3) or six (Ki67) non-overlapping fields was performed and examined at a 200x magnification. The number of blood vessel (CD34) and the percentage of positive cells as proportion of all counted cells (Ki67; Caspase-3) was determined and used for further analysis. Expression of pAkt and pERK was semi-quantitatively scored according to the following distinctions: no positive cells (0), low (1), medium (2) or high (3) staining intensity in >10% of the tumor. Digital images of hematoxylin and eosin (H&E) stained slides at 40x magnification (VisionTek^{TM}, Sakura, version 2.6, East Dundee, IL, USA) were used to determined mitotic activity in ten non-overlapping fields. Mean scores were used to compare the treatment groups. Differences were determined with an unpaired Student's t-test in GraphPad Prism Version 5.03 Software. $p<0.05$ were considered significant.

3. Supplementary figures



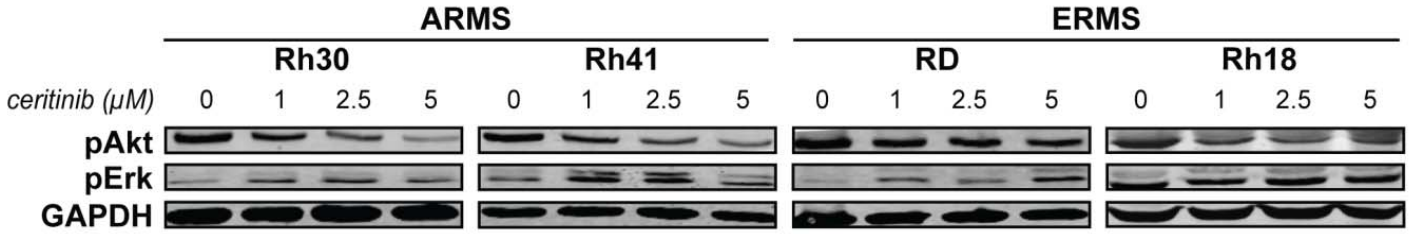
Position	Protein	Phosphorylation site
A1-2	Positive control	-
A3-4	FGFR1	pan-Tyr
A5-6	TrkA/NTRK1	pan-Tyr
A7-8	ALK	pan-Tyr
A9	Positive control	-
A10-11	EphA1	pan-Tyr
A12-13	EphB4	pan-Tyr
A14-15	Akt/PKB/Rasc	Thr308
A16-17	IRS-1	pan-Tyr
A18	Positive control	-
B1-2	EGFR/ErbB1	pan-Tyr
B3-4	FGFR3	pan-Tyr
B5-6	TrkB/NTRK2	pan-Tyr
B7-8	PDGFR	pan-Tyr
B9	Positive control	-
B10-11	EphA2	pan-Tyr
B12-13	Tyro-3/Dtk	pan-Tyr
B14-15	Akt/PKB/Rasc	Ser473
B16-17	Zap-70	pan-Tyr
B18	Positive control	-
C1-2	Her2/ErbB2	pan-Tyr
C3-4	FGFR4	pan-Tyr
C5-6	Met/HGFR	pan-Tyr
C7-8	cKit/SCFR	pan-Tyr
C9	Negative control	-
C10-11	EphA3	pan-Tyr
C12-13	Axl	pan-Tyr
C14-15	p44/42 MAPK (ERK1/2)	Thr202/Tyr204
C16-17	Src	pan-Tyr
C18	STAT3	Tyr705
D1-2	Her3/ErbB3	pan-Tyr
D3-4	InsR	pan-Tyr
D5-6	Ron/MST1R	pan-Tyr
D7-8	FLT3/Flk2	pan-Tyr
D9	Negative control	-
D10-11	EphB1	pan-Tyr
D12-13	Tie2/TEK	pan-Tyr
D14-15	S6 ribosomal protein	Ser235/236
D16-17	Lck	Pan-Tyr

D18	STAT3	Tyr705
E1-2	Positive control	-
E3-4	IGF1R	pan-Tyr
E5-6	Ret	pan-Tyr
E7-8	M-CSFR/CSF-1R	pan-Tyr
E9	Positive control	-
E10-11	EphB3	pan-Tyr
E12-13	VEGFR2/KDR	pan-Tyr
E14-15	c-Abl	Thr308
E16-17	IRS-1	pan-Tyr
E18	Positive control	-

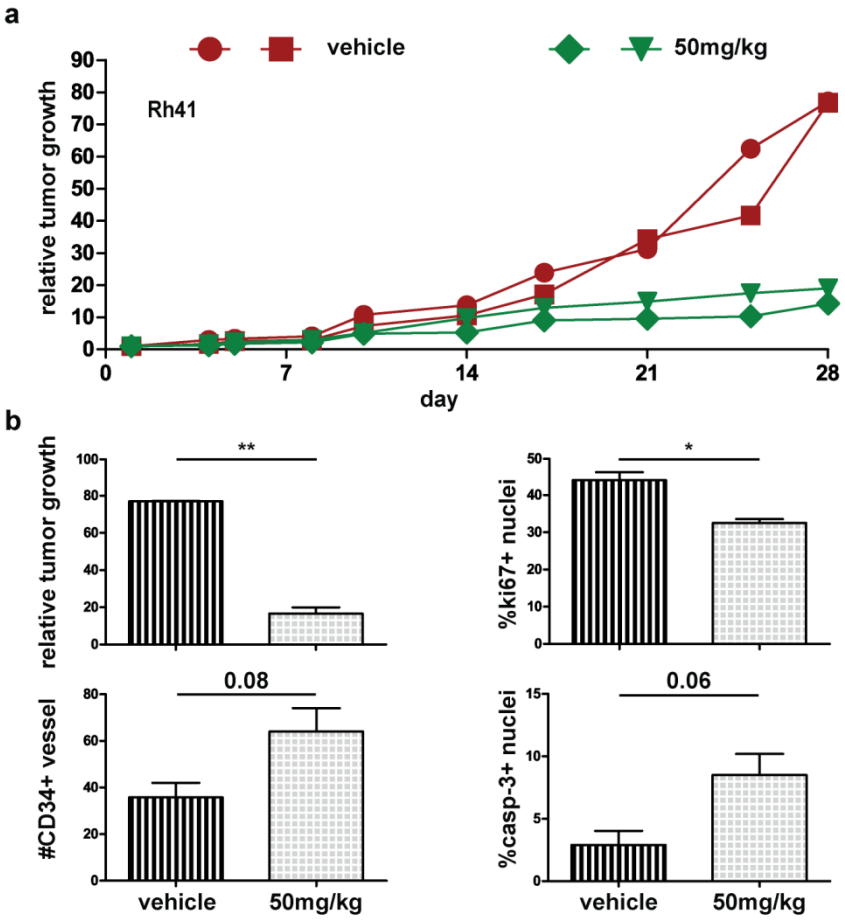
Supplementary Figure 1. An overview of all the targets present in the PathScan analysis and their location on the array.

	Rh30		Rh41		RD		Rh18	
ceritinib	-	+	-	+	-	+	-	+
ALK	2	2	2	1	1	2	0	0
Axl	2	2	2	3	0	1	1	1
c-Kit/SCFR	7	7	8	7	6	7	1	1
EGFR/ErbB1	5	3	5	4	2	4	1	1
EphA1	14	12	20	12	16	13	1	2
EphA2	6	5	10	6	5	6	2	2
EphA3	7	6	8	6	3	6	2	3
EphB1	5	4	4	3	1	3	1	1
EphB3	1	2	3	1	0	2	0	1
EphB4	3	3	10	7	1	2	3	4
FGFR1	9	8	9	8	8	8	3	3
FGFR3	17	12	13	10	12	11	5	5
FGFR4	12	10	32	13	7	8	3	3
FLT3/Flk2	4	2	6	4	3	2	2	2
Her2/ErbB2	7	5	9	6	4	6	2	2
Her3/ErbB3	4	3	8	5	3	9	3	3
IGF1R	2	0	17	2	11	2	2	0
InsR	4	3	5	4	2	4	1	1
M-CSFR/CSF-1R	1	1	2	1	1	1	1	1
Met/HGFR	9	9	19	8	3	5	1	1
PDGFR	4	5	6	5	3	4	1	1
Ret	12	9	12	9	10	8	3	2
Ron/MST1R	7	6	9	7	6	7	2	2
Tie2/TEK	3	3	5	5	1	3	2	3
TrkA/NTRK1	3	3	4	3	2	3	1	1
TrkB/NTRK2	13	11	13	11	11	11	2	2
Tyro-3/Dtk	10	8	13	11	8	8	1	1
VEGFR2/KDR	1	1	1	2	0	1	1	2
Akt (Ser473)	179	61	124	60	87	61	41	14
Akt (Thr308)	35	25	37	33	24	30	15	15
c-Abl (pan-Tyr)	4	4	6	4	2	7	1	1
IRS-1 (pan-Tyr)	12	15	18	16	22	43	9	13
Lck (pan-Tyr)	4	3	4	3	2	5	0	1
Erk1/2 (Thr202/204)	9	8	12	11	10	15	11	19
S6 (Ser235/236)	80	25	37	23	69	47	132	157
Src (pan-Tyr)	34	40	301	190	46	74	79	82
STAT1 (Tyr701)	10	10	9	7	6	11	4	5
STAT3 (Tyr705)	20	21	8	5	9	17	5	6
Zap-70 (pan-Tyr)	10	12	13	13	8	11	2	2

Supplementary Figure 2. Effects of ceritinib treatment on relative RTK/signaling protein phosphorylation in ARMS and ERMS cell lines. Relative phosphorylation values of the PathScan-examined RTKs and signaling proteins for each cell line before and after ceritinib treatment. Boxes represent the RTKs/signaling proteins that were further examined.



Supplementary Figure 3. Effect of 24h ceritinib treatment on PI3K/Akt and MEK/Erk signaling pathway activity. In order to determine the mechanism of action of ceritinib in RMS cells, the concentration that had an effect on intracellular signaling following 24h treatment was determined. Cells were treated with a range of ceritinib concentrations (0-5μM) for 24h and the activity of the two main intracellular signaling pathways: PI3K/Akt (pAkt; 60kDa) and MEK/ERK (pERK; 44-42kDa), was assessed.



Supplementary Figure 4. Treatment effects on ARMS tumor growth –outliers. **a** Relative tumor volume of the outliers of the control and 50 mg/kg treatment groups (n=2 per group) (relative growth 77.0 ± 0.3 vs. 16.6 ± 3.3, $p<0.01$**). b** Graphic representation of the relative tumor volume for the vehicle and ceritinib (25 or 50 mg/kg) groups. Quantitative scoring of Ki67, CD34, and Caspase-3 levels for the outliers. Values are presented as mean ± SD, *$p<0.05$, **$p<0.01$

4. Supplementyary References

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