The role of constitutive activation of FMS-related tyrosine kinase-3 and *NRas/KRas* mutational status in infants with *KMT2A*-rearranged acutelymphoblastic leukemia

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Supplemental methods

Clinical samples

Clinical samples (bone marrow (BM) or peripheral blood) were obtained from 167 pediatric patients with *KMT2A*-rearranged ALL before treatment enrolled in multicenter trials ALL-BFM 86, 90, 95, 2000, and AEIOP-BFM ALL 2009 as well as German patients enrolled in Interfant-99/-06 after informed consent was obtained in accordance with the Declaration of Helsinki. 95 patients were infants younger than 12 month (91 B-lineage, 2 biphenotypic, 2 unknown phenotype) and 72 were children older than 1 year (61 B-lineage, 1 biphenotypic, 10 T-cell phenotype) (Supplemental Table S1). In addition, we analyzed relapse samples of 8 infants (all B-lineage ALL). Samples of 15 patients with *BCR-ABL* t(9;22) positive B-cell precursor ALL and bone marrow of 8 healthy donors served as controls in our gene expression analysis. Genomic DNA was extracted from BM or peripheral blood samples according to standard procedures.

Quantification of FLT3 gene expression

For gene expression analyses BM or peripheral blood samples had to contain more than 70% blasts, as assessed morphologically before gradient centrifugation. RNA isolation, cDNA synthesis and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) were performed as previously described[1] using QuantiTect Primer Assays for *FLT3* [QT00071316] and *succinate dehydrogenase complex subunit A* (*SDHA)* [QT00059486] for normalization; QIAGEN). Contaminating DNA was routinely removed from RNA samples by using the DNA-*free*™ Kit (Ambion). Each sample was tested in duplicate. The expression ratio was calculated as 2^n, where *n* was the C_T value difference normalized by the C_T difference of a calibrator sample. *FLT3* transcript level of the cell line RS4;11 (purchased from DSMZ) was used as a calibrator[2].

Detection of FLT3 and N/KRAS mutations

FLT3 juxtamembrane domain (JMD) and *FLT3*-TKD mutations were detected by PCR and restriction fragment length polymorphism-mediated (RFLP) PCR assays as previously described[3, 4]. For the examination of *FLT3*-JMD mutations, exon 14 to 15 were amplified by using the primers R5 and R6 for cDNA and 11F and 12R for genomic DNA templates[4, 5]. Exon 20 of the *FLT3*-TKD was amplified using the primers 17F and 17RC or 17F and TK-R1,

respectively. Amplified *FLT3*-TKD PCR products were subjected to digestion with *Eco*RV. All PCR products were first separated with the QIAxcel system (QIAGEN). Products corresponding to mutated alleles were electrophoresed on a 3% agarose gel, cut, purified and sequenced.

NRAS and *KRAS* genes were screened for mutations in codons 12, 13, and 61 by high resolution melting analysis (HRMA) using the LightCycler 480 System and the Gene Scanning Software (Roche). DNA fragments spanning these codons were amplified by PCR in 96-well plates using the primers and conditions previously described by Yang et al[6]. For the identification of sequence variants by HRMA, plates were heated from 65 °C up to 95 °C with a ramp rate of 0.1 °C/s. Melting profiles were normalized, grouped and displayed as fluorescence *versus* temperature plots or as derivative plots as the negative derivative of the fluorescence relative to the temperature (-dF/dT) *versus* temperature. All positive results of HRMA were confirmed by direct sequencing of larger PCR-amplicons as previously described[6].

Wet-lab and animal experimentation

NOD.Cg-Prkdc^{scid} Il2rgtm1Wjl/SzJ (NSG) mice were purchased from Charles River and bred. Mice were maintained as approved by the governmental animal care and use committees. Patient-derived xenografts were established by intrafemoral injection of 1x10^6 ALL cells per animal. Experiments were terminated upon clinical signs of engraftment (activity loss, weight loss, hind limb paralysis) or detection of >80% human blasts in the peripheral blood by human CD19/CD45 staining and FACS analysis. In some experiments animals were fed with BrdU one hour before euthanasia and cell cycle analyses were performed using FACS analysis (BD Accuri) as previously described[7]. For Western Blotting, cells were recovered from target organs and processed as previously described[7, 8]. Antibodies for p-FLT3 (Y591), p-ERK (Thr202/Tyr204), ERK, pAkt (Ser308), Akt, p21 and GAPDH were purchased from Cell Signaling. Lestaurtinib (LC Laboratories) was applied by intraperitoneal injection of 10 mg/kg body weight daily Monday through Friday alone or in combination with chemotherapy mimicking an ALL induction regimen (dexamethasone 70 µg orally days 1-5, vincristine 10 µg intravenously day 1 and PEG-asparaginase 100 IU intravenously day 1). This cycle was repeated every 28 days.

Statistical analysis

Patients: Event-free-survival (EFS) was defined as the time from diagnosis to the date of the last follow-up or the first event. Events were resistance to therapy, relapse, secondary neoplasm or death from any cause. Failure to achieve remission due to early death or resistance was considered as event at time zero. The Kaplan-Meier method was used to estimate survival rates, differences were compared with the log-rank test. Cox proportional hazards model was used for uni- and multivariate analyses. Cumulative incidence functions of relapse (CIR) for competing events were constructed by the method of Kalbfleisch and Prentice and were compared with Gray's test. Differences in initial characteristics were analyzed by the χ^2 and Fisher's exact tests for categorical and Mann-Whitney-U test or Wilcoxon signed-rank test for continuous variables.

Xenografts: Statistical analyses were performed using GraphPad Prism (La Jolla, CA, USA). Statistical significance was assessed using the Mann-Whitney U-test. Survival was analyzed using the Kaplan-Meier method and log-rank statistics. A p-value of <0.05 was considered statistically significant.

Supplementary References

1. Cario G, Izraeli S, Teichert A, Rhein P, Skokowa J, Moricke A, et al. High interleukin-15 expression characterizes childhood acute lymphoblastic leukemia with involvement of the CNS. J Clin Oncol. 2007; **25**(30): 4813-20.
2. Stong RC, Korsmeyer SJ, Parkin JL, Arthur DC, Kersey JH. Human acute leukemia cell line with the t(4;11) chromosomal rearrangement exhibits B lineage and monocytic characteristics. Blood. 1985; **65**(1): 21-31.
3. Taketani T, Taki T, Sugita K, Furuichi Y, Ishii E, Hanada R, et al. FLT3 mutations in the activation loop of tyrosine kinase domain are frequently found in infant ALL with MLL rearrangements and pediatric ALL with hyperdiploidy. Blood. 2004; **103**(3): 1085-8.
4. Chillon MC, Fernandez C, Garcia-Sanz R, Balanzategui A, Ramos F, Fernandez-Calvo J, et al. FLT3-activating mutations are associated with poor prognostic features in AML at diagnosis but they are not an independent prognostic factor. Hematol J. 2004; **5**(3): 239-46.
5. Kiyoi H, Naoe T, Nakano Y, Yokota S, Minami S, Miyawaki S, et al. Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. Blood. 1999; **93**(9): 3074-80.
6. Yang X, Qian J, Sun A, Lin J, Xiao G, Yin J, et al. RAS mutation analysis in a large cohort of Chinese patients with acute myeloid leukemia. Clin Biochem. 2013; **46**(7-8): 579-83.
7. Alsadeq A, Strube S, Krause S, Carlet M, Jeremias I, Vokuhl C, et al. Effects of p38alpha/beta inhibition on acute lymphoblastic leukemia proliferation and survival in vivo. Leukemia. 2015; **29**(12): 2307-16.
8. Krause S, Pfeiffer C, Strube S, Alsadeq A, Fedders H, Vokuhl C, et al. Mer tyrosine kinase promotes the survival of t(1;19)-positive acute lymphoblastic leukemia (ALL) in the central nervous system (CNS). Blood. 2015; **125**(5): 820-30.

Table S1: Cohort assembly of *KMT2A*-rearranged ALLs according to immunophenotypes and KMT2A-fusion partners.

		KMT2A-fusion						
	Total	**AF4**	**ENL**	**AF9**	**AF10**	**AF6**	**AF1**	**unknown**
Infants	**95**	**40**	**26**	**18**	**3**	**1**	**3**	**4**
BCP-ALL	88	39	24	14	3	1	3	4
B-ALL	3	-	-	3	-	-	-	-
Biphenotypic ALL	2	-	1	1	-	-	-	-
Unknown	2	1	1	-	-	-	-	-
Children >1 yr	**72**	**34**	**12**	**13**	**4**	**3**	**-**	**6**
BCP-ALL	57	33	5	11	2	-	-	6
B-ALL	2	-	1	-	1	-	-	-
B-lineage ALL	2	-	1	-	1	-	-	-
Biphenotypic ALL	1	1	-	-	-	-	-	-
T-ALL	10	-	5	2	-	3	-	-

BCP indicates B-cell precursor

Table S2: Multivariate Cox regression analysis of prognostic variables including *FLT3*^{low}, for event-free survival and relapse incidence in infants treated according to the Interfant protocols.

	EFS			Relapse incidence		
Feature	**Hazard ratio**	**95% CI**	**p**	**Hazard ratio**	**95% CI**	**p**
FLT3^{low} (RQ <2)	3.80	1.71-8.42	0.001	6.68	2.37-18.8	<0.0001
Age <6 month	3.37	1.32-8.62	0.011	4.08	1.27-13.11	0.018
WBC ≥300,000/µl	0.80	0.34-1.87	0.60	0.85	0.29-2.45	0.76
Prednisone response	0.81	0.33-2.01	0.65	0.64	0.21-1.95	0.43
Day 33 non remission	3.07	0.99-9.55	0.053	3.90	0.97-15.8	0.056

EFS indicates event-free survival; CI, confidence interval; RQ, relative quantification ($2^{-\Delta\Delta CT}$); WBC, white blood cell

Table S3: Multivariate Cox regression analysis of prognostic variables including *FLT3*^{high} vs. *FLT3*^{low}, *RAS* mutation vs. wild-type, and the interaction of *FLT3*^{high} and *RAS* mutation, for event-free survival and relapse incidence in infants treated according to the Interfant protocols.

	EFS			Relapse incidence		
Feature	**Hazard ratio**	**95% CI**	**p**	**Hazard ratio**	**95% CI**	**p**
Interaction *FLT3*^{high}/*RAS* WT	0.19	0.03-1.11	0.065	0.04	0.01-0.37	0.005
FLT3^{low} (RQ <2)	1.10	0.25-4.95	0.90	0.81	0.15-4.46	0.81
RAS mut.	0.99	0.29-3.30	0.98	1.12	0.26-4.91	0.88
Age <6 month	3.61	1.32-9.90	0.012	6.08	1.51-24.46	0.011
WBC ≥300,000/µl	0.91	0.38-2.19	0.83	1.05	0.34-3.26	0.93
Prednisone response	0.81	0.32-2.08	0.66	0.57	0.18-1.88	0.36
Day 33 non remission	2.31	0.77-6.97	0.15	2.96	0.78-11.29	0.11

EFS indicates event-free survival; CI, confidence interval; RQ, relative quantification ($2^{-\Delta\Delta CT}$); WBC, white blood cell