t(6;9)(p22;q34)/*DEK-NUP214*-rearranged pediatric myeloid leukemia: an international study of 62 patients

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Patients

The inclusion criteria comprised age of 0-18 years and a diagnosis between 1 January 1993 and 31 December 2011 of *de novo* AML or MDS with t(6;9)/*DEK-NUP214*, as determined by G-, Q-, or R-banding, fluorescence in situ hybridization (FISH) and/or reverse transcription polymerase chain reaction (RT-PCR).

The study was conducted within the I-BFM study group cooperation and 24 study groups and treatment centers participated. They contributed background data for frequency analysis and submitted clinical and genetic data on 62 patients in the study: The Spanish Pediatric Hematology Society (SHOP; Spain, n=1), the Taiwan Pediatric Oncology Group (TPOG; Taiwan, n=3), the European Working Group of myelodysplastic syndrome (MDS) and juvenile myelomonocytic leukemia (JMML) in Childhood (EWOG-MDS, n=3), the Dutch Childhood Oncology Group (DCOG; the Netherlands, n=5), the St Jude Children's Research Hospital (United States, n=6), the Associazione Italiana de Ematologia e Oncologia Pediatrica (AIEOP; Italy, n=3), the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG; Japan, n=9), the Leucémies Aiguës Myéloblastiques de l'Enfant Cooperative Group (LAME and Hôpital Saint-Louis,Paris, France, n=9), the Hong Kong Pediatric Hematology & Oncology Study Group (HKPHOSG; Hong Kong, n=2), the Nordic Society for Pediatric Hematology and Oncology (NOPHO; Denmark, Finland, Iceland, Norway, and Sweden, n=8), the Medical Research Council (MRC; United Kingdom, n=7), the Schneider Children's Medical Center (Israel, n=1), the Berlin-Frankfurt-Münster Study Group (BFM; Germany, n=4, Austria, and Switzerland), the Australian and New Zealand Children's Cancer Study Group (ANZCCSG; Australia and New Zealand), nine Brazilian centers (Brazil), the Czech Pediatric Hematology Working Group (CPHWG; Czech Republic), the Argentine Group for the Treatment of Acute Leukemia (GATLA; Argentina), the Hungarian Pediatric Oncology Network (Hungary), the National Program for Antineoplastic Drugs for Children (PINDA; Chile), and the European Organization for Research and Treatment of Cancer– Children's Leukemia Group (EORTC-CLG; Belgium, n=1).

In addition to the clinical data, the participating groups contributed smears, BM biopsies, and material for gene expression analysis. Patients were treated according to national AML trials, and the treatment protocols were approved according to local ethical committees and national regulations. Clinical and genetic characteristics and data on therapy and follow-up (FU) were

collected. All karyotype strings were centrally reviewed following the International System for Human Cytogenetic Nomenclature 2009[1] by three of the co-authors (J.H., B.J., and E.F.). Available diagnostic smears and BM biopsies were reviewed by co-author G.K.

Isolation of leukemic blasts, RNA isolation and GEP

Viably frozen cells were requested from participating study groups and the leukemic cells were isolated and enriched as previously described.[2] All samples accepted for GEP contained 80% or more blasts, as determined by May-Grünwald-Giemsa (Merck, Darmstadt, Germany) stained cytospins. A minimum of $2x10^6$ leukemic cells were lysed in Trizol reagent (Gibco BRL/Life Technologies, Breda, The Netherlands) and stored at –80°C. Isolation of total cellular RNA was performed as described before.[3] The integrity of total RNA was checked using the Agilent 2100 Bio-analyzer (Agilent, Santa Clara, CA, USA). cDNA and biotinylated cRNA were synthesized, hybridized, and processed on the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's guidelines as previously described.[4]

Gene expression profiling (GEP) was performed on 297 pediatric AML patient samples including eight t(6;9)-positive samples. The GEP data on the full cohort have been previously published but an individual analysis of t(6;9)/*DEK-NUP214* was not yet performed.[4] Original data are available in the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo; accession GSE17855). The gene expression profiles of t(6;9)-positive cases were compared with those of other representative pediatric AML samples.[4] Top scoring differentially expressed genes were presented in a top-list rated according to significance based on log-fold change and number of positive probe sets.

Quantitative real time PCR (RT-qPCR)

From the top-list of differentially expressed genes generated from the GEP, four genes were selected for further mRNA expression validation by RT-qPCR based on statistical significance, occurrence of multiple probes in the top-list, and log-fold change, combined with potential biological relevance; *EYA3 (*eyes absent homolog 3, Drosophila*), SESN1 (*sestrin 1), *PRDM2*, also known as *RIZ (*PR domain containing 2, with ZNF domain), and *HIST2H4 (*histone cluster 2, H4).

RT-qPCR was performed on t(6;9)/*DEK-NUP214*-positive samples from 17 patients (eight from the GEP profiled subset and nine additional available samples with acceptable quality from the

included patients) and one cell line (FKH-1) and compared with AML without t(6;9)/*DEK-NUP214* (31 patient samples and 13 cell lines). The ABI PRISM 7900HT sequence detector (Applied Biosystems, Foster City, CA, USA) was used. The primers applied are described in Supplementary Table S1. SYBRgreen dye was used to detect PCR products in the expression analysis of target genes. The expression of the genes was compared with that of the reference gene *GAPDH*, with primers and probe as previously described (Supplementary Table S1).[5] The average cycle threshold (Ct) value was used to calculate mRNA expression levels of target genes relative to the expression level of *GAPDH* using the comparative Cycle time (ΔCt) method.[6]

FLT3-ITD detection

FLT3-ITD analysis was performed by PCR as previously described (Primer sequences are shown in the Supplementary Table S1)[7,8] on submitted t(6;9) cases with unknown *FLT3*-ITD status and available material. After electrophoresis on 1% agarose gel the samples were sequenced for presence of ITD.

Statistical analyses

The Kaplan-Meier method was used to estimate the 5-year probabilities of OS (pOS) and the 5-year event-free survival (pEFS). The 5-year cumulative incidence of relapse (pCIR) was calculated by the method of Kalbfleisch and Prentice.[9] The survival estimates were compared using the log-rank test and for CIR the gray test was used. Patients diagnosed with MDS were excluded from the survival analysis. OS was measured from the date of diagnosis to the date of death of any cause or the date of last FU. The pEFS was calculated from the date of diagnosis to the first event (resistant disease, relapse, secondary malignancy or death) or to the date of last FU. Patients without events were censored at time of last FU. CIR was defined as time between diagnosis and relapse and resistant disease was included as an event on day 0. The median time to HSCT in CR1 was 150 days with 98 days as a minimum. Patients with an event within 150 days of treatment were excluded from the analysis comparing the effect of HSCT in CR1 with conventional chemotherapy alone. In the comparison of differences in subgroups the Chi-squared or Fisher exact test was used. For continuous variables, the Mann-Whitney U test was applied. For multivariate analysis, the Cox

proportional-hazard regression model was used. *P* values were considered significant if less than 0.05. Statistical analyses were conducted using SPSS for Mac, Version 20, (SPSS Science, Chicago, IL).

The GEP data acquisition was performed using Expresso (Bioconductor package Affy). Probe-set intensities were normalized using the variance stabilization normalization (Bioconductor package VSN) in the statistical data analysis environment R, version 2.11.[10] Expression levels were log-transformed during this normalization. An empirical Bayes linear regression model was used to compare the signatures for the t(6;9)-positive cases with the other AML cases.[11] Moderated T-statistics *P*-values were corrected for multiple testing using the False Discovery Rate (FDR) method, as defined by Benjamini and Hochberg,[12] with values <0.05 considered as significant.

For comparison of the gene expression of the target genes between t(6;9)-positive and negative groups, the Mann-Whitney or Kruskal-Wallis test was used. For assessment of correlation of the RNA expression levels from Affymetrix profiling analysis and RT-qPCR, Spearman's correlation coefficient was applied.

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Supplementary Table S1: Primers and probe sequences

Primer/probe	Sequence (5'-3')
DEK-exon 8 FW	AGC-AGC-ACC-ACC-AAG-AAG-A
NUP214-exon 19 RV	TGG-CTA-GGG-TGT-TAA-ACA-GTG
EYA3_2_FW	GGG-CAA-GAG-GAA-AGC-TGA
EYA3_2_RV	GCC-ACT-GAA-ACC-ATC-TGT-TG
SESN1_1_FW	TGG-CAA-TGC-ACA-AAG-ATG-T
SESN1_1_RV	GCG-GGT-AAT-GGC-TCT-CAG
RIZ_PR_2_FW	TGG-GCC-ACT-AAA-CCA-ATT-T
RIZ_PR_2_RV	TGG-CAT-CAA-TGC-ACA-TCC
RIZ_CR_1_FW	AGC-CAG-ATT-TCA-CCT-CTG-C
RIZ_CR_1_RV	CTT-CCC-CCA-AAT-CAT-TCA-C
HIST2H4_1_FW	GCC-CAG-TGC-CTG-AAA-AGT-AT
HIST2H4_1_RV	GCA-ACC-CGA-TTG-CTA-AGT-AC
GAPDH Forward	GTC-GGA-GTC-AAC-GGA-TT
GAPDH Reverse	AAG-CTT-CCC-GTT-CTC-AG
FLT3-exon 14 FW	GCA-ATT-TAG-GTA-TGA-AAG-CCA-GC
FLT3-exon 15 RV	CTT-TCA-GCA-TTT-TGA-CGG-CAA-CC

PCR program used for all reactions: 2' 50°C, 10' 95°C, 40 cycles of 15'' 95°C and 1' 60°C

Supplementary Table S2: Karyotypes with aberrations in addition to the t(6;9)(p22;q34)

Patient No.	Karyotype
1	46,XX,t(6;9)(p22;q34)[14]/47,idem,+13[6]
2	46,XY,t(6;9)(p22;q34)[6]/45,idem,-Y[19]
3	47,XXXc,der(6)t(6;9)(p22;q34)t(6;6)(q21;p22),der(9)t(6;9)(q21;q34)[20]
4	46,X,-Y,t(6;9)(p22;q34),der(16)t(13;16)(q21;p12),+mar
5	48-49,XY,der(4)t(4;6)(q21;p21),der(6)t(6;9)(p22;q34),+8,der(9)t(6;9)t(4;6),+13,+13[19]
6	46,XY,t(6;9)(p22;q34),t(8;21)(q22;q22),+mar,inc
7	46,XY,t(6;9)(p22;q34)[2]/45,idem,-Y[13]
8	46,XY,t(6;9)(p22;q34)[21]/45,idem,-12[3]
9	46,XX,t(6;9)(p22;q34)[27]/48,idem,+8,+13[19]
10	46,XX,t(6;9)(p22;q34)[2]/47,idem,+8[18]
11	46,XX,der(6)t(6;9)(p22;q34),der(9)?inv(9)(q21q22)del(9)(q34)[25]

Supplementary Table S3. Characteristics of children with t(6;9)(p22;q34) AML treated with HSCT included in the survival analysis (n=33)

	CR1 (n=18)	After relapse (n=14)
Number of blasts at HSCT		
<5%	18	6
5% - 19%	0	0
>20%	0	1
Data missing	0	7
Donor		
Sibling	5	1
Related other	1	0
Unrelated	11	12
Data missing	1	1
HLA match		
Matched (9 or 10/10)	15	10
Mismatched	1	0
Data Missing	2	4
Source of stem cell		
BM	12	6
PB	3	1
Cord blood	1	5
Data missing	2	2

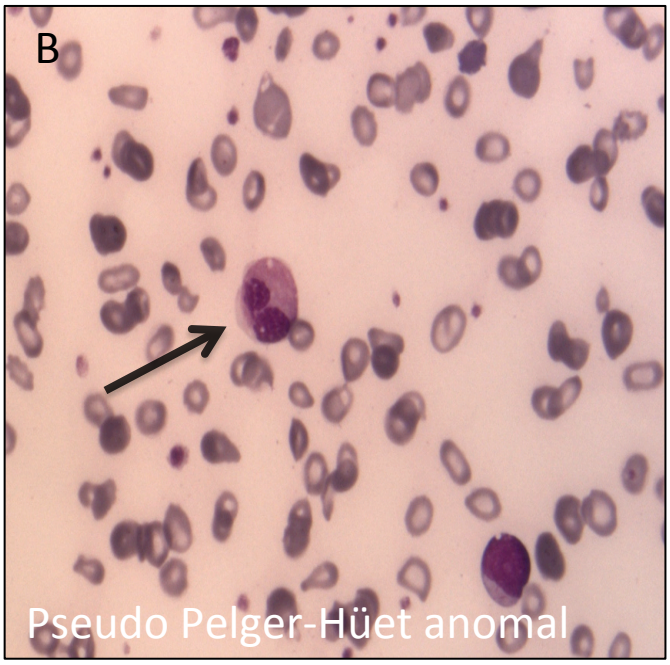
HSCT: hematopoietic stem-cell transplantation, CR: complete remission, RD: resistant disease, HLA: human leukocyte antigen, BM: bone marrow, PB: peripheral blood.

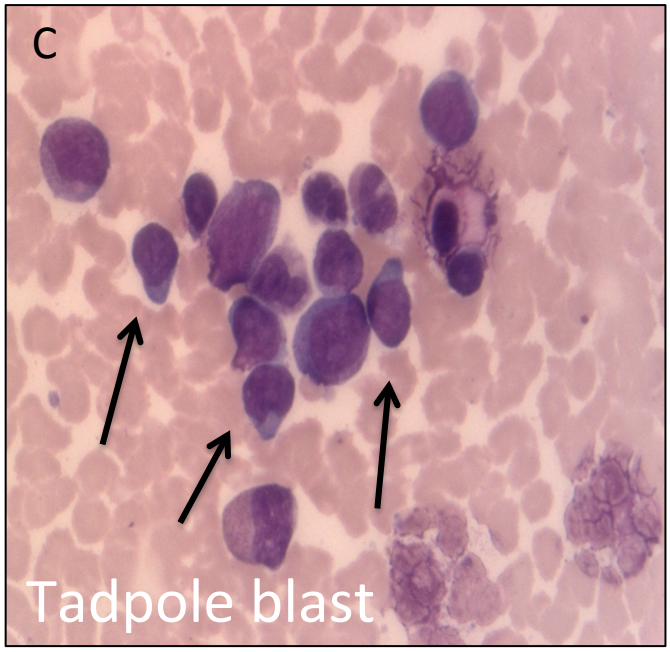
Supplementary Table S4: Multivariate analysis of pediatric t(6;9)/*DEK-NUP214* rearranged AML on survival parameters

	OS			EFS		
	Hazard ratio	95% CI	*P*	Hazard ratio	95% CI	*P*
HSCT in CR1						
No	1.0	reference		1.0	reference	
yes	0.4	0.1-2.8	0.33	0.1	0.0-0.8	0.03
FLT3-ITD						
Negative	1.0	reference		1.0	reference	
Positive	2.7	0.6-10.0	0.18	2.3	0.8-6.6	0.14

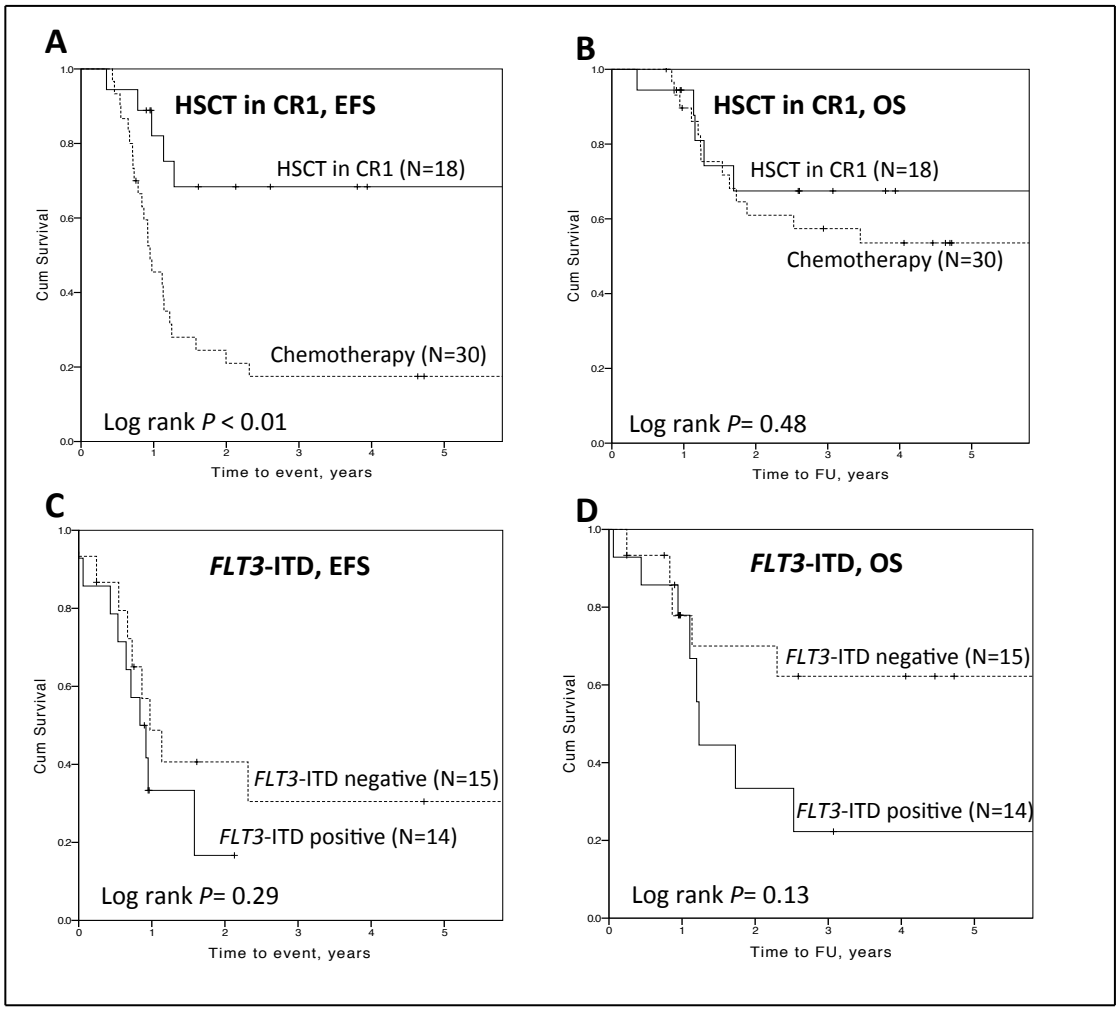
HSCT: hematopoietic stem cell transplantation, CR1: first complete remission, OS: overall survival, EFS: event free survival

A	Number of patients
Peripheral blood (n=11)	
Anisopoikilocytosis	10
Schistocytes	2
Macrocytosis	1
Pseudo-Pelger	11
Giant platelets	6
Tear drop cells	1
Bone Marrow (n=15)	
Auer rods	0
Dyserythropoiesis (n=15)	
Absent	0
Mild(1-10%)	6
Moderate (11-50%)	6
Severe(>50%)	0
Not evaluable	3
Dysmyelopoiesis (n=15)	
Absent (<1%)	0
mild (1-10%)	10
Moderate (11-50%)	3
Severe(<50%)	0
Not evaluable	2
Dysmegakaryopoiesis (n=15)	
Absent (<1%)	2
mild (1-10%)	6
Moderate (11-50%)	0
Severe (>50%)	0
Not evaluable	7

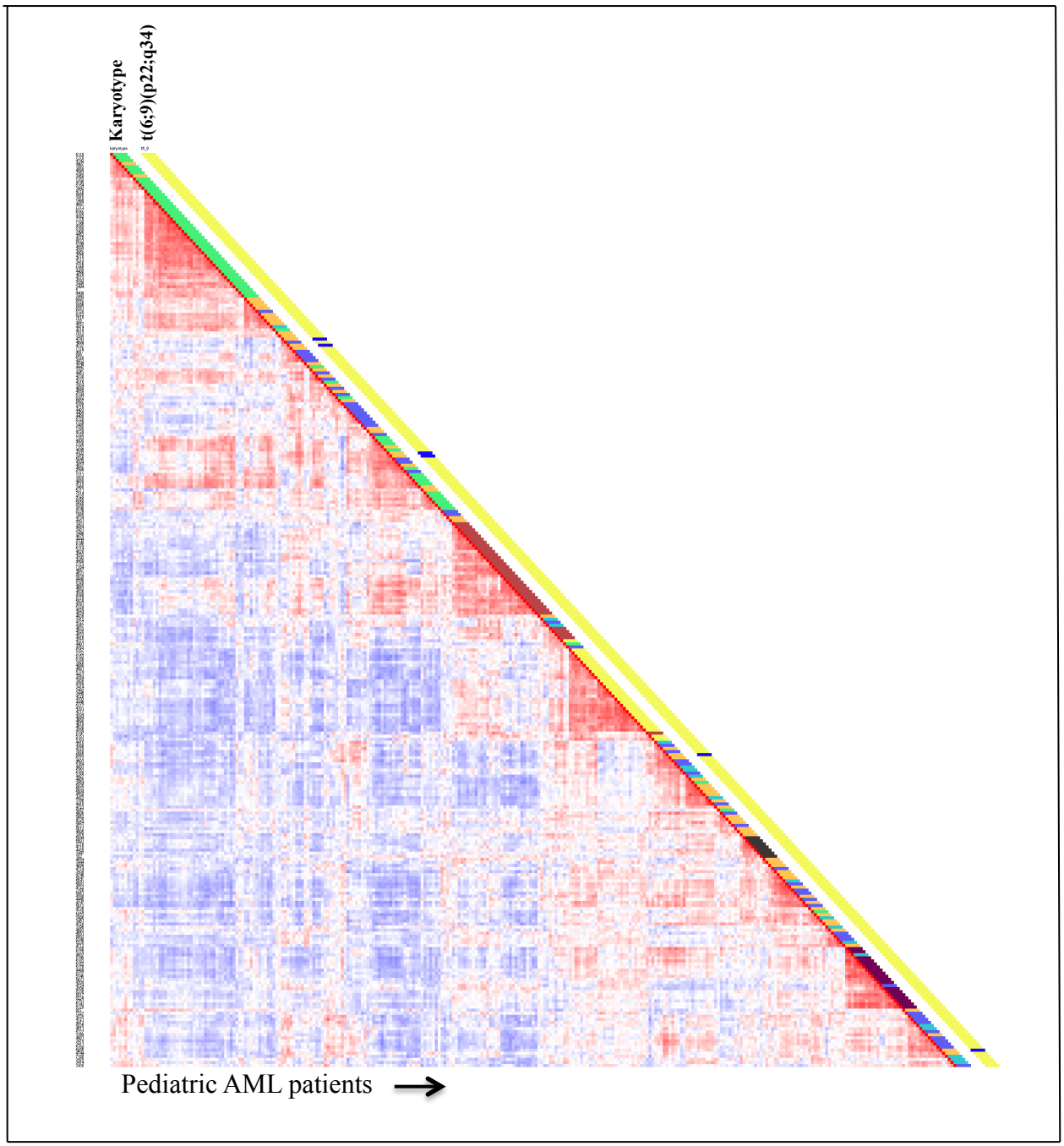




Supplementary Figure 1. Morphologic characteristics and features of t(6;9)(p22;q34)-positive pediatric myeloid malignancies, as ascertained by a central review. A) Table of characteristics. B) Pelger-Huët anomaly. C) Tadpole blasts indicated by arrows. (A+B Wright-Giemsa)



Supplementary Figure 2. Event free survival (EFS) and overall survival (OS) of pediatric t(6;9)(p22;q34)-positive AML patients treated with hematopoietic stem cell transplantation (HSCT) in first complete remission (CR1) vs. AML patients treated with conventional chemotherapy only (A+B). Patients with an event within the first 150 days (median time to HSCT) were excluded from the analysis. C+D) *FLT3*-ITD positive AML patients vs. *FLT3*-ITD negative (wildtype and one point mutation) AML patients.



Supplementary Figure 3: Unsupervised clustering analysis of 297 samples of pediatric AML samples, visualizing the pairwise correlation between the patients (colored by Pearson's correlation coefficient). The values with deeper colors indicate higher positive (red) or negative (blue) correlations. Cytogenetic data are given in the columns along the diagonal of the heatmap. The cytogenetic subgroups are depicted in the first column (MLL rearrangement, green; inv(16)(p13q22), red; t(8;21)(q22;q22), yellow; t(15;17)(q22;q21), purple; t(7;12)(q36;p13), brown; cytogenetically normal, blue; other, orange; unknown, aqua). t(6;9)/*DEK-NUP214* positive cases are illustrated in the second column: (t(6;9)/*DEK-NUP214,* blue and not t(6;9)/*DEK-NUP214,* yellow).

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