

Acute lymphoblastic leukemia with aleukemic prodrome: preleukemic dynamics and possible mechanisms of immunosurveillance

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Supplementary materials for:

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PATIENTS AND METHODS

Patient samples

The cohort comprises all available cases with pre-diagnostic bone marrow sample that did not fulfil the diagnostic criteria of ALL for other than technical reasons.

Standard diagnostics, including molecular genetics, karyotyping and fluorescence in-situ hybridization (FISH) were performed according to the standard practice of local diagnostic laboratories. Basic clinical/outcome data were collected from treating centers. Diagnostic and treatment procedures and protocols were approved by local Institutional Review Boards. Informed consent was obtained in accordance with the Declaration of Helsinki.

Bone marrow/peripheral blood samples taken during pre-diagnostic period and at diagnosis were collected into EDTA. Mononuclear cells were isolated via Ficoll-Paque density gradient centrifugation according to recommendation of manufacturer (Ficoll-Paque, Pharmacia, Uppsala, Sweden). Isolated mononuclear cells were stored in -80°C. DNA from mononuclear cells was isolated using QIAamp[®] DNA Blood Mini Kit/QIAamp[®] DNA Blood Micro Kit (Qiagen, Germany).

Following the definitive diagnosis of leukemia, all patients were given standard therapy according to the current leukemic treatment protocols.

Backtracking of leukemic markers using quantitative PCR

Immunoglobulin (IG) and T-cell receptor (TR) gene rearrangements (12 rearrangements in 8 patients), genomic breakpoint of *ETV6-RUNX1* (1 patient) and *BTG1* intragenic deletion (1 patient) were identified in leukemic cells at diagnosis and were retrospectively investigated in pre-diagnostic bone marrow/peripheral blood samples using quantitative real-time PCR (qPCR). The IG/TR clonal rearrangements were detected in the diagnostic samples using multiplex PCR as described previously (1). Backtracking and quantification of leukemospecific IG/TR gene rearrangements was performed according to the standard approach used for minimal residual disease monitoring (2). Quality and quantity of DNA was assessed by quantification of ALB housekeeping gene (3).

Detection and backtracking of ETV6-RUNX1 breakpoint on genomic level

Amplification of *ETV6-RUNX1* genomic breakpoint fusion (patient UPN1300) was performed by long distance PCR (LD-PCR). The primers used for amplification were described previously (4). To backtrack the genomic *ETV6-RUNX1* fusion by PCR, the following patient-specific pair of primers was used: forward primer 5'-CAGCTCTGCCAGTCTGTAGTCTGACAG-3', reverse primer 5'-GCCTCAGTTTGAATTCTCTCACAAC-3'.

Investigation of T-cell receptor gamma repertoire

T-cell receptor gamma (TRG) gene rearrangement profiling by next generation sequencing was performed at the DNA level via immunoSEQ assay (Adaptive Biotechnologies, Seattle, USA) as described previously (5).

Real-time quantitative PCR detection of the specific V γ 9-J γ 1.2 clone defined by the V-J fusion sequence 5'-TGTGCCTTGTGGGAGGTGCAAGAGTTGGGCAAAA-3' was performed using 400 ng DNA template and 500nM each of forward and reverse primer, 200nM of probe and 2x TaqMan Universal Master Mix II (Applied Biosystems, USA). Following primers and probe were used: forward primer 5'-CACAATGTAGAGAAACAGGACATAGCTAC-3', reverse primer 5'-GCTTTGTCCGGACCAA-3', probe 6Fam-TGGGAGGTGCAAGAGTTGGGCAA-TAMRA. The levels of the V γ 9-J γ 1.2 cells were assessed using cloned plasmid standards. To assess level of the V γ 9-J γ 1.2 clone only within non-malignant cells, the relative quantification of the clone was normalized to levels of the housekeeping gene (ALB) recalculated to the percentage of non-malignant cells in the sample. The relative proportion of non-malignant cells was determined using flow cytometry at diagnosis and by quantification of leukemia-specific IG/TR gene rearrangements by qPCR in follow-up samples.

To evaluate the levels of non-malignant clonal cells harboring defined V γ 9-J γ 1.2 rearrangement, bone marrow samples from 138 leukemia patients and 10 healthy controls were analyzed. The patients cohort included 101 childhood ALL (*ETV6-RUNX1* (n=30), hyperdiploidy (n=20), *TCF3-PBX1* (n=9), *KMT2A* rearrangement (n=5), *BCR-ABL1* (n=4), B-others (n=19), T-ALL (n=14)), 29 childhood AML (*KMT2A* rearrangement (n=9), *CBFB-MYH11* (n=9), *PML-RARA* (n=7), *RUNX1-RUNX1T1* (n=4)) and 8 adult ALL. In 14 childhood ALL patients, samples from diagnosis plus 4 different timepoints during the treatment (day 8, day 15, day 33 and week 12 of treatment) were analyzed.

Detection of leukemic markers via SNP array

Leukemic DNA from diagnostic bone marrow samples was genotyped using single nucleotide polymorphism (SNP) array (HumanOmni Express BeadChip, Illumina, San Diego, USA). DNA labeling and hybridization were performed according to the recommended Infinium HD assay Ultra protocol from Illumina. The analysis of DNA copy number alterations (CNA) and regions of uniparental disomy (UPD) were performed using CNV Partition 2.4.4. algorithm plug-in within the GenomeStudio followed by visual inspection in the Illumina Chromosome Browser and manual correction. Identified CNA/UPD were mapped against human genome assembly GRCh37/hg19.

Assessment of clonal evolution via Fluorescence In Situ Hybridization

Fluorescence *in situ* Hybridization (FISH) was retrospectively performed on fixed bone marrow cells from patient UPN1300 with *ETV6-RUNX1* positive leukemia. The Vysis LSI ETV6(TEL)/RUNX1(AML1) ES Dual Color Translocation Probe Kit (Abbott Molecular) labeled with Spectrum Orange/Spectrum Green was used in combination with bacterial artificial chromosome probes (BlueGnome) for the regions of interest (14q24, 12q24.32, 12q24.11, 1p33, 4q31). Hybridization was carried out according to the recommendations of manufacturer. Usually 200 interphase nuclei and at least 5 with the *ETV6-RUNX1* fusion were evaluated in each hybridization. The presence of the signals for the investigated regions was evaluated with respect to the *ETV6-RUNX1* status. A cell was considered *ETV6-RUNX1* positive if the *ETV6-RUNX1* fusion signal together with small extra signal from the 5' part of translocated RUNX1 gene were present.

Statistics

Mann-Whitney U test or one-way ANOVA with Kruskal-Wallis multiple comparison test (Graphpad Prism, Graphpad Software Inc.) were used to compare difference between analyzed groups and its significance. The p values <0.05 were considered statistically significant.

References:

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SUPPLEMENTARY TABLE S1:**IG/TR Targets used for the (pre)leukemia backtracking:**

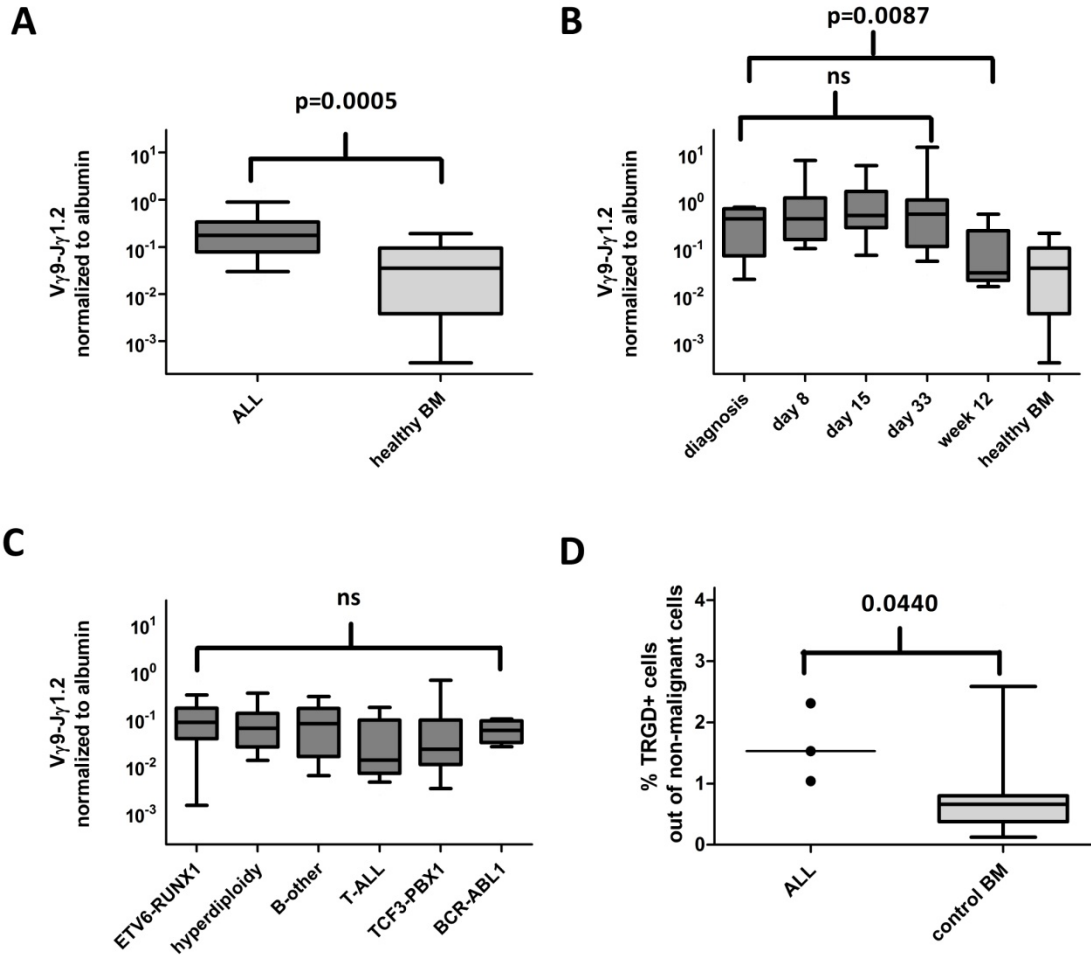
Patient	Target 1	QR/Sensitivity	Target 2	QR/Sensitivity
UPN 63	IGH (VH3)	$10^{-3}/10^{-4}$		
UPN484	IGH (VH3)	$10^{-4}/10^{-4}$	IGK (IK4)	$5 \times 10^{-4}/10^{-4}$
UPN1071	IGH (VH3)	$5 \times 10^{-4}/10^{-4}$	IGK (intron)	$10^{-4}/10^{-4}$
UPN1171	IGH (VH3)	$10^{-4}/10^{-5}$	TRD (VD2)	$5 \times 10^{-4}/10^{-4}$
UPN1300	TRG (VG1)	$10^{-4}/10^{-4}$		
UPN1775	IGH (VH1)	$10^{-4}/10^{-4}$		
UPN1909	Incomplete IGH (DH2)	$10^{-3}/10^{-4}$	IGH (VH3)	$10^{-4}/10^{-4}$
UPN_UK	TRD/A (VD9/JA29)	$5 \times 10^{-4}/10^{-4}$		

IGH – immunoglobulin heavy chain, IGK – immunoglobulin kappa, TRG – T-cell receptor gamma, TRD – T-cell receptor delta, TRA – T-cell receptor alpha; QR – quantitative range

Interpretation criteria according to van der Velden et al., Leukemia 2007.

SUPPLEMENTARY FIGURE S1:

Evaluation of TR-gamma/delta lymphocytes in leukemic and non-leukemic bone marrow.



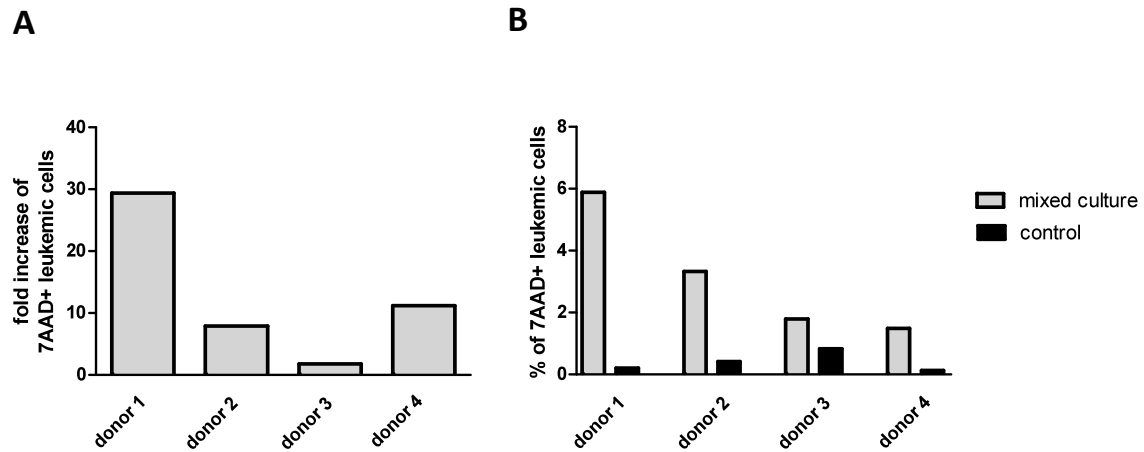
Representation of the T-lymphocytic clone (defined by the specific $V_{\gamma 9}\text{-}J_{\gamma 1.2}$ rearrangement) within non-malignant cells of patients with acute lymphoblastic leukemia (ALL) normalized to housekeeping gene (ALB) in bone marrow taken (A) at diagnosis of ALL (n=98) and in bone marrow from healthy donors (n=10); (B) at the diagnosis and at defined timepoints during the ALL treatment of 14 selected patients and in bone marrow from healthy donors (n=10); (C) in diagnostic samples from different leukemia subtypes (*ETV6-RUNX1* (n=30), hyperdiploid ALL (n=20), B-other ALL (n=19), T-ALL (n=14), *TCF3-PBX1* (n=9), *BCR-ABL1* (n=4)). (D) Percentage of TR-gamma/delta positive T-lymphocytes within non-malignant cells in bone marrow of newly diagnosed ALL (n=3) and in non-leukemic bone marrow controls (n=15) assessed by flow cytometry.

ns=

non-significant.

SUPPLEMENTARY FIGURE S2:

Evaluation of cytotoxic effect of IPP expanded $\gamma\delta$ T-lymphocytes on REH leukemic cell line.



The $\gamma\delta$ T-lymphocytes were magnetically separated from peripheral blood of healthy donors and subsequently expanded by IPP. Following 10-14 days of cultivation, $\gamma\delta$ cells were seeded with Cell Trace violet stained REH leukemic cell line at 10:1 effector:target ratio and cultivated for 5 hours. (A) The cytotoxicity was determined as a fold increase of dead leukemic cells (7AAD+ Cell Trace+) in mixed culture vs. control (REH cells without $\gamma\delta$ T-lymphocytes); (B) percentage of dead leukemic cells (7AAD+ Cell Trace+) in mixed culture (depicted in grey) and in control (depicted in black).