Supplementary information
Low-level RQ-PCR MRD in adult ALL is of prognostic value which can be further enhanced by NGS analysis

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Material & Methods

Treatment protocol

Within the GMALL 07/2003 protocol, all Philadelphia-chromosome (Ph) negative patients received an intensive, pediatric-based 2 phase induction therapy (in phase I: dexamethasone, vincristine, daunorubicin, PEG-asparaginase; in phase II: 6-mercaptopurine, cytosine arabinoside and cyclophosphamide) followed by consolidation I with dexamethasone, vindesine, etoposide, high-dose methotrexate and high-dose cytarabine¹.

After Consolidation I, patients with Ph-negative ALL who achieved CR after induction therapy were stratified according to the conventional risk factors:

Standard risk (SR): No risk factor

High risk (HR): At least one risk factor

- BCP-ALL
- CR only achieved after Induction II or
- WBC > 30,000/µl or
- pro-B immunophenotype or t(4;11)
- Early T or mature T-ALL

HR patients with an available donor were scheduled for an allogeneic stem cell transplantation (SCT) after Consolidation I. Patients in the SR group were scheduled to receive further intensive consolidation chemotherapy. Patients in the SR group with persistent MRD >10⁻⁴ until week 16 were candidates for transplantation in first CR as well.

The GMALL 07/03 protocol recommended not to change the scheduled treatment in case of low MRD positivity as this was regarded as a sort of gray area which did not fit into the categories of complete molecular response or molecular failure.

Interpretation of real-time quantitative PCR MRD data

A sample was considered MRD positive if at least one replicate generated a specific amplification curve with the lowest threshold cycle (CT) at least 1 CT lower than the lowest CT of the polyclonal control and maximally 4 CTs higher than the last specific dilution step (according to the EuroMRD guidelines for definition of MRD positivity for the treatment reduction setting²). Separately, all follow-up samples that were considered to be low-level MRD positive were cross-checked for correct amplicon length to enhance specificity as published by Fronkova and colleagues³.

Identification of leukemia-specific rearrangements at diagnosis

For all 178 MolNE patients, sequences of dominant IG/TR rearrangements at the time of diagnosis were available. Those were obtained within routine marker screening employing the classical Biomed-2 approach⁴. For 110 of 178 MolNE patients, sufficient amounts of left-over DNA from initial diagnosis and/or w+16 was available and at least one clonal IGH-VJ (n=71) and/or TRB-VJ (n=46) MRD marker was identified within routine diagnostics. Six of these patients showed both IGH-VJ and TRB-VJ clonal markers and 1 patient had 2 IGH-VJ clonal markers. In 96 of these patients, EuroClonality NGS-based
marker screening employing IGH-VJ-FR1 and TRB-VJ primers\(^5\) was performed to confirm the results of the routine low-throughput marker screening and as the basis for the NGS-based MRD quantification in w+16 samples. All rearrangements with abundance >5% of total reads were considered as leukemic markers.

**MRD quantification by NGS**

MRD at w+16 was quantified by NGS in 86 patients with available DNA with IGH-VJ-FR1 (60 rearrangements) and TRB-VJ (33 rearrangements) EuroClonality primers and 1-step PCR\(^5\,^6\) to avoid contaminations. Twelve of the clonal markers could not be confirmed by NGS due to the unavailability of the diagnostic material. In these cases, clonal markers identified within routine Sanger-sequencing-based markerscreening were used. Three replicates, each containing 500ng DNA were analyzed for each sample. To prevent unbalanced amplification in aplastic samples, 100ng of polyclonal mononuclear DNA (buffy coat, BC) was added to each reaction. On top of that, a certain number of DNA copies of three different cell lines (Supplementary Table 1) with known rearrangements were added to each replicate to enable calculation of read coverage per molecule. Of the 86 analyzed samples, 19 (22%) were RQ-PCR-negative with insufficient sensitivity, 66 (69%) were positive below QR and 1 (1%) was positive below 1E-04. For 5 of the patients only a limited amount of w+16 DNA was available, which limited the sensitivity of the NGS-MRD analysis. E.g. if only 100ng DNA (~15,000 cells) are analyzed, the sensitivity cannot exceed 1×10\(^{-4}\). For more details on these samples, see Supplementary Table 2.

**Statistical analysis**

Survival analysis was performed with the Kaplan-Meier method. Overall survival (OS) was calculated from the date of diagnosis to the date of death or date of last follow-up. Probability of continuous complete remission (CCR) was calculated from the date of CR to the date of relapse or date of last follow-up. Patients with death in CR or withdrawal were censored at the respective dates. Comparisons of survival curves were performed with log rank tests. Analyses were performed with the SAS program (SAS-PC, Version 9, SAS Institute, Cary, NC).
Results

NGS-based markerscreening

To confirm the results of the routine low-throughput marker screening in the 96 patients with available material, 102 sequencing libraries (42 TRB-VJ and 60 IGH-VJ) were prepared and sequenced on the Illumina MiSeq. The identification of a clonal marker was successful in 101/102 (99%) libraries. In 1 patient (1%) the marker from the routine diagnostics was not detected and no additional marker was found. In this case, the marker obtained within routine diagnostics was used for MRD quantification at w+16. In 3 patients (3%), a TRB-VJ rearrangement from routine diagnostics was not detected by NGS, but a new dominant TRB-VJ rearrangement was identified. In these 3 cases, the newly identified clonal markers were used for MRD detection at w+16. Two of them were negative by NGS and RQ-PCR, and one case was positive by both methods (< QR by RQ-PCR).

NGS – based MRD detection

An NGS-based MRD analysis was performed in w+16 samples of 86 patients using 93 clonal markers (33 TRB-VJ and 60 IGH-VJ). The median coverage per library was 391,154 reads (range 72,795-763,086). Even a single read with a sequence of a clonal marker was considered as MRD-positivity. In 18/19 (95%) RQ-PCR-MRD-negative cases MRD-negativity was confirmed also by NGS; in 1/19 (5%) patients MRD was detected at a level of 1.2E-05 by NGS. In 1 patient with quantifiable RQ-PCR-MRD below 1E-04, MRD at a level of 3.3E-05 was detected by NGS. In 40/66 (61%) patients with RQ-PCR-MRD below quantitative range, the MRD positivity was confirmed by NGS. In the remaining 26/66 (39%) patients the clonal marker was not detected in the w+16 sample (see also Supplementary Figure 1).
Supplementary Table 1

**IGH-VJ mix**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Copies per reaction</th>
<th>Malignancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Namalwa</td>
<td>300</td>
<td>Burkitt Lymphoma</td>
</tr>
<tr>
<td>SMS-SB</td>
<td>100</td>
<td>ALL</td>
</tr>
<tr>
<td>RCH-ACV</td>
<td>10</td>
<td>BCP-ALL</td>
</tr>
</tbody>
</table>

**TRB-VJ mix**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Copies per reaction</th>
<th>Malignancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>JB6</td>
<td>300</td>
<td>ALCL</td>
</tr>
<tr>
<td>Karpas299</td>
<td>100</td>
<td>ALCL</td>
</tr>
<tr>
<td>Molt13</td>
<td>10</td>
<td>ALL</td>
</tr>
</tbody>
</table>

**Supplementary Table 1: Composition of cell line mix** which was added to every sample to enable calculation of coverage per molecule.
**Supplementary Table 2**

<table>
<thead>
<tr>
<th>Patient</th>
<th>ng DNA for NGS-MRD analysis</th>
<th>NGS-MRD</th>
<th>RQ-PCR MRD</th>
</tr>
</thead>
<tbody>
<tr>
<td>S18081966FBM</td>
<td>220</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>F18101985MRM</td>
<td>60</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>G01091970ISM</td>
<td>160</td>
<td>negative</td>
<td>low positive</td>
</tr>
<tr>
<td>N22081973MSM</td>
<td>100</td>
<td>positive</td>
<td>low positive</td>
</tr>
<tr>
<td>M28121973SRM</td>
<td>100</td>
<td>positive</td>
<td>low positive</td>
</tr>
</tbody>
</table>

**Supplementary Table 2: Samples with limited amount of DNA from w+16.** NGS-MRD analysis was performed with lower DNA input, as indicated in the table. Only 1 sample was negative by NGS, although low positive by RQ-PCR.
Supplementary Figure 1: **Comparison of RQ-PCR and NGS MRD results in a subgroup of MolNE patients.** MRD, minimal residual disease; real-time quantitative PCR, RQ-PCR; NGS, next-generation sequencing; pos, positive; neg, negative; QR, quantitative range.
Supplementary Figure 2: Prognostic impact of minimal residual disease (MRD) levels at end of consolidation I (week 16), as shown by Kaplan-Meier estimates of overall (OS) and disease-free survival (DFS). OS and DFS according to NGS-MRD in patients who were MolNE (<10^{-4}: non-quantifiable MRD positivity or a quantifiable MRD positivity at levels <10^{-4}) by RQ-PCR, thereby excluding RQ-PCR MRD negative patients with insufficient assay sensitivity. X-axis: years; Y-axis: survival probability; 5y-OS, 5-year overall survival; 5y-DFS, 5-year disease-free survival.
References


