Minimal residual disease (MRD) detection in acute lymphoblastic leukaemia based on fusion genes and genomic deletions: towards MRD for all

Roland P. Kuiper,1,2 Patricia G. Hoogeveen,3 Reno Bladergroen,1 Freerk van Dijk,1 Edwin Sonneveld,1 Frank N. van Leeuwen,1 Judith Boer,1,4 Irina Sergeeva,5 Harma Feitsma,5 Monique L. den Boer1,4,6 and Vincent H. J. van der Velden3

1Princess Máxima Center for Pediatric Oncology, 2Department of Genetics, University Medical Center Utrecht, 3Department of Immunology, Laboratory Medical Immunology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, 4Oncode Institute, 5Cergentis, Utrecht, and 6Department of Pediatric Oncology, Erasmus MC – Sophia Children’s Hospital, Rotterdam, the Netherlands

Received 24 May 2021; accepted for publication 18 July 2021
Correspondence: Vincent H. J. van der Velden, Department of Immunology, Erasmus MC, Laboratory Medical Immunology, Wytemaweg 80, 3015 CN, Rotterdam, the Netherlands.
E-mail: v.h.j.vandervelden@erasmusmc.nl

Introduction

The use of standardised multiagent chemotherapy regimens with risk-adapted intensity has greatly contributed to the progressive improvements in survival rates of children with acute lymphoblastic leukaemia (ALL). Initial treatment response by serial quantitative measurements of minimal residual disease (MRD) has proven to be one of the strongest independent prognostic factors for paediatric ALL and has been implemented in most treatment protocols currently used. In the Netherlands, MRD monitoring forms the primary basis for risk group stratification since 2004 and is performed using real-time quantitative polymerase chain reaction (RT-qPCR) analysis of rearranged immunoglobulins (IG) and T-cell receptor (TR) genes. The methodology has been highly standardised in international consortia. However, in 5–10% of cases MRD classification is not feasible because a PCR-detectable target cannot be identified or because the target does not reach the required sensitivity. In addition, IG/TR rearrangements can be oligoclonal and consequently can be lost during the course of the disease. Consequently, the MRD-based stratification is suboptimal for these patients, with a risk of under- or over-treatment. In the present study, we demonstrate that genomic breakpoint sequences of fusion genes (FG) and driving deletions provide excellent alternative targets for MRD monitoring in paediatric ALL when standard IG/TR targets cannot be obtained, resulting in adjusted risk-group stratifications in more than half of the cases.

Summary

Minimal residual disease (MRD) diagnostics are implemented in most clinical protocols for patients with acute lymphoblastic leukaemia (ALL) and are mostly performed using rearranged immunoglobulin (IG) and/or T-cell receptor (TR) gene rearrangements as molecular polymerase chain reaction targets. Unfortunately, in 5–10% of patients no or no sensitive IG/TR targets are available, and patients therefore cannot be stratified appropriately. In the present study, we used fusion genes and genomic deletions as alternative MRD targets in these patients, which retrospectively revealed appropriate MDR stratification in 79% of patients with no (sensitive) IG/TR target, and a different risk group stratification in more than half of the cases.

Keywords: minimal residual disease, fusion genes, leukaemia, ALL.
these genomic fusion breakpoints are independent of gene activity and thus have comparable quantitative dynamics compared to standard IG/TR targets. Several recent studies have demonstrated proof-of-principle for using such genomic alterations for MRD in paediatric ALL.\(^5\)–\(^7\) However, whereas we expect that there is a wide range of gene rearrangements and deletions that can be used as MRD targets, their frequency is low and new strategies need to be developed to systematically identify suitable candidate targets. Historically, the use of genomic breakpoints of these driving lesions as targets has been hampered by the fact that their occurrence and precise genomic location is highly variable. However, technological improvements of diagnostic pipelines in many centres, including the use of RNA sequencing for subtype stratification, makes the application of implementation of FG as targets more realistic.

The use of FG or deletions for MRD monitoring requires the identification of the (intronic) genomic breakpoints for these structural variants, which are unique for each patient. These breakpoints can be identified in a direct and unbiased manner based on whole genome sequencing (WGS) data, followed by Sanger sequencing validation. In a subset of cases, a suitable FG has already been identified by standard karyotyping, fluorescent in situ hybridisation (FISH) or RNA sequencing, which allows a targeted approach for genomic breakpoint sequencing, like targeted locus amplification (TLA). TLA is a strategy to selectively amplify and sequence regions of >100 kb around a preselected primer pair by cross-linking of physically proximal genomic sequences and is highly suitable for the detection of (balanced) chromosomal rearrangements in leukaemia samples.\(^8\)–\(^10\) Combined with next-generation sequencing, the TLA technique directly reveals genomic breakpoints that can be used to design genomic targets for MRD.\(^8\)

In the present study, we aimed to determine the applicability of genomic breakpoints from leukaemia-specific FG and deletions (FG/DEL) for MRD. We analysed the efficacy of target identification in a selected series of samples as well as the performance of these targets compared to the classical IG/TR targets.

Three groups of Dutch ALL10-treated paediatric ALL cases were considered for our study, namely those for which classical MRD target identification failed or for which no sufficient or sufficiently sensitive targets were available (Group 1), patients with standard-risk (SR) MRD stratification who nevertheless relapsed (Group 2), and a group of relapsed cases with successful MRD risk stratification that could be used for comparison of MRD performance (Group 3). From a total of 106 cases that fulfilled at least one of these criteria, we managed to include 74 cases for which diagnostic and follow-up material was available (Fig 1A). Suitable genomic breakpoints were identified through various routes. First, we identified 34 such cases, which carried ETS variant transcription factor 6 (\(ETV6\))-Run-related transcription factor 1 (\(RUNX1\)) fusions, rearrangements of lysine methyltransferase 2 family [\(KMT2A\), mixed-lineage leukaemia (\(MLL\))] or transcription factor 3 (\(TCF3\)), and focal deletions in Ikaros Family Zinc Finger 1 (\(IKZF1\)) (Table SI). In addition, we performed RNA sequencing for 27 cases followed by FG detection using STAR-fusion,\(^11\) which revealed another five FG. All cases with one or more fusions (\(n = 32\)) were subjected to TLA,\(^8\) which revealed the exact genomic breakpoint for 34 FG/DELs in 32 cases. Secondly, we performed WGS on genomic DNA from 19 cases, which revealed the genomic breakpoints of 10 FG and three deletions. Taken together, this resulted in 47 genomic breakpoints in FG/DEL

Fig 1. Genomic breakpoint identification in selected patients with acute lymphoblastic leukaemia (ALL). (A) Workflow of cases included in this study. Two cases revealed multiple breakpoints, as outlined in detail in Tables SII and SIII. (B) Overview of recurrent fusion gene (blue) and deletion breakpoints determined in this study (see also Table SIII). [Colour figure can be viewed at wileyonlinelibrary.com]
in 44 out of 74 cases (59%; Fig 1A). Strikingly, from the 28 cases where classical IG/TR-based MRD detection failed, 22 revealed a FG/DEL (79%). In all, 31 of the 44 cases carried primary driver FGs, representing mutually exclusive ALL subtypes, which most likely represent leukaemia-initiating lesions (Fig 1B and Tables SII and SIII). Among the remaining targets were FG for which this leukaemia-initiating status is less certain (FLI1, BCL11A-ELK3, PHF6-RUNX1, DDX3X-MLLT10), and 11 secondary driver aberrations (e.g. deletions in IKZF1 or PAX5, Table SIII).

An RQ-PCR assay was developed for the 47 identified FG/DEL. A quantitative range (QR) of at least $10^{-4}$ was obtained in 37/47 breakpoints (78%), a sensitivity of at least $10^{-4}$ was reached in 44/47 breakpoints (94%; Fig 2A; Table SIV). These numbers are significantly higher than those generally obtained for IG/TR targets.12 For 16 patients, both diagnosis and relapse samples were available; IG/TR rearrangements and FG/DEL were stable in 13 (81%) and 15 cases (94%), respectively (Fig 2B). The single case with both IG/TR rearrangements and FG/DEL lost at relapse was a late T-ALL relapse (>5 years after initial diagnosis), which was most likely a second T-ALL.13 Therefore, FG/DEL generally perform well in RQ-PCR analysis and are highly stable between diagnosis and relapse.

We subsequently compared MRD data obtained by IG/TR and FG/DEL analysis in 285 follow-up samples from 44 patients. As shown in Fig 2C, a strong correlation was found between both approaches: 252/285 (88%) samples showed a qualitative concordance and for samples quantifiable by both approaches the Spearman correlation coefficient was 0.97 ($P < 0.0001$). In all, 24 samples (8%) were negative by one approach while low-level positive (not quantifiable) by the other approach. Four samples (1%) could be quantified by one approach due to excellent QR of $10^{-5}$ but were not quantifiable by the other (QR $10^{-4}$). Truly discordant data were observed in only nine (3%) samples; seven of these were from a single patient (M4A-011) that became MRD negative by analysis of T-cell receptor gamma (TRG), TR beta (TRB) and IKZF1, but remained high level positive by two genomic breakpoints of a complex KMT2A-translocation. Remarkably, all five evaluated markers were strongly present in the relapse sample. The single case being IG/TR positive ($2 \times 10^{-4}$) and FG negative was monitored using a PAX5 deletion, a known secondary driver. These data

Fig 2. (A) Quantitative range of immunoglobulin/T-cell receptor (IG/TR) targets and fusion genes and deletions (FG/DEL) targets. The IG/TR data are based on routinely obtained data in the total ALL10 protocol ($n = 1837$), the FG/DEL data from this study ($n = 47$). (B) Stability of IG/TR and FG/DEL data between diagnosis and relapse ($n = 16$). (C) Comparison between MRD data obtained by IG/TR data (maximum MRD level of tested targets) and by FG (blue; $n = 227$) or DEL (orange; $n = 72$) analysis ($n = 285$ samples from 44 patients). Two patients (M4A-011, nine samples; and M4A-098, five samples) were tested by both FG and DEL; both data are included in the figure. For example, 13 samples were positive non-quantifiable (PNQ) based on IG/TR data and negative by FG ($n = 11$), DEL ($n = 1$) or FG and DEL ($n = 1$; M4A-098). For analysis of the qualitative concordance, the highest MRD value as determined by FG or DEL was used. PNQ, positive below quantitative range of the assay. [Colour figure can be viewed at wileyonlinelibrary.com]
provide data; Roland P. Kuiper and Vincent H. J. van der Velden wrote the paper; all authors reviewed and approved the final paper.

**Conflict of interest**

Irina Sergeeva and Harma Feitsma are employed by Cergenetis. The other authors declare no conflict of interest.

**Supporting Information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table SI.** Genomic breakpoints identified by targeted locus amplification (TLA).

**Table SII.** Genomic breakpoints used for real-time quantitative polymerase chain reaction (RQ-PCR).

**Table SIII.** Genomic breakpoint positions and breakpoint-spanning sequences.

**Table SIV.** Polymerase chain reaction (PCR)-based minimal residual disease (MRD) analysis and risk group stratification based on fusion genes and deletions (FG/DEL) and immunoglobulin and/or T-cell receptor (IG/TR) targets.

**References**

1. Pieters R, de Groot-Kruseman H, Van der Velden V, Fiocco M, van den Berg H, de Bont E, et al. Successful therapy reduction and intensification for childhood acute lymphoblastic leukemia based on minimal residual disease monitoring: study ALL10 from the Dutch Childhood Oncology Group. *J Clin Oncol.* 2016;34:2591–601.

2. Szczepanski T, Willemse MJ, Brinkhof B, van Wering ER, van der Burg M, van Dongen JMJ. Comparative analysis of Ig and TCR gene rearrangements at diagnosis and at relapse of childhood precursor-B-ALL provides improved strategies for selection of stable PCR targets for monitoring of minimal residual disease. *Blood.* 2002;99:2315–23.

3. van der Velden VHJ, Bruggemann M, Hoogeveen PG, de Bie M, Hart PG, Raff T, et al. TCRB gene rearrangements in childhood and adult precursor-B-ALL: frequency, applicability as MRD-PCR target, and stability between diagnosis and relapse. *Leukemia.* 2004;18:1971–80.

4. van der Velden VHJ, Willemse MJ, van der Schoot CE, Hähnle K, van Wering ER, van Dongen JIJ. Immunoglobulin kappa deleting element rearrangements in precursor-B acute lymphoblastic leukemia are stable targets for detection of minimal residual disease by real-time quantitative PCR. *Leukemia.* 2002;16:928–36.

5. Venn NC, van der Velden VHJ, de Bie M, Waanders E, Giles JE, Law T, et al. Highly sensitive MRD tests for ALL based on the IKZF1 Δ3-6 microdeletion. *Leukemia.* 2012;26:1414–6.

6. Hoffmann J, Krumbholz M, Gutiérrez HP, Fillies M, Szymansky A, Bleckmann K, et al. High sensitivity and clonal stability of the genomic fusion as single marker for response monitoring in ETV6-RUNXI-positive acute lymphoblastic leukemia. *Pediatr Blood Cancer.* 2019;66:e27780.

7. Zur Stadt U, Alawi M, Aadd M, Indenbirken D, Escherich G, Horstmann MA. Characterization of novel, recurrent genomic rearrangements as sensitive MRD targets in childhood B-cell precursor ALL. *Blood Cancer J.* 2019;9:86.

8. de Vree PJF, de Wit E, Yılmaz M, van de Heijningen M, Klous P, Verstegen MJAM, et al. Targeted sequencing by proximity ligation for comprehensive variant detection and local haplotyping. *Nat Biotechnol.* 2014;32:1019–25.
9. Hottentot QP, van Min M, Splinter E, White SJ. Targeted locus amplification and next-generation sequencing. *Methods Mol Biol*. 2017;1492:185–96.

10. Alimohamed MZ, Johansson LF, de Boer EN, Splinter E, Klous P, Yilmaz M, et al. Genetic screening test to detect translocations in acute leukemias by use of targeted locus amplification. *Clin Chem*. 2018;64:1096–103.

11. Haas BJ, Dobin A, Li B, Stranzky N, Pochet N, Regev A. Accuracy assessment of fusion transcript detection via read-mapping and de novo fusion transcript assembly-based methods. *Genome Biol*. 2019;20:213.

12. van der Velden VHJ, van Dongen JJM. MRD detection in acute lymphoblastic leukemia patients using Ig/TCR gene rearrangements as targets for real-time quantitative PCR. *Methods Mol Biol*. 2009;538:115–50.

13. Szczepański T, van der Velden VHJ, Waanders E, Kuiper RP, van Vlierberghe P, Gruhn B, et al. Late recurrence of childhood T-cell acute lymphoblastic leukemia frequently represents a second leukemia rather than a relapse: first evidence for genetic predisposition. *J Clin Oncol*. 2011;29:1643–9.

14. Peham M, Konrad M, Harbott J, König M, Haas OA, Panzer-Grümmayer ER. Clonal variation of the immunogenotype in relapsed ETV6/RUNX1-positive acute lymphoblastic leukemia indicates subclone formation during early stages of leukemia development. *Genes Chromosomes Cancer*. 2004;39:156–60.

15. Hovorkova L, Zaliova M, Venn NC, Bleckmann K, Trkova M, Potuckova E, et al. Monitoring of childhood ALL using genomic breakpoints identifies a subgroup with CML-like biology. *Blood*. 2017;129:2771–81.