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

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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.

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 (RQ-PCR) analysis of rearranged immunoglobulins (IG) and T-cell receptor (TR) genes. The methodology has been highly standardised in international consortia. However, in ~5% 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.[1] In addition, IG/TR rearrangements can be oligoclonal and consequently can be lost during the course of the disease.[2–4] 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.

Fusion genes and gene deletions frequently act as primary drivers of leukaemogenesis and, as such, can be very stable during disease progression, and suitable as alternative genomic MRD PCR targets. In contrast to fusion transcripts,

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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 selected genomic DNA or viable cells from patients for which a FG/DEL was already identified through routine diagnostics. In total, we identified 34 such cases, which carried ETS variant transcription factor 6 (*ETV6*)-Runt-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

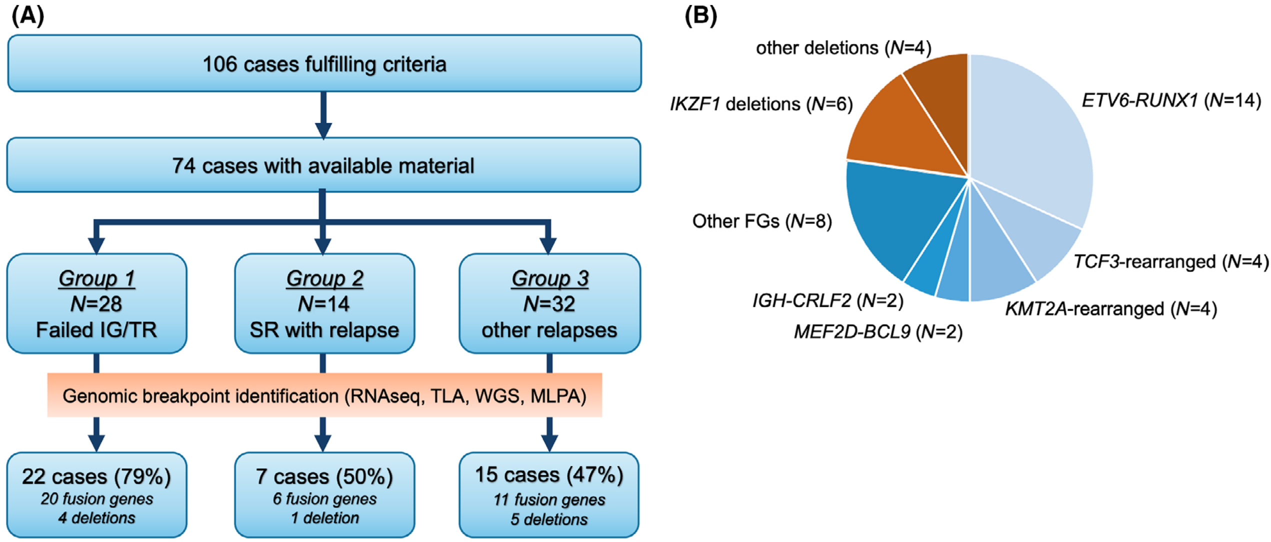

Fig 1. Genomic breakpoint identification in selected patients with acute lymphoblastic leukaemia (ALL). (A) Workflow of cases included in this study. Two cases revealed multiple breakpoints, as outlined in detail in Tables SII and SIII. (B) Overview of recurrent fusion gene (blue) and deletion breakpoints determined in this study (see also Table SIII). [Colour figure can be viewed at wileyonlinelibrary.com]

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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 *IGH-CEBPE*), 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 ($P < 0{\cdot}0001$). 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 (2×10^{-4}) and FG negative was monitored using a *PAX5* deletion, a known secondary driver. These data

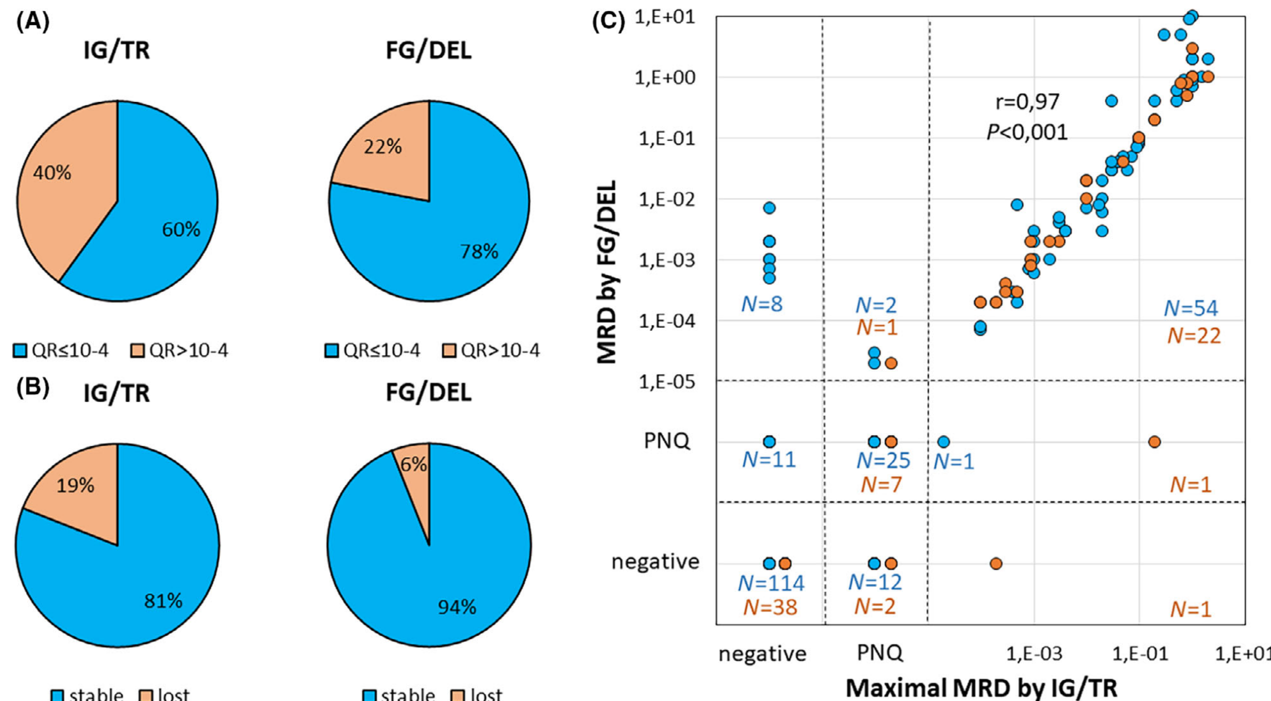

Fig 2. (A) Quantitative range of immunoglobulin/T-cell receptor (IG/TR) targets and fusion genes and deletions (FG/DEL) targets. The IG/TR data are based on routinely obtained data in the total ALL10 protocol (n = 1837), the FG/DEL data from this study (n = 47). (B) Stability of IG/TR and FG/DEL data 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]

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indicate that not all FG/DEL are equally suitable and that FG are preferred above deletions as MRD PCR markers.

Finally, we evaluated the potential impact of MRD analysis by FG/DEL on risk-group classification. In Group 2 (relapsed SR patients), three out of six evaluable patients would have been stratified as medium risk (MR) by FG analysis and consequently would have received more intensive treatment, possibly preventing the relapse. In Group 1 [no (sensitive) IG/TR targets and therefore treated according to MR], 17 out of 20 evaluable cases (85%) could be classified by FG/DEL analysis: eight of the 17 cases would have been classified as SR (no relapses) and two of 17 as high risk (one relapse). Overall, these data indicate that applying FG/DEL MRD data will allow MRD monitoring in the vast majority of patients not classifiable by IG/TR analysis. Additionally, our present data suggest that FG/DEL MRD data allow appropriate risk-group classification, possibly even better than IG/TR-based risk-group classification, although this obviously should be confirmed in a much larger and prospective study.

In conclusion, our present data show that genetic lesions can be identified in a significant number of patients with ALL by using TLA, RNAseq and/or WGS. Such genetic lesions generally perform well in RQ-PCR analysis and provide data highly comparable to IG/TR data, confirming previous studies evaluating *ETV6-RUNX1*, *BCR-ABL1* and *IKZF1* deletions,[5,6,14,15] although a fraction of *BCR-ABL1*-positive ALL cases may give discordant results due to a chronic myeloid leukaemia-like phenotype.[15] Applying FG/DEL MRD data will allow MRD monitoring in a proportion of the patients not classifiable by IG/TR data. Furthermore, in our present study FG/DEL showed excellent stability between diagnosis and relapse and provided reliable MRD data and classification, at least as good as IG/TR-based data. We therefore recommend including FG/DEL analysis for MRD analysis in patients with ALL for whom no (sensitive) MRD target is available and to compare IG/TR and FG/DEL data in other patients to obtain more detailed information about which FG and genomic deletions are most suitable as targets for MRD testing.

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Author contributions

Roland P. Kuiper, Monique L. den Boer and Vincent H. J. van der Velden designed the study; Reno Bladergroen, Freerk van Dijk, Patricia G. Hoogeveen and Judith Boer performed experiments; Edwin Sonneveld and Frank N. van Leeuwen provided 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 Cergentis. 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.

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