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Highly sensitive MRD tests for ALL based on the IKZF1 Δ3–6 microdeletion

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Current clinical trials for patients with acute lymphoblastic leukemia (ALL) depend upon the measurement of minimal residual disease (MRD) at early stages of therapy to determine the risk of relapse for each patient who is being used for treatment stratification.[1] PCR-based MRD tests are usually designed to detect the specific rearrangements of immunoglobulin and T-cell receptor (Ig/TCR) genes found in the leukemic clone. We now present evidence supporting the hypothesis that the most common deletion in the *IKZF1* gene in ALL also provides the basis for highly sensitive MRD tests that give MRD results in close agreement with Ig/TCR MRD markers.

Mullighan *et al.*[2] reported that DNA copy-number alterations (CNAs) in the *IKZF1* gene, which codes for the lymphoid transcription factor IKAROS, were very common in leukemic DNA from Philadelphia chromosome (Ph)-positive ALL patients (83%). CNAs were not detected in the *IKZF1* gene at diagnosis in 23 patients with chronic myeloid leukemia but *IKZF1* CNAs were found after blast crisis (that is, conversion to acute form of leukemia) in 4 out of 15 patients including 3 out of 4 patients with lymphoid blast crisis.[2] A subsequent study showed CNAs in *IKZF1* in 29% of high risk, Ph-negative ALLs.[3] One quarter to one third of patients with *IKZF1* alterations had a deletion of exons 4–7; corresponding to loss of coding exons 3–6 and resulting in the expression of a dominant-negative IKAROS isoform, IK6.[2] The deletion breakpoints for these *IKZF1*Δ3–6 alterations are usually located within a few nucleotides, and have characteristic N regions comparable to Ig/TCR gene rearrangements.

These findings suggested the feasibility of designing MRD assays based on real-time quantitative (RQ) PCR in the same way as Ig/TCR-based MRD tests. One possibility was the use of two primers and a probe, with one primer specific for the unique N regions at the breakpoint fusion site. Another possibility was the use of a generic *IKZF1*Δ3–6 RQ-PCR assay based on one probe and two primers that all bind to germline sequences and create a PCR product that bridges the breakpoint. Given the assumption that these *IKZF1* rearrangements are absent or rare in normal lymphocytes, such a germline assay might be specific for leukemia. These approaches were assessed by comparison with standard Ig/TCR-based MRD tests.

Patients carrying the *IKZF1*Δ3–6 deletion were identified by two alternate methods. PCR analysis of diagnosis leukemic samples with published *IKZF1* primers[2] was used to identify 28 patients (6%) in a set of 458 patients enrolled on the Australian and New Zealand Children's Haematology Oncology Group (ANZCHOG) Study 8 clinical trial. For patients enrolled on the Dutch Clinical Oncology Group (DCOG) ALL9 trial, an *IKZF1* focused multiplex ligation-dependent probe amplification test was used and revealed a range of CNAs involving the *IKZF1* gene in 15/34 (44%) of relapse cases and 18/131 (14%) of unselected patients.[4,5] Some of the patients with the *IKZF1* Δ3–6 deletion were used in this study. This research was approved by the institutional bioethics committees at University of New South Wales and Erasmus Medical Centre with informed consent from parents of children for the use of the samples for research.

The subsequent RQ-PCR analyses for MRD were performed using different sets of primers and probes to detect the *IKZF1* deletion, which are shown in Figure 1. MRD was measured by RQ-PCR in triplicate using standards made by serial dilution (10^{-1}, 10^{-2}, 10^{-3}, 5×10^{-4}, 10^{-4}, 5×10^{-5}, 10^{-5}) of the patients' diagnosis DNA diluted in normal mononuclear cell DNA in 25 μl volumes in 96-well plates. The RQ-PCR of ANZCHOG patients was performed using set A or set B on 500-ng DNA with KAPA Biosystems (Boston, MA, USA) mastermix using a touchdown program (with extension temperature dropping by 1°C for 10 cycles from 71°C to 61°C and then a further 45 cycles) on the Biorad (Hercules, CA, USA) IQ5 or CFX96 platform. The RQ-PCR of DCOG patients was performed using set C on 600 ng DNA with Universal mastermix (Roche, Indianapolis, IN, USA) on an Applied Biosystems (ABI, Foster City, CA, USA) StepOne plus cycler. MRD tests using set A primers and probe were performed on 61 follow-up bone marrow samples from eight patients, set B on 57 samples from seven patients (four in common with set A) and set C on 44 samples from seven patients. All MRD data were assessed according to EuroMRD guidelines.[6]

The set A primers and probe were designed to detect all 28 *IKZF1* Δ3–6 rearrangements in ANZCHOG Study 8 patients allowing for the most truncated intron 2 and intron 6 sequences, and has a forward primer and a minor groove binding probe both binding to intron 2 upstream of the breakpoint and a reverse

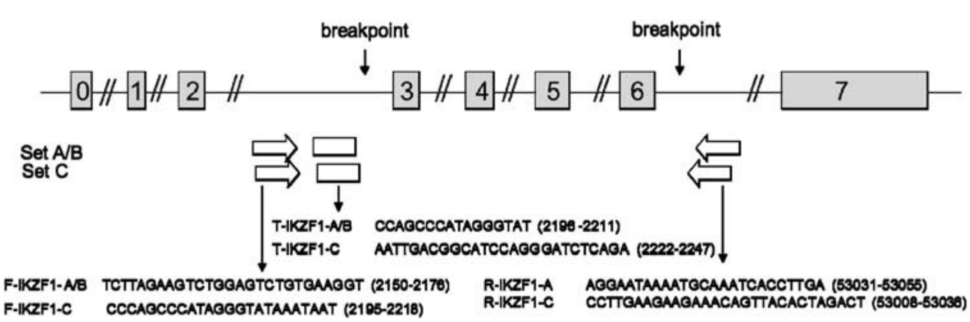

Figure 1. Primers and probes used for RQ-PCR analysis of IKZFΔ3–6 deletions. The gene coding for Ikaros has eight exons (gray boxes 0–7), of which 1–7 are coding. The sequence between the two breakpoint arrows is deleted in *IKZF1*Δ3–6, and this gene alteration can be detected by RQ-PCR using primer sets A, B or C (horizontal arrows, and F-IKZF1 and R-IKZF1 sequences) and probes (open boxes and T-IKZF1 sequences). For both sets A and C, the 5′ primer and probe match sequences before the breakpoint in intron 2 and the 3′ primer binds after the breakpoint in intron 6. Set B used the same probe and 5′ primer as set A in combination with a different allele-specific primer for each patient which bridged the unique breakpoint sequence. Both probes were synthesized by Applied Biosystems, but with different chemical structures: T-IKZF1-A/B has a minor groove binding capacity, and T-IKZF1-C is a TAMRA Taqman probe. The location of primer and probe sequences shown in brackets are according to NT_033968.6.

primer downstream in intron 6. Set B has the same probe and forward primer as set A, but was used in combination with an allele-specific reverse primer (positioned over the breakpoint fusion). Set C was used in the Dutch cohort and has primers and a Taqman probe in similar positions as set A but all closer to the breakpoint giving a shorter product. The random deletions and insertions of bases at the breakpoint fusion cause variations in the size of the PCR products between the patients, but the size for the RQ-PCR product with no insertion or deletion is 171 base pairs for set A and 109 base pairs for set C. Set C was therefore potentially a more efficient RQ-PCR assay than A, but it would not be suitable for about 25% of patients with *IKZF1* Δ3–6 rearrangements.

An overall comparison of MRD results obtained with the *IKZF1*Δ3–6 marker tested on the same samples as earlier MRD analyses using Ig/TCR markers is shown in Figure 2a. The close concordance of results was confirmed by the Spearman's coefficient of rank correlation (rho) of 0.985 (0.979–0.989 95% confidence interval; $P<0.0001$). This scattergram also illustrates the limits to MRD testing with less reliable results obtained for MRD levels $<10^{-4}$ (1 in 10 000 cells). The results are highly correlated with a slope of the linear regression of the log10 MRD values approximating 1 (0.98 ± 0.13). The *IKZF1* results were as close to the Ig/TCR results as an earlier study in which we examined reproducibility by repeating MRD tests for samples using Ig/TCR markers.[7]

To assess the three different primer/probe *IKZF1* sets used to measure MRD, Bland–Altman analyses[8] were performed for each set, comparing the difference in MRD level for the *IKZF1* marker and Ig/TCR markers, regarded as the current gold standard (Figures 2b–d). On the basis of this analysis, all three *IKZF1* MRD sets of reagents provided highly suitable MRD tests, generating MRD results in close concordance with Ig/TCR MRD results tested on the same samples. In each set, the average difference and regression lines were not significantly different from zero and there was no real difference in standard deviations. Samples from four of the patients were tested using both Set A and Set B, and the concordance of results was also high (data not shown).

The three *IKZF1* MRD sets all showed high specificity with no or very-low levels of background amplification observed for control mononuclear cell samples from individuals without leukemia, which were included in every patient assay. Established EuroMRD (ESG-MRD-ALL) guidelines were used to assess all MRD data[6] and the assay slopes, quantitative ranges and sensitivities are shown in the Supplementary Information for the paper. The slopes for set A amplification curves (3.40 ± 0.16, mean ± s.d.) were not higher than set C MRD tests (3.55 ± 1.3), suggesting that the longer RQ-PCR product did not reduce the efficiency of the assay. With a single exception, (quantitative range of 5×10^{-4} for one set B assay), all the *IKZF1* MRD assays would meet current clinical trial requirements with quantitative ranges and sensitivities between 10^{-4} and 10^{-5} corresponding to the detection of a single leukemic cell in 10 000 to 100 000 normal cells.

Given the relatively small number of patients and the high level of concordance of MRD results, it is not possible to identify one of the methods as superior. All three *IKZF1* sets have given highly acceptable MRD results in comparison to regular Ig/TCR-based MRD tests. Set A is applicable to more patients with the *IKZF1*Δ3–6 deletion including those with slightly longer trunca-

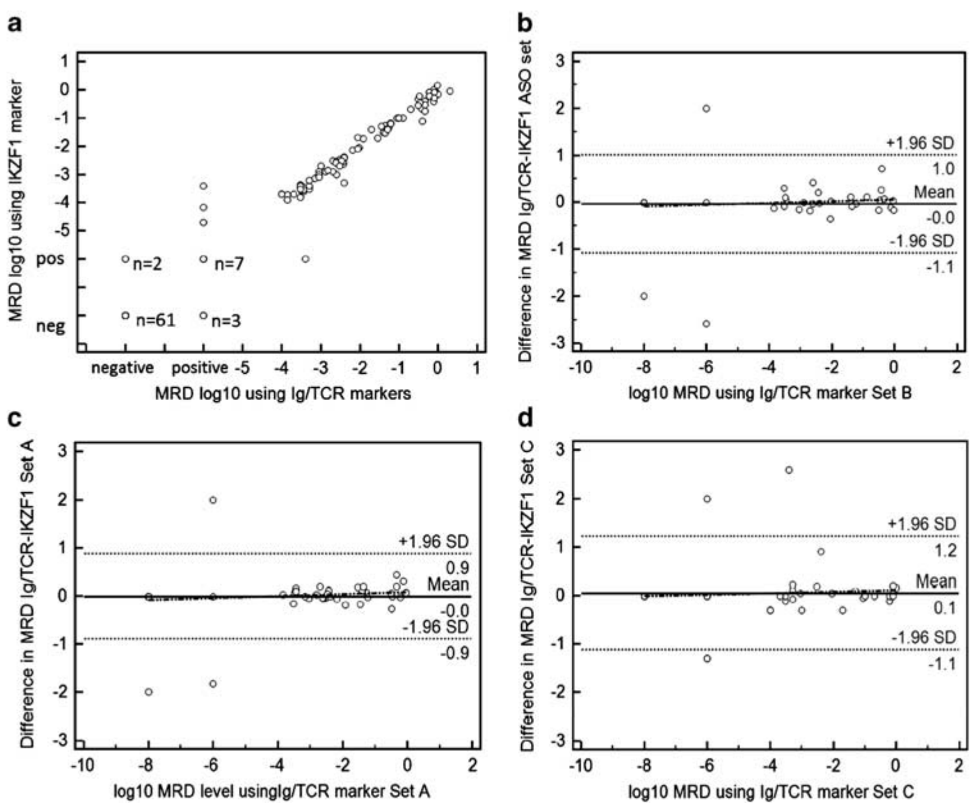

Figure 2. Measurement of MRD using markers based on *IKZF1* Δ3–6 deletions compared with Ig/TCR rearrangements. The RQ-PCR MRD data were interpreted using EuroMRD guidelines[6] and the analyses use the log10MRD of the dilution of diagnosis sample giving the same amplification as each sample. (**a**) Scatterplot comparing overall MRD results on 164 bone marrow samples from 16 patients tested using both methods. (**b**–**d**) Bland–Altman analysis for the three different *IKZF1* MRD tests displayed as the difference in results for the *IKZF1* and Ig/TCR markers against the Ig/TCR gold standard.[7] All negative MRD results were coded as log10 MRD of −8 and non-quantitative results as −6 as standardized by the EuroMRD. Figure 2b shows data for IKZF1 set B obtained with patient/allele assays each designed so that one primer binds to the specific breakpoint sequence, Figure 2c shows data for Set A germline primer/probe combination (with same probe as Set B) and Figure 2d shows Set C data for a different germline primer/probe set.

tions, and the germline sets (A and C) have the advantage of not requiring DNA sequencing or specific custom-made primers.

Two DNA MRD markers with high sensitivity (at least 10^{-4}) are generally required in MRD intervention clinical trials,[1,9] and in a large cohort of 2854 pediatric precursor B ALL patients, 20% of patients had only one sensitive marker and 8% had none.[9] Four of the 16 cases evaluated in this study had only one sensitive Ig/TCR marker so that availability of *IKZF1*-based MRD testing would have been useful for their risk stratification. Using routine PCR, *IKZF1*Δ3–6 rearrangements were identified in 6% of ALL patients in the ANZCHOG cohort in this study, so inclusion of this marker in standard screening for MRD targets would be an easy way to provide more patients with two sensitive markers.

The concept of using disease-related markers for MRD testing has been already established for fusion transcripts such as BCR-ABL and for gene rearrangements such as for *SIL-TAL1* in T-ALL and for *MLL* rearrangements in infant ALLs.[10] Kuiper *et al.*[4] in an analysis of paired diagnosis and relapse samples from 34 patients found *IKZF1* deletions and nonsense mutations in 14 (41%) patients at diagnosis and showed that all were conserved at relapse, in contrast to other recurrent genetic lesions found at diagnosis such as *PAX5*, *CDKN2A* and *EBF1*. It is therefore likely that this *IKZF1* marker will be at least as stable as Ig/TCR rearrangements, although this will need to be confirmed in more extensive studies.

In summary, we have assessed three ways to measure MRD levels by RQ-PCR for the most common deletion of the *IKZF1* gene found in ALL and demonstrated that all three methods provided robust and sensitive MRD assays for patients with this arrangement. The two primer and probe sets based on germline sequences could be used within a few days of diagnosis to provide quantitative measures of very-early responses to therapy. We expect that *IKZF1* gene deletions (*IKZF1*Δ3–6 and probably others) will provide a useful addition to the repertoire of MRD markers currently available for monitoring MRD in ALL.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

Prognostic factors for acute myeloid leukemia patients with t(6;9)(p23;q34) who underwent an allogeneic hematopoietic stem cell transplant

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Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is often selected as a curative treatment strategy for acute myeloid leukemia (AML). In particular, AML patients with poor cytogenetics at diagnosis are considered for allo-HSCT as the first-line therapy.[1–3] Recently, we have reported that AML with the t(6;9)(p23;q34) abnormality, which predicts a very poor prognosis in patients treated with chemotherapy,[4] is associated with an