

IGH and IGK gene rearrangements as PCR targets for pediatric Burkitt's lymphoma and mature B-ALL MRD analysis

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We recently reported that minimal residual disease (MRD) and minimal disseminated disease (MDD), assessed by long-distance PCR (LD-PCR) for t(8;14), are negative prognostic factors in mature B-cell acute lymphoblastic leukemia (B-ALL) and in Burkitt's lymphoma (BL). However, t(8;14) is detectable in only about 70% of patients, thus preventing MRD studies by this approach in the remaining patients. At present, no molecular assays have been reported for MRD and MDD analysis in t(8;14)-negative patients. The aim of our study was to evaluate the characteristics of patient-specific immunoglobulin (Ig) gene rearrangements as RQ-PCR targets for MRD analysis, in order to extend MRD studies to those patients who are not eligible for the LD-PCR assay. The study was performed according to the guidelines of the European Study Group on MRD detection in ALL (ESG-MRD-ALL). Overall, 36 B-ALL and 19 BL cases were analyzed. Multiple PCR reactions were performed for each sample to identify heavy and kappa light-chain rearrangements. A total of 97 RQ-PCR targets (62 for B-ALL, 35 for BL) were analyzed for sensitivity. The rearrangement pattern identified was similar to that reported for normal peripheral blood lymphocytes. In 88% of the targets, a sensitivity of at least 10^{-4} was achieved. In 87% of patients (84% of B-ALLs, 95% of BLs) at least one sensitive target was available. All PCR targets identified at diagnosis were preserved at relapse. Our results suggest that MDD and MRD can be successfully studied using a single sensitive Ig target in the great majority of B-ALL and BL cases. The combination of LD-PCR and Ig-based assays will allow MRD analysis in virtually all of the patients.

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Mature B-cell lymphoblastic leukemia (B-ALL) and Burkitt's lymphoma (BL) of childhood are often considered to be different manifestations of BL rather than different diseases.¹ Both are characterized by the expression of mature B-cell surface markers, including CD19, CD20 and immunoglobulin (Ig)M.² The chromosomal translocation t(8;14)(q24;q32) can be identified in about 70% of B-ALL and 70–75% of BL cases.^{3,4}

The amplification by long-distance PCR (LD-PCR) of this translocation breakpoint is currently used to monitor minimal residual disease (MRD) in patients enrolled in the Italian Association of Pediatric Hematology-Oncology (AIEOP) national protocol NHL-97. Using this approach, we have

recently reported that minimal disseminated disease (MDD) in BL has a negative prognostic impact on the outcome, being often associated with advanced stage disease and high levels of serum LDH.⁵ In addition, using multivariate analysis, we demonstrated that MRD was predictive of higher risk of failure in children with mature B-ALL.⁶

All together, these data suggest that, similar to other subtypes of ALL, a MRD-based risk group classification could be conceived for BL and mature B-ALL, and that this information might be used for the design of new protocols with MRD-based stratification of treatment.⁷

However, the application of the LD-PCR assay is prevented in patients in whom the typical chromosomal translocation

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cannot be detected. To date, no molecular assays have been reported for MRD and MDD analysis in t(8;14)-negative patients.

In this study, we describe the Ig rearrangement pattern in BL and in mature B-ALL of childhood, in comparison with previous findings on peripheral blood B-lymphocytes (PBLs) and B-cell precursor-ALL (BCP-ALL), and evaluated the characteristics of patient-specific Ig gene rearrangements as MDD and MRD PCR targets in these malignancies.

MATERIALS AND METHODS

Samples and DNA Isolation

A total of 36 cases of B-ALL and 19 cases of BL were included in the study. Patients were treated with a chemotherapy regimen, including a pre-phase and four to six high dose-intensity cycles, according to specific risk groups.⁵

Bone marrow (BM) nucleated cells were isolated by differential lysis. The tumor tissue of diagnostic biopsy specimens was incubated overnight at 56°C with proteinase K solution. High-molecular-weight genomic DNA was prepared using the QIAmp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Seven to 10 µg of diagnosis DNA and 4–5 µg of DNA of follow-up samples were required for reliable MRD analysis of Ig rearrangements.

Identification of PCR Targets

A set of PCR reactions was performed on the BM at diagnosis of B-ALL or on diagnostic biopsy samples of BL to identify complete and incomplete IGH rearrangements, IGKV deletional rearrangements and IGKV-J rearrangements. IGH rearrangements were identified using five VH and seven DH family primers, in combination with one JH consensus primer, as published previously.⁸ If no clonal IGHV-D-J band was detected, the sample was amplified using three multiplex primer sets, corresponding to the three IGHV FR regions, according to the BIOMED-2 guidelines.⁹ BIOMED-1 and BIOMED-2 primer sets were used for the amplification of IGK deletional and IGKV-J rearrangements, respectively.^{9,10}

To define whether an amplified gene rearrangement was clonal, PCR products were subjected to a heteroduplex analysis.¹¹ In case a single homoduplex band was identified, the PCR product was directly sequenced, whereas in cases of heteroduplex bands, the PCR product was cloned and at least 10 colonies were sequenced, or the PCR products were excised, eluted and re-amplified before sequencing.¹²

Sequences of complete IGH and IGKV-J rearrangements were aligned to the IMGT database (<http://imgt.cines.fr/>), while IGH incomplete rearrangements and IGK deletional rearrangements were compared with the Nucleotide Blast directory (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and manually inspected.

The χ^2 test was used to compare specific gene family frequencies in our series with previously reported data on Ig gene rearrangements.

ASO Primer Design and Ig-Based MRD PCR Assay

At least one allele-specific oligonucleotide (ASO) primer was designed complementary to the junctional region of each potential MRD PCR target, either manually or using Primer Express software (Applied Biosystems, Foster City, CA). Each target was tested for specificity and sensitivity. RQ-PCR analysis was performed on ABI PRISM Sequence Detection System 7000 (Applied Biosystems, Foster City, CA) by using sequence-specific TaqMan hydrolysis probes, as published previously.¹³ A standard curve was made by serially diluting the diagnostic DNA specimen in DNA obtained from mononuclear cells (MNCs) from a pool of four healthy donors (HDs). To determine the background of the RQ-PCR assay (ie, the amplification of comparable Ig gene rearrangements in normal cells), HD-MNC DNA samples were run in six-fold per experiment. The quantitative range and sensitivity of each MRD PCR target were determined according to the guidelines of the European Study Group on MRD detection in ALL (ESG-MRD-ALL).¹⁴

RESULTS

Features of IGH and IGK Rearrangements

In 94% of the 55 analyzed patients, at least two Ig gene rearrangements were detected (Table 1).

IGH rearrangements were found in 98% of the patients, with 87% represented by IGHV-D-J and 49% by IGHD-J. In four patients (7%), both productive and unproductive IGHV-D-J rearrangements were identified. In 44% of the patients, complete productive and incomplete IGH rearrangements were co-detected (Figure 1a). No relationship could be established between co-detected rearrangements, as a common D-J stem was never identified. In two cases a clonal band was detected with the FR2 multiplex primer set, but the rearrangements could not be identified, as DNA specimens were insufficient to perform the necessary amplifications.

Table 1 Frequency and sensitivity of IGH and IGK gene rearrangements as MRD RQ-PCR targets in mature B-ALL and BL patients

Targets/patient	Mature B-ALL (n = 36)	BL (n = 19)	All (n = 55)
No target	3 (8%)	0 (0%)	3 (6%)
≥ One target	33 (91%)	19 (100%)	52 (94%)
≥ Two targets	33 (91%)	19 (100%)	52 (94%)
≥ Three targets	22 (61%)	15 (79%)	33 (60%)
No sensitive ^a target	3 (8%)	1 (5%)	4 (7%)
One sensitive target	8 (23%)	3 (16%)	11 (20%)
Two sensitive targets	22 (61%)	15 (79%)	37 (67%)

B-ALL, B-cell lymphoblastic leukemia; BL, Burkitt's lymphoma; IGH, immunoglobulin heavy chain; IGK, immunoglobulin kappa light chain; MRD, minimal residual disease.

^aMRD targets with a detection limit $\leq 10^{-4}$ by RQ-PCR analysis.

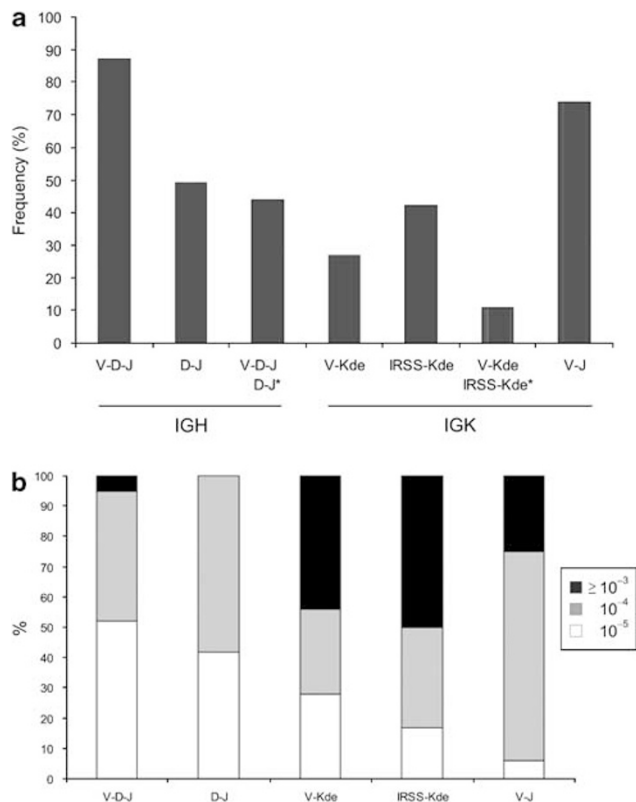


Figure 1 Frequencies (a) and sensitivity (b) of identifiable IGH and IGK rearrangements in B-ALL and BL patients. Rearrangements co-detected in the same patient are indicated with an asterisk (*).

IGK deletional rearrangements were found in 58% of the patients (27% IGKV-Kde, 42% IRSS-Kde, 11% both IGKV- and IRSS-Kde). IGKV-J rearrangements were detected in 74% of the patients. In one patient this rearrangement could not be analyzed because of the poor quality of the DNA. In 6 patients (11%) two IGKV-J rearrangement could be detected, and in 16 patients (29%) IGKV-J and IRSS-Kde rearrangements were co-detected.

Oligoclonal rearrangements (defined as ≥ 3 PCR bands for the same locus) were not found.

On comparing the prevalence of specific IGHV, D and J gene families in B-ALL and BL with that in previously published studies on PBLs,¹⁵⁻¹⁷ no statistically significant difference was found. In addition, the frequencies of IGHD and IGHJ gene segments were not significantly different between complete and incomplete rearrangements. On the other hand, the frequencies of IGHD and IGHJ gene families in IGH incomplete rearrangements appeared to be significantly different from those reported for BCP-ALL⁸ ($P=0.02$ for IGHD, $P=0.04$ for IGHJ). In BCP-ALL, a prevalence of the IGHJ6 segment (61% of BCP-ALL vs 26% in our series) was observed, predominantly rearranged to IGHD segments from the more upstream part of the IGHD region.⁸

As for heavy-chain complete rearrangements, the frequencies of IGKV and IGKJ families in IGKV-J rearrange-

ments were not significantly different from the data reported for PBLs.^{18,19} Moreover, unlike BCP-ALL,²⁰ no considerable difference was found in IGKV family frequencies between deletional and IGKV-J rearrangements.

Sensitivity of Ig Rearrangements as RQ-PCR Targets and MRD Analysis

A total of 97 RQ-PCR targets (62 for mature B-ALL, 35 for BL) were analyzed for sensitivity (Figure 1b). For 85/97 targets (88%) a sensitivity of at least 10^{-4} was reached (10^{-4} in 50% of the cases and 10^{-5} in 38%). The percentage of targets with a sensitivity $\geq 10^{-4}$ ranged from 100% for IGHD-J to 50% for IRSS-Kde rearrangements. In 20% of the patients only one sensitive target was available, and in 13% either no target was available or the target had an inadequate sensitivity (detection limit $\leq 10^{-4}$) (Table 1). In two cases, one BL and one B-ALL, the diagnostic specimen was insufficient to perform a second RQ-PCR assay. These patients were considered as having a single sensitive target.

The application of the RQ-PCR assay detected BM MRD positivity during chemotherapy in 6/36 patients affected by B-ALL and BM involvement at diagnosis in 6/19 BLs. Five B-ALL patients became MRD negative after the first cycle of chemotherapy, but they turned positive at the end of therapy and four of them subsequently relapsed. The sixth patient maintained MRD positivity during treatment. In 5/6 BL patients MDD was detected by both targets at the same level, while in a single case minimal BM infiltration could be measured only by the more sensitive target (sensitivity 10^{-5} vs 10^{-3}). All patients turned negative before the second cycle of chemotherapy and one patient just after the pre-phase.

In all, 32/36 B-ALLs and 12/19 BLs were positive for t(8;14). In most cases, the two methods detected MRD with similar results. Only in one case of B-ALL and in two cases of BL did the Ig-based approach detect MRD in the absence of a positive LD-PCR.

DISCUSSION

The features of IGH gene rearrangements identified in our cohort of patients were those expected for B-cell with a mature phenotype.²¹ In fact, in 53% of the patients both IGH alleles were rearranged and no relationship could be established between co-detected rearrangements. Furthermore, the potentially functional rearrangements of kappa light chain were frequently found (74%). As for normal PBLs, N-regions, when present, were very short (1-2 nucleotides), as the activity of Terminal deoxynucleotidyl Transferase is decreased or absent at the time of light-chain rearrangement.²² In addition, the frequencies of specific gene families were similar to those reported for normal PBLs.¹⁵⁻¹⁹

The prevalence of specific IGHD and IGHJ gene segments was significantly different from that reported for BCP-ALL.⁸ The remarkable predominance of incomplete rearrangements involving IGHD genes from the more upstream part of the IGHD region and the most downstream IGHJ6 segment

suggests that, in BCP-ALL, most IGHD-J rearrangements likely represent secondary recombinations, deleting pre-existing D–J joinings. In our series, the frequency of the IGHD and IGJ gene segments was not significantly different between complete and incomplete rearrangements, possibly suggesting that IGHD-J joining simply represents the first step of the heavy-chain rearrangement process. In addition, these rearrangements were often oligo-clonal in BCP-ALL,⁸ whereas in our series oligoclonality was never detected.

The problems of oligoclonality, secondary rearrangements and ongoing rearrangements might generate false-negative results, and this is the reason why the current guidelines recommend that at least two PCR targets must be monitored, preferentially representing two different gene loci.²³ However, considering the mature phenotype of B-ALL and BL cells, that likely harbor end-stage Ig rearrangements, and the similarity with PBL's rearrangement pattern, our preliminary results suggest that MDD and MRD could be studied with a single sensitive target in these malignancies, pending confirmation on larger patient series.

In three patients no clonal rearrangements were identified. This finding could be explained by the fact that consensus primers or family primers were used. Such primers may not be suitable to amplify all gene segment combinations with the same efficiency, leading to non-detectability of a portion of clonal rearrangements.

Regarding the Ig-based MRD assay, at least one sensitive RQ-PCR target was available in 87% of patients (84% of B-ALLs and 95% of BLs). The sensitivity level of this approach was considerably high, being at least 10^{-4} in 88% of the targets. In addition, PCR targets identified at diagnosis were preserved in all the four relapsed patients.

In four cases, although at least two clonal rearrangements were detected, the sensitivity level achieved was 10^{-3} or lower. These results are possibly due to the characteristics of each patient-specific junctional region: a limited number of junctional (N/P) or deleted nucleotides reduces the specificity of the ASO primer, giving a higher background amplification. Moreover, the sequence of the junction itself may be inadequate for primer design, as in the case of high-percentage GC or homopolymeric stretches.

Comparing the LD-PCR with Ig-based RQ-PCR, in most of the cases a similar sensitivity was achieved. However, in one case of B-ALL and in two cases of BL, a sensitivity of 10^{-5} was reached with the Ig-based assay, thus detecting the presence of MRD at the end of therapy in the B-ALL and BM involvement at diagnosis in the two BLs, despite their negativity by LD-PCR.

Our results suggest that Ig-based RQ-PCR assay and LD-PCR for t(8;14) can be successfully used for MRD analysis in mature B-ALL and BL patients. The LD-PCR method is simple, fast and relatively inexpensive, but the t(8;14) translocation can be detected only in about 70% of the cases. In addition, the sensitivity achievable by this method depends, in part, on the PCR product size.⁵ On the other hand,

the Ig rearrangements can be considered as universal targets in B-cell malignancies, covering about 95% of the patients, and the PCR amplification of such targets is highly sensitive and quantitative. However, this method is laborious and costly.²³

Given that our results showed the feasibility of the study, we plan to prospectively determine the relative prognostic value of these two molecular approaches for MRD detection in B-ALL and BL.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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