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Research paper

Next-generation antigen receptor sequencing of paired diagnosis and relapse samples of B-cell acute lymphoblastic leukemia: Clonal evolution and implications for minimal residual disease target selection

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ABSTRACT

Antigen receptor gene rearrangements are frequently applied as molecular targets for detection of minimal residual disease (MRD) in B-cell precursor acute lymphoblastic leukemia patients. Since such targets may be lost at relapse, appropriate selection of antigen receptor genes as MRD-PCR target is critical. Recently, next-generation sequencing (NGS) – much more sensitive and quantitative than classical PCR-heteroduplex approaches – has been introduced for identification of MRD-PCR targets. We evaluated 42 paired diagnosis-relapse samples by NGS (IGH, IGK, TRG, TRD, and TRB) to evaluate clonal evolution patterns and to design an algorithm for selection of antigen receptor gene rearrangements most likely to remain stable at relapse. Overall, only 393 out of 1446 (27%) clonal rearrangements were stable between diagnosis and relapse. If only index clones with a frequency > 5% at diagnosis were taken into account, this number increased to 65%; including only index clones with an absolute read count > 10,000, indicating truly major clones, further increased the stability to 84%. Over 90% of index clones at relapse were also present as index clone at diagnosis. Our data provide detailed information about the stability of antigen receptor gene rearrangements, based on which we propose an algorithm for selecting stable MRD-PCR targets, successful in > 97% of patients.

1. Introduction

Antigen receptor (immunoglobulin (IG) and T-cell receptor (TR)) gene rearrangements can be considered as DNA fingerprints of B-cell precursor acute lymphoblastic leukemia (BCP-ALL) cells. Consequently, they are frequently used to monitor minimal residual disease (MRD) in BCP-ALL patients [1]. However, IG/TR gene rearrangements can be lost during the course of the disease due to outgrowth of subclones, ongoing rearrangements or secondary rearrangements, thereby resulting in false-negative MRD results [2–8]. We previously have shown that monoclonal IG/TR targets are more likely to be stable between diagnosis and relapse than oligoclonal IG/TR rearrangements, and provided a strategy to select appropriate MRD-PCR targets [4].

In previous studies, IG/TR gene rearrangements were analyzed by using PCR-based methods (sometimes in combination with Southern blot), which allowed identification of clonal IG/TR gene rearrangements down to the level of 1–5% [9,10]. More recently, highly sensitive next generation sequencing (NGS) has been introduced for identification of IG/TR gene rearrangements in BCP-ALL patients at diagnosis. These studies show many more clonal IG/TR gene rearrangements than using the classical approaches and indicate a much higher level of oligoclonality than suggested before [7,11,12]. Therefore, it needs to be determined which of the multiple IG/TR gene rearrangements identified by NGS are suitable as MRD-PCR target and should be selected for monitoring. Several studies already applied NGS for analysis of MRD as well, with very promising data often at least equivalent to current MRD technologies [8,13–19]. However, within the EuroMRD network standardized protocols for accurate quantification of MRD are still under development and consequently NGS is not yet routinely used for MRD analysis [20,21]. Until then, NGS can be used instead of the classical approaches for identification of MRD-PCR targets, which subsequently can be used to monitor MRD with the well-

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Table 1
Number of clonal IG/TR rearrangements identified using NGS at diagnosis.

	All rearrangements			Rearrangements > 5%			%positive patients by classical PCR[b]
	Number of rearrangements		%positive patients[a]	Number of rearrangements		%positive patients[a]	
	median	range		median	Range		
IGH Vh-Jh	4,5	0–79	93%	1	0–5	90%	80–85%
IGH Dh-Jh	0	0–99	38%	0	0–4	33%	20%
IGK Vκ-Jκ	0	0–10	43%	0	0–3	26%	30%
IGK Intron-Kde	0	0–3	12%	0	0–1	7%	15–25%
IGK Vκ-Kde	1	0–8	57%	0	0–2	43%	45%
IGK total	1.5	0–19	69%	1	0–4	60%	60–75%
TRB Vβ-Jβ	1	0–19	64%	0	0–2	45%	25–30%
TRD Dδ-Dδ	0	0–8	26%	0	0–1	12%	40%
TRD Vδ-Dδ	2	0–69	69%	1	0–4	60%	
TRD total	2	0–71	69%	1	0–4	64%	40%
TRG Vγ-Jγ	2	0–102	76%	1	0–5	67%	50–60%
Total	20	4–311	100%	6	2–14	100%	100%

[a] Percentage of patients with ≥ one rearrangement for the indicated locus.
[b] Based on reference [22].

established patient-specific RQ-PCR method. In both cases, however, appropriate selection of MRD-PCR targets is crucial.

To design a strategy for MRD-PCR target selection based on NGS-based data, we evaluated 42 paired diagnosis-relapse samples to evaluate the stability of IG/TR clones. Our data show that NGS allows the identification of possible MRD-PCR targets in all patients, and that selection of MRD-PCR targets with a high frequency (> 5%) and a high absolute read count (> 10,000), via the proposed algorithm, results in at least one MRD-PCR target remaining stable at relapse in > 97% of patients.

2. Materials and methods

2.1. Patient and control samples

Bone marrow (BM) or peripheral blood (PB) samples were obtained at both diagnosis and relapse from 42 children with BCP-ALL (*Supplementary Table S1*). Genomic DNA was isolated from BM or PB mononuclear cells (MNCs) by the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). All samples were obtained according to the guidelines of the local Medical Ethics Committees (MEC 2004-203 and MEC 2012-287) and in line with the Declaration of Helsinki Protocol.

2.2. Classical PCR-heteroduplex-Sanger sequencing method

Primers and protocols for the detection of immunoglobulin heavy chain (IGH; Vh-Jh and Dh-Jh), immunoglobulin kappa (IGK; Vκ-Jκ, Vκ-Kde, Intron-Kde) light chain, T cell receptor gamma (TRG; Vγ-Jγ), T cell receptor delta (TRD; Dδ-Dδ, Vδ-Dδ, Vδ-Jα) gene rearrangements, and T cell receptor beta (TRB; Vβ-Jβ, Dβ-Jβ) rearrangements have been described previously [22]. Clonality of PCR products was confirmed by heteroduplex analysis and Sanger sequencing was performed with BigDye™ Primer Sequencing Kit (Thermo Fisher, MA, USA).

2.3. Next-generation sequencing method

NGS-based immunosequencing (performed by Sequenta, USA) was performed as published [13]. Briefly, a DNA quantity of 120–180 ng, corresponding to 20,000–30,000 MNCs, was used for the amplification of complete IGH gene rearrangements (Vh-Jh; three separate multiplex PCRs), incomplete IGH gene rearrangements (Dh-Jh), IGK gene rearrangements (Vκ-Jκ, Vκ-Kde, Intron-Kde), TRG gene rearrangements (Vγ-Jγ), TRD gene rearrangements (Vδ-Dδ, Dδ-Dδ) and TRB gene rearrangements (Vβ-Jβ). These rearrangements were amplified in a first PCR reaction of 25 cycles, using locus-specific primer sets [13]. Next, 1:100 of these PCR products was further amplified in a second PCR reaction of 14 cycles, using universal primers complementary to the adaptors that were linked to the locus-specific primers with sample-identifiers. The final PCR products were sequenced using the Illumina HiSEQ platform. Low-quality reads were filtered out and sequences with a single read were excluded [13]. Also sequences below previously-established thresholds, which might reflect non-leukemic rearrangements, were excluded from the analysis [12]. For each sequence, the frequency and the absolute read count (ARC), i.e. the read count corrected for PCR amplification by the spike-in method, were calculated [12,13]. In line with previous reports [13,14,23,24], an index clone was arbitrarily defined as a clonal IG/TR gene rearrangement with a frequency > 5%. The PRecISe Clonal Analysis (PRISCA) tool in Galaxy was used to compare sequences from paired diagnosis-relapse samples [12,25]. Details are provided in the Supplement.

3. Results

3.1. Identification of IG/TR gene rearrangements at diagnosis

Using NGS, we identified a median of 20 (range: 4–311) clonal IG/TR gene rearrangements per patient. VH-JH gene rearrangements were detected most, followed by TRG and Vδ-Dδ (Table 1). Frequencies of identified clonal rearrangements however varied significantly. A common pattern was the presence of many small clones and a more limited number of larger clones, particularly for IGH, TRD and TRG (Table 1). If only clonal IG/TR gene rearrangements with a frequency of at least 5% (i.e. index clones) were taken into account, the median number of rearrangements detected per patient was 6 (range: 2–14) with VH-JH, TRG and Vδ-Dδ again being most frequent (Table 1). Thus, using NGS at least two major IG/TR clonal rearrangements could be identified per patient, allowing selection of at least two possible MRD-PCR targets in all evaluated patients.

Evaluation of the percentage of patients positive for a particular IG/TR gene rearrangement (irrespective of its frequency) showed VH-JH rearrangements to be most frequent (93% of patients), followed by TRG (76%) (*Supplementary Table S2*). If only index clones were taken into account, still 90% of patients were positive for VH-JH rearrangements, whereas the frequency of TRG was decreased to 67%. Index clones for IGK, TRB, and TRD rearrangements could be identified in 60%, 45%, and 64% of patients, respectively (Table 1).

3.2. Concordance between NGS and classical PCR identified MRD-PCR targets

Comparison of IG/TR targets identified by classical PCR methods and NGS methods generally showed concordant results. Overall, 156 out of 172 (91%) IG/TR gene rearrangements identified by classical PCR were also detected by NGS (*Supplementary Table S2*). Of note, the applied NGS system did not contain Dβ-Jβ and Vδ2-Jα primers and therefore these rearrangements could not be detected and were excluded from this comparison. In comparison with classical PCR, NGS detected an additional 114 clonal rearrangements with a frequency > 5%, 66 (57%) of these however having an ARC < 1000 suggesting minor subclones (which likely are below the detection limit of the applied PCR-heteroduplex technology; *Supplementary Table S2*). Of the 48 clones with an ARC above 1000, 19 concerned IGH (Vh-Jh) rearrangements. The vast majority of these cases (17/19; 89%) could be explained by the VH1/7 and VH4/6 primers used in the PCR-heteroduplex analysis, which are not able to detect all V gene segments (e.g. VH1.14, VH4.34, VH6.1*02; see *Supplementary Table S3*) [22,26].

3.3. Stability of IG/TR gene rearrangements between diagnosis and relapse

We next evaluated whether IG/TR gene rearrangements detected at diagnosis were preserved at relapse. Overall, out of 1446 clonal rearrangements identified at diagnosis (irrespective of their frequency at diagnosis), only 393 (27%) were stable at relapse (Table 2 and Fig. 1). TRB rearrangements were most stable (63/82; 77%), whereas all other clonal gene rearrangements had a stability < 50% (Table 2). If only index clones present at diagnosis were considered, 165 out of 253 (65%) rearrangements remained stable (Table 2). Except for clonal IGH Dh-Jh rearrangements, all major IG/TR rearrangements had a stability of at least 50%. Thus, index clones were more stable than minor clones. Furthermore, 165 out of 181 (91%) stable index clones present at relapse were already present at diagnosis as an index clone. It should be noted that clones detected both at diagnosis and relapse showed different kinetics, some being relatively stable, whereas other were much more frequent in one of the two disease stages (Fig. 1).

Since *KMT2A*-rearranged patients are known to be highly oligoclonal and instable [27,28], we also evaluated the *KMT2A*-negative and *KMT2A*-rearranged patients separately. Indeed, in *KMT2A*-rearranged patients many more clonal rearrangements could be detected, the vast majority however showed frequencies < 5% and were lost at relapse (*Supplementary Table S4A and 4B*).

Since a high frequency of a clonal rearrangement does not necessarily imply that the clonal rearrangement is present in a large part of the leukemic cells, we further evaluated the stability of clonal rearrangemement not only based on their frequency but also based on their ARC. As shown in Fig. 2, highest stability was found for clonal rearrangements with an ARC > 10,000 (overall 134/160, 84%; all having a frequency > 5%), whereas clonal rearrangements with a frequency > 5% but an ARC < 10,000 had much lower stability (55/117; 47%). Thus, major clones (ARC > 10,000) were most stable, whereas both large subclones (1000 < ARC < 10,000) and minor subclones (ARC < 1000) were generally much less stable. High frequency (> 5%)/low ARC (< 10,000) clonal rearrangements more frequently concerned TR genes (59/103; 57%), of which particularly TCRB rearrangements showed good stability (65% versus 41% for TRG and 17% for TRD; Fig. 2E–H). High frequency/low ARC clonal IG rearrangements were slightly less common; of these fair stability was observed for IGK (63%) but not for IGH (14%) (Fig. 2A–D).

Table 2
Stability of clonal IG/TR rearrangements identified using NGS between diagnosis and relapse in 42 BCP-ALL patients.

Locus	All		> 5%		> 5% & ARC[a] > 10,000	
	Stable/All	% stable	Stable/All	% stable	Stable/All	% stable
IGH Dh-Jh	19/169	11%	9/22	41%	6/9	67%
IGH Vh-Jh	94/468	20%	48/66	73%	47/54	92%
IGK Vκ-Jκ	17/38	45%	12/16	75%	7/10	70%
IGK Intron-Kde	3/7	43%	2/3	67%	1/2	50%
IGK Vκ-Kde	18/50	36%	15/24	63%	12/16	75%
TCRB Vβ-Jβ	63/82	77%	17/25	68%	4/5	80%
TCRD Dδ-Dδ	5/31	16%	3/5	60%	3/4	75%
TCRD Vδ-Dδ	65/250	26%	26/41	63%	24/30	80%
TCRG Vγ-Jγ	109/353	31%	33/52	63%	22/25	88%
Total	393/1446	27%	165/253	65%	126/152	83%

[a] Absolute read count.

3.4. Emerging subclones at relapse

Next, we evaluated how many new clones emerged at relapse. In total, 321 clonal sequences were only detected at relapse, of which 59 (18%) were index clones (Fig. 1). Although new clones were detected in all different loci, new clonal TR rearrangements were most frequent (64% of rearrangements with a frequency > 5%) (*Supplementary Table S5*). Furthermore, the vast majority (70%) of these new high-frequency TR clonal rearrangements had an ARC below < 10,000 (suggesting subclones), whereas the majority (52%) of new high-frequency IG rearrangements had an ARC > 10,000 (suggesting major index clones). Thus, emerging index clones generally concerned small TR clones and major IG clones.

3.5. Strategy for selecting stable IG/TR gene rearrangements

We then evaluated how to select MRD-PCR targets from clonal IG/TR rearrangements detected by NGS at diagnosis, in such a way that the chance of selecting at least one stable IG/TR rearrangement is highest. Using the algorithm shown in Fig. 3, at least one stable IG/TR rearrangement was selected in 41 out of 42 (98%) of patients (Fig. 4). In one patient (6974; 14 year, *KMT2A*-rearranged pro-B-ALL) no index clones with an ARC > 10,000 were identified. In eight patients only one clonal IG/TR rearrangement could be selected; in all cases this target was however stable at relapse. Detailed analysis of the six selected VH-JH rearrangements that were lost at relapse showed persistence of a DH-JH stem at relapse in one patient (Fig. 4).

Using the classical PCR approach, also no IG/TR target could be identified in patient 6974. In addition, also in three other patients (5257, 6190, and 11,938) all selected MRD-PCR targets were lost. Of note, two of these patients (6974 and 11,938) could be monitored using the stable *KMT2A* rearrangement as RQ-PCR target [28].

In the 30 patients in whom (RQ-PCR-based) MRD diagnostics were actually performed, 40/57 (70%) of the applied MRD-PCR targets would also have been selected if the NGS approach had been used (Fig. 4B). The remaining rearrangements were either detected (ARC > 10,000) but not selected (n = 4, 3 stable), not identified by NGS (n = 6, including a Vλ-Jλ, Vκ-Vκ, Dβ-Jβ and two DH7.27-JH rearrangements not identifiable by the applied NGS method; 4 stable) or had an ARC < 10,000 (n = 7; 6 stable).

4. Discussion

MRD analysis using IG/TR gene rearrangements is being used for risk group stratification since many years, as this technology is highly sensitive, applicable to the vast majority of ALL patients, and well standardized across multiple European and non-European countries [1,29,30]. In addition, quality assurance is organized via regular quality control rounds by EuroMRD. The development of NGS-based analysis of IG/TR gene rearrangements has opened new possibilities for MRD analysis. Although it is anticipated that NGS eventually will also be used for the actual MRD analysis, the development of standardized

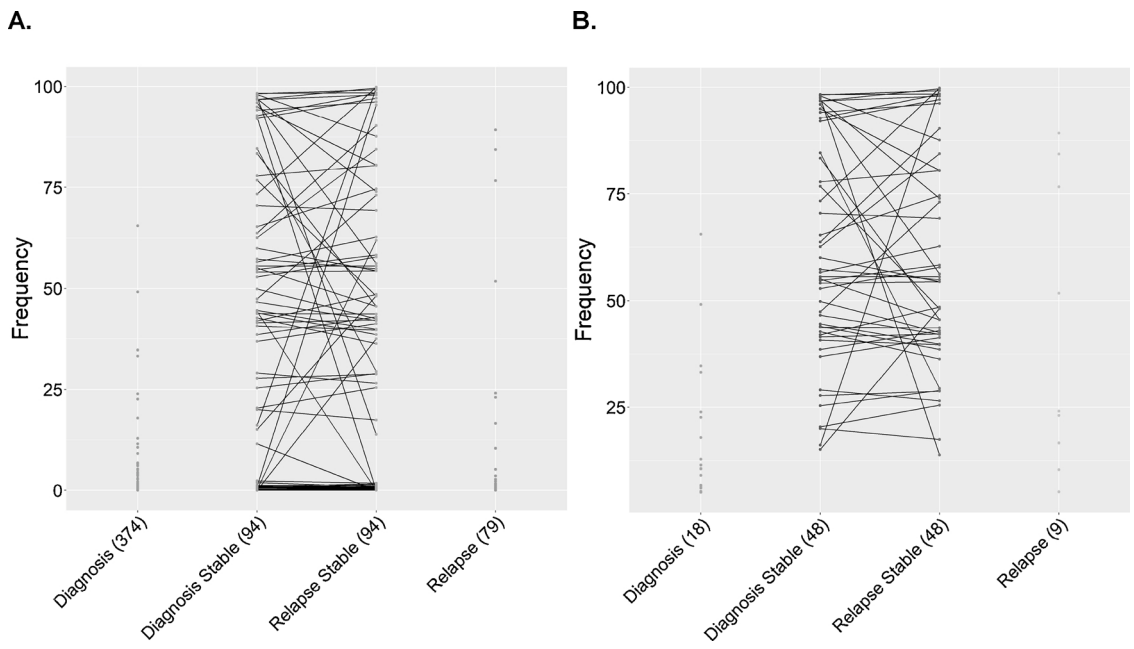

Fig. 1. Number and frequency of clonal IGH (Vh-Jh) gene rearrangements identified in relapsed BCP-ALL patients at diagnosis only, both at diagnosis and relapse, or at relapse only. A. All clonal Vh-Jh gene rearrangements are shown, independent of their frequency at diagnosis or relapse. 94 out of 468 (20%) Vh-Jh rearrangements detected at diagnosis could also be detected at relapse. **B.** Only clonal Vh-Jh gene rearrangements with a frequency of at least 5%, i.e. index clones, are shown. 48 out of 66 (73%) Vh-Jh rearrangements detected at diagnosis could also be detected at relapse.

and validated protocols for NGS-based MRD analysis has not yet been completed within EuroMRD. However, NGS may already be an attractive alternative for the classical PCR-heteroduplex-Sanger sequencing approaches used to identify MRD-PCR targets at diagnosis. Given the deep sequencing capacity of NGS, such method will provide much more information about IG/TR clonal rearrangements present at diagnosis, also in a more quantitative manner. Indeed, using NGS we identified a median of 20 clonal rearrangements per patient, although many of these having either a low frequency and/or a low ARC. Therefore, in line with previous reports [7,11,12,16] most BCP-ALL patients seem to be oligoclonal based on NGS data. In contrast, previous studies using PCR and Southern blotting reported only 40% of the BCP-ALL patients being oligoclonal, with maximally 9 leukemic rearrangements per patient [9,22,31]. Since oligoclonal rearrangements are more likely to be lost during relapse [4], appropriate selection of MRD-PCR targets is crucial. In this study we therefore used NGS to analyze clonal IG/TR rearrangements in paired diagnosis-relapse samples of 42 BCP-ALL patients, to evaluate their stability and to define an algorithm for optimal selection of MRD-PCR targets.

Most clonal rearrangements detected by classical PCR-heteroduplex analysis and Sanger sequencing were also identified by NGS, whereas NGS detected many additional clonal sequences. The vast majority of these additional rearrangements (even if frequencies > 5%) had relatively low ARC, suggesting minor subclones. Next to inherent differences in sensitivity between both approaches, differences were related to applied primer systems (which may not detect all specific gene segments) and to singleplex versus multiplex PCR reactions, the latter more prone to disproportional amplification in case multiple rearrangements are present. Overall, if particularly clonal rearrangements with a frequency > 5% (i.e. index clones) were taken into account, potential MRD-PCR targets could be identified by NGS in all patients, and the percentage of patients positive for a particular rearrangement generally was higher than published data on PCR-heteroduplex analysis [22,28].

Comparison of paired diagnosis-relapse samples showed an overall stability of only 393 out of 1446 (27%) clonal rearrangements. If only index clones were taken into account, this percentage increased to 65%; including only index clones with an ARC > 10,000 even further increased the stability to 84%. This percentage is significantly higher than the overall stability observed using classical PCR-heteroduplex analysis (71%) and comes close to the stability of monoclonal rearrangements (89%) [4]. These data indicate that in most patients, the large clone present at diagnosis will remain at relapse, an observation also made by Bashford-Rogers et al for *IGH* in a small series of ten relapsed ALL patients [7]. In line with this, 91% of index clones present at relapse were also present as index clones at diagnosis. Nevertheless, clonal evolution is common, with some clones lost at relapse and new clones emerging at relapse. Since many emerging clones concerned TR rearrangements with relatively low ARC, it may well be that (at least part of) these rearrangements are not present in the BCP-ALL cells but reflect reactive T-cell clones. This is supported by the finding that high frequency (> 5%) low ARC (< 10,000) TR clones were often stable, whereas such minor IG clones were generally lost at relapse. Therefore, one should be cautious when selecting NGS-identified TR gene rearrangements as MRD-PCR target, as particularly the clones with low ARC might represent (oligo)clonal T-cell proliferations and therefore possibly are no appropriate MRD-PCR targets.

Based on the observed stability, we designed an algorithm that allows selection of MRD-PCR targets from which at least one remains stable at relapse. This algorithm would have been successful in > 95% of patients, only in one patient (a highly oligoclonal pro-B-ALL) no appropriate MRD-PCR target would have been identified. Of note, also using the classical approach monitoring of this patient was not successful. In an additional three patient the selected MRD-PCR targets by the classical approach were lost at relapse, favoring the use of NGS in target identification and selection. In eight patients only one MRD-PCR target could be selected by our algorithm. One logically always can decide to select additional clonal rearrangements (i.e. clonal rearrangements with an ARC < 10,000, but preferably with high frequency) to increase the chance of obtaining an appropriate RQ-PCR for sensitively monitoring MRD, since the final MRD-PCR target selection is not only based on expected stability, but also on expected sensitivity of the RQ-PCR [22].

In our study we only focused on comparison of diagnosis and relapse

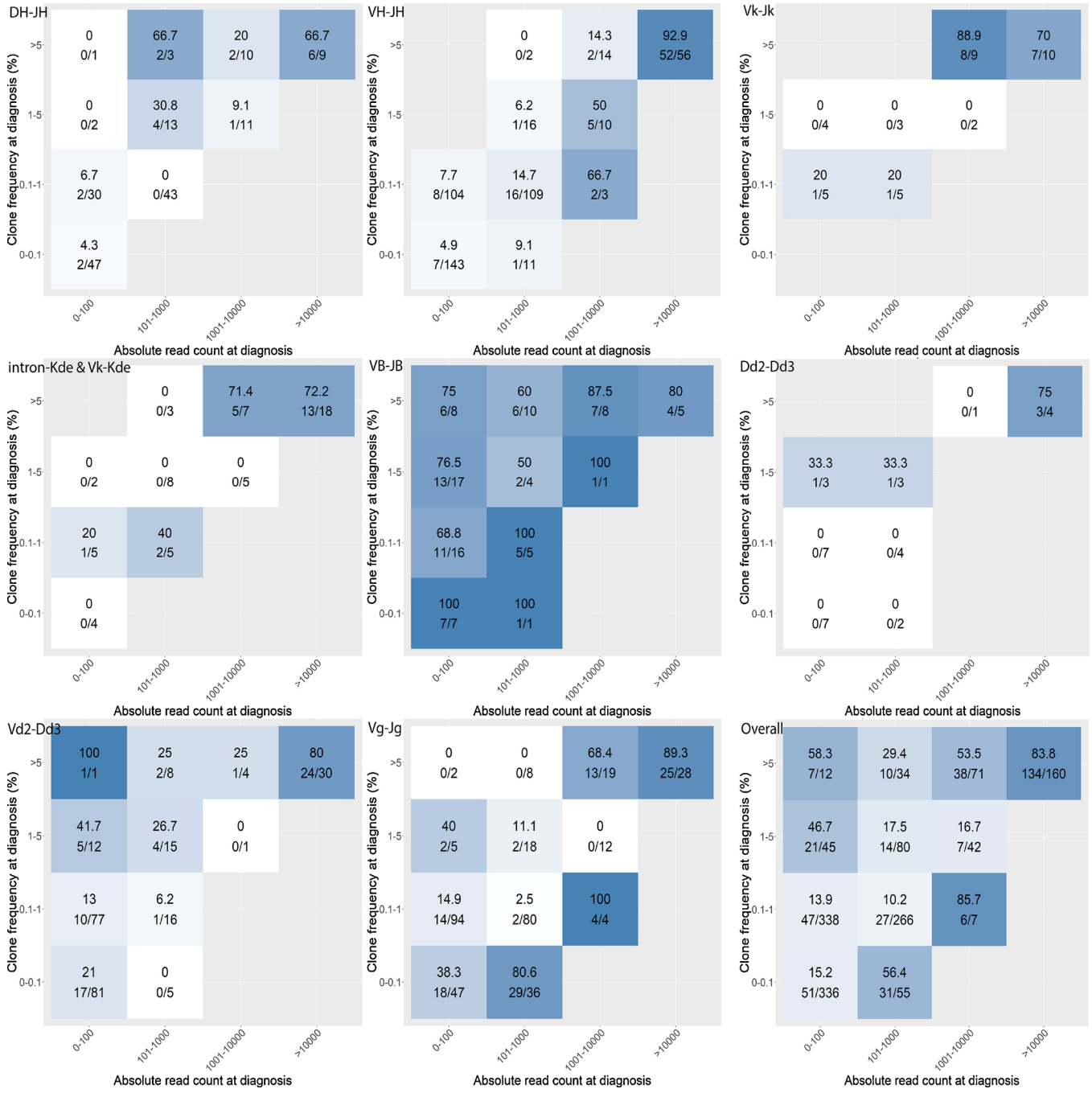

Fig. 2. Stability of clonal IG/TR rearrangements between diagnosis and relapse, depending on frequency and absolute read count at diagnosis. A. Dh-Jh, **B.** Vh-Jh, **C.** Vκ-Kde and Intron-Kde, **D.** Vκ-Jκ, **E.** Vβ-Jβ, **F.** Vδ-Dδ, **G.** Dδ-Dδ, **H.** Vγ-Jγ **I.** all different types of IG/TR rearrangements together. The numbers refer to the percentage of stable rearrangements (number on top of each cell) and the number of stable rearrangements / total number of rearrangements with specified absolute read count (ARC; x-axis) and frequency at diagnosis (y-axis). The white to blue colors reflect the level of stability (the bluer the more stable) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

pairs, and did not evaluate actual MRD measurements in follow-up samples based on the newly-generated NGS data. Nevertheless, the available MRD data showed that, in most patients (27/30), the targets remaining stable at relapse could also be detected in bone marrow samples obtained after the first and/or second course of chemotherapy. However, three patients were classified as standard risk by MRD diagnostics (i.e. were MRD negative at both time points), but nevertheless relapsed with confirmed stability of the applied IG/TR gene rearrangements. The MRD-PCR targets actually used were similar to the ones that would have been selected using the NGS approach, and therefore these patients would also have been classified as standard risk using the NGS approach. Clearly such (rare) relapsing standard risk patients need to be studied in more detail to understand why they suffer from a relapse and how such relapse may be predicted.

In this study, we applied the NGS method as designed by Sequenta [13]. Although the use of other NGS approaches (such as the one currently being developed within EuroClonaility/EuroMRD) may not results in fully identical data (e.g. due to the use of other primer sets), the overall conclusions will likely remain the same, that is that the major clonal rearrangements are the ones most likely to remain stable at relapse. The ARC threshold of 10,000 used in our algorithm needs to be verified in other NGS systems, but it is expected that this will mainly be

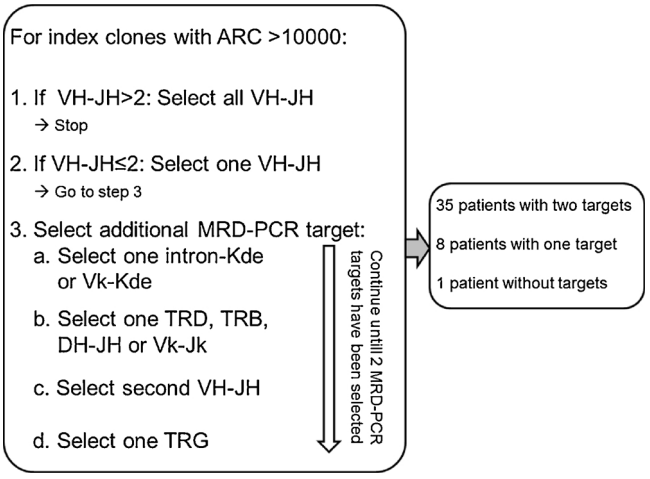

Fig. 3. Proposed algorithm for selection of NGS-defined clonal rearrangements at diagnosis as MRD-PCR targets. In patients in which more than two complete IGH gene rearrangements with an ARC > 10,000 are detected, all these rearrangements are selected (in our series, this maximally concerned three IGH VH-JH). In patients with 1 or 2 IGH VH-JH, one VH-JH is selected, and additionally a second IG/TR rearrangements is selected (intron-Kde / VK-Kde > TRD / TRB / DH-JH / Vk-Jk > second VH-JH > TRG). Once two MRD-PCR targets have been selected, the algorithm can be stopped, otherwise one continues with the next step. In patients without VH-JH, one immediately goes to step 3.

impacted by the amount of DNA used for the NGS analysis and the percentage of ALL cells present in the sample (~150 ng and > 60% in this study). Based on a DNA input of ~150 ng (derived from ~25,000 cells) in our assay, the ARC of 10,000 would correspond with ~40% of cells harboring the clonal IG/TR gene rearrangement. This number could be used if other amounts of DNA are being used to calculate the corresponding ARC. If the percentage of ALL cells in the diagnostic sample is low (e.g. < 50%), also lower ARC thresholds may be needed to identify possible MRD-PCR targets. In that respect the ARC threshold of 10,000 should not be considered as a strict threshold but rather as an extra parameter (next to frequency) to identify clonal rearrangements truly present in the vast majority of the BCP-ALL cells and thus most likely to remain stable.

5. Conclusions

To our best knowledge, this is the largest diagnosis-relapse study using NGS analysis of multiple IG/TR loci. Our data show that NGS provides more detailed and quantitative information on IG/TR rearrangements, allowing a better identification of truly major leukemic clones which are most likely to be stable at relapse, and provide a rationale for selecting appropriate MRD-PCR targets, favoring the use of NGS analysis in target identification at diagnosis. Within the EuroClonality/EuroMRD network, the NGS method is currently being standardized and quality assurance programs have been initiated [21], which will allow application of NGS for the actual MRD assessment in the near future as well.

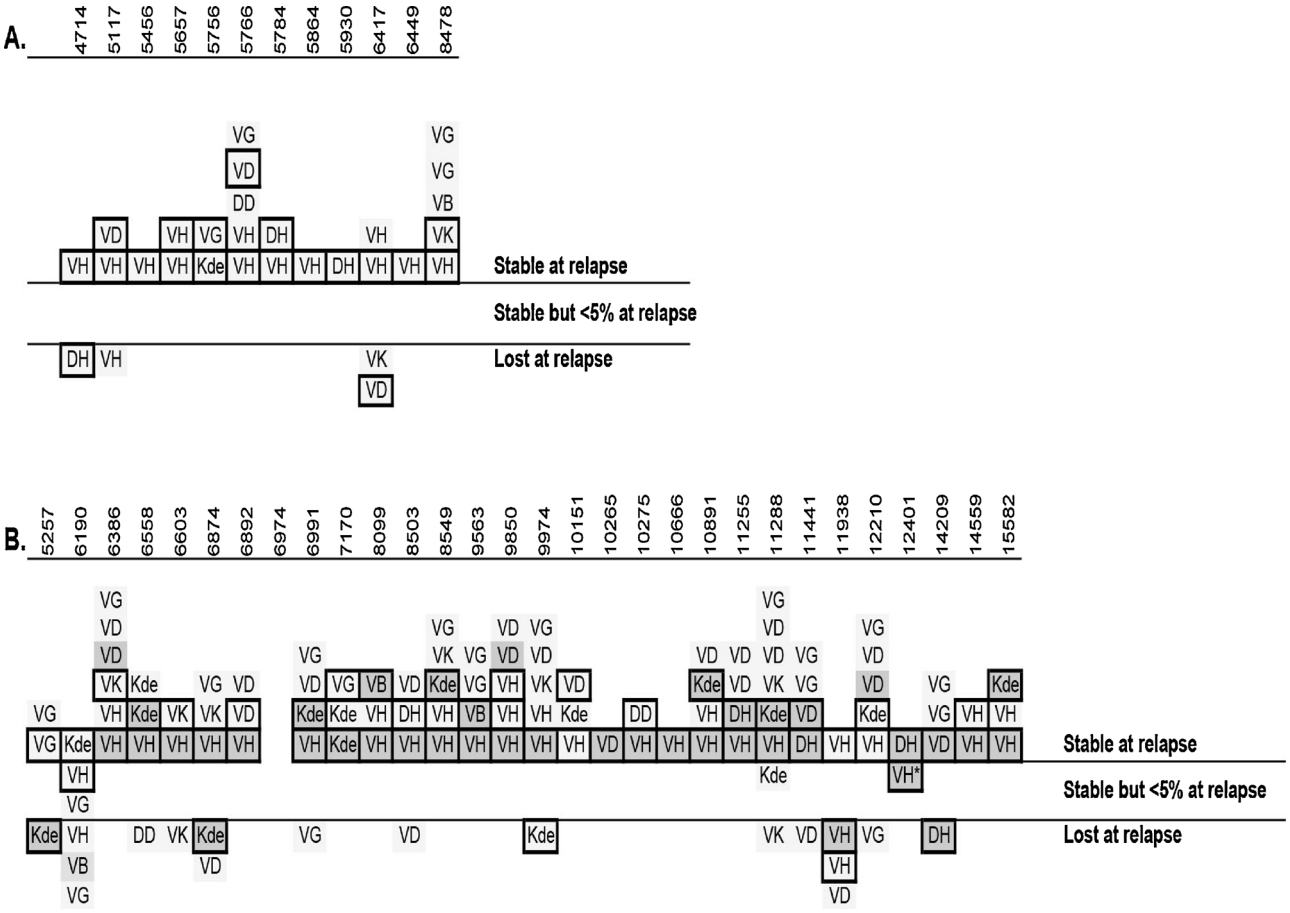

Fig. 4. Overview of NGS-identified IG/TR gene rearrangements present at diagnosis and their stability at relapse. A. 12 patients for which MRD diagnostics were not performed. **B.** 30 patients for whom MRD diagnostics were performed. In the plots, rearrangements shown in the upper part were stable, the ones in the bottom part were lost at relapse, and those in-between were still present at relapse but at frequency < 5%. The bordered rearrangements are those that were selected according to the algorithm in Fig. 3. The darker-colored rearrangements were used for actual MRD analysis. *: common stem remained at relapse as major clone. VH: Vh-Jh; DH: Dh-Jh; Kde: Vκ-Kde and Intron-Kde; VK: Vκ-Jκ; VB: Vβ-Jβ; VD: Vδ-Dδ; DD: Dδ-Dδ; VG: Vγ-Jγ.

Declaration of interest

VHJvdV: contract research for Amgen, Roche, Pfizer, Janssen, and BD Biosciences; consultancy fees from Celgene and Amgen (none of these related to the content of this manuscript). The other authors declare no conflict of interest.

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VHJvdV designed the research study; PMJT and MdB performed the research; MdB, DvZ, VdH, APS, and VHJvdV analyzed the data; VHJvdV wrote the paper; all authors revised the paper and approved the final version.

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Appendix A. Supplementary data

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