

## Detection of Minimal Residual Disease in B Lymphoblastic Leukemia by High-Throughput Sequencing of *IGH*

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### Abstract

**Purpose:** High-throughput sequencing (HTS) of immunoglobulin heavy-chain genes (*IGH*) in unselected clinical samples for minimal residual disease (MRD) in B lymphoblastic leukemia (B-ALL) has not been tested. As current MRD-detecting methods such as flow cytometry or patient-specific qPCR are complex or difficult to standardize in the clinical laboratory, sequencing may enhance clinical prognostication.

**Experimental Design:** We sequenced *IGH* in paired pretreatment and day 29 post-treatment samples using residual material from consecutive, unselected samples from the Children's Oncology Group AALL0932 trial to measure MRD as compared with flow cytometry. We assessed the impact of ongoing recombination at *IGH* on MRD detection in post-treatment samples. Finally, we evaluated a subset of cases with discordant MRD results between flow cytometry and sequencing.

**Results:** We found clonal *IGH* rearrangements in 92 of 98 pretreatment patient samples. Furthermore, while ongoing recombination of *IGH* was evident, index clones typically prevailed in MRD-positive post-treatment samples, suggesting that clonal evolution at *IGH* does not contribute substantively to tumor fitness. MRD was detected by sequencing in all flow cytometry-positive cases with no false-negative results. In addition, in a subset of patients, MRD was detected by sequencing, but not by flow cytometry, including a fraction with MRD levels within the sensitivity of flow cytometry. We provide data that suggest that this discordance in some patients may be due to the phenotypic maturation of the transformed cell.

**Conclusion:** Our results provide strong support for HTS of *IGH* to enhance clinical prognostication in B-ALL. *Clin Cancer Res*; 20(17); 4540–8. ©2014 AACR.

### Introduction

B lymphoblastic leukemia/lymphoma (B-ALL) is an aggressive, immature B-cell neoplasm in which substantial progress in pediatric and adult outcomes has been achieved (1). In addition to conventional prognostic factors, such as cytogenetics and karyotype, post-treatment detection of minimal residual disease (MRD) in B-ALL is important for

identifying patients who may require treatment intensification (2–7). MRD measurement is accepted for prognostication in pediatric B-ALL, and may also surpass other prognostic markers in adult B-ALL (2–4, 7–9). However, although MRD assessment by flow cytometry or qPCR has been incorporated widely in clinical studies, broader implementation has been limited by the challenges of ensuring accurate test performance in the clinical laboratory. For flow cytometry, MRD assessment is confounded by observer variation due to difficulty of consistently interpreting complex multidimensional data in the context of therapy-induced immunophenotypic drift and marrow regeneration. For qPCR, in which patient-specific clonal rearrangements of the immunoglobulin heavy chain (*IGH*) or T-cell receptors (*TRG*, *TRB*, *TRD*, and *TRA*) are tracked, MRD assessment requires significant laboratory and institutional commitment to maintain staffing to ensure continuity and quality of testing.

We recently reported the first application of high-throughput sequencing (HTS) for the assessment of MRD in acute T-lineage lymphoblastic leukemia, demonstrating the potential of sequencing to improve clinical

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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### Translational Relevance

This study evaluates the potential for high-throughput sequencing (HTS) of the immunoglobulin heavy-chain gene, *IGH*, to detect minimal residual disease (MRD) in patients with acute precursor B lymphoblastic leukemia (B-ALL). We show that MRD can be readily detected by sequencing with no false-negative results—as compared with flow cytometry—by using unselected, consecutive paired pretreatment and day 29 post-treatment samples from patients enrolled in Children’s Oncology Group ALL00932 trial. As prior work exploring next-generation sequencing in B-ALL included only patients with prior confirmed evidence of a clonal *IGH* rearrangement, our results show that sequencing of *IGH* alone is broadly applicable for unselected patients with B-ALL. Furthermore, we measure *IGH* repertoire during therapy and show that ongoing recombination at *IGH* does not contribute to enhanced tumor fitness that represents day 29 relapse. We contend that the enhanced specificity of HTS of *IGH* for MRD detection should greatly improve patient prognostication.

prognostication (10). Subsequently, Faham and colleagues demonstrated the feasibility of sequencing for MRD detection in B-ALL (11). However, their study did not seem to evaluate the potential, widespread applicability of sequencing *IGH* for MRD detection in B-ALL, because they required all samples to have a known clonal *IGH* gene rearrangement for inclusion. Gawad and colleagues (12) recently reported evidence for massive clonal evolution (ongoing recombination) in *IGH* as detected by deep sequencing. However, they focused primarily on diagnostic B-ALL samples and did not systematically evaluate the impact of this ongoing recombination of *IGH* on MRD detection.

Here, we report on HTS of *IGH* for MRD in B-ALL using residual material from unselected samples derived from the patients enrolled in Children’s Oncology Group (COG) clinical trial AALL0932. We show that HTS of *IGH* is broadly applicable in most patients with B-ALL (91 of 98 patients considered), and find that sequencing detected MRD in all cases that were positive by flow cytometry, with no false-negative results. We further demonstrate the use of a synthetic immune system to minimize bias and ensure accurate results in multiplexed PCR. Notably, given the enhanced precision and specificity of HTS, we provide the first comprehensive assessment of clonal evolution of *IGH* during induction therapy. Finally, we show that in rare cases, index clonal *IGH* sequences may be identified within an immunophenotypically mature B-cell population in post-treatment samples, suggesting that in some patients, tumor cells may undergo therapy-induced immunophenotypic maturation. Taken together, our data suggest that HTS of *IGH* could significantly improve clinical prognostication in B-ALL.

## Materials and Methods

### Clinical samples

Clinical samples (pretreatment: peripheral blood or bone marrow aspirate; and day 29 post-treatment: bone marrow aspirates) were collected from 99 individuals with informed consent by local clinicians, as part of enrollment into COG Study AALL0932 (registered as #NCT01190930; ref. 13). All patients had a diagnosis of B-ALL established locally, and then COG-coded pre- and post-treatment samples were submitted to the University of Washington Hematopathology Laboratory for routine flow cytometry analysis for MRD. Samples were not otherwise preselected, such as on the basis of having prior evidence of a clonal *IGH* gene rearrangement (11).

### Flow cytometry

Multiparametric flow cytometry was performed at the University of Washington as part of the routine evaluation for MRD (14). Samples were labeled with the antibody combinations: CD20 FITC, CD10 PE, CD38 PerCP-Cy5.5, CD19 PE-Cy7, CD58 APC, and CD45 APC-H7 and CD9 FITC, CD13/CD33 PE, CD34 PerCP-Cy5.5, CD19 PE-Cy7, CD10 APC, and CD45 APC-H7, and processed using NH<sub>4</sub>Cl + 0.25% formaldehyde for red cell lysis with >750,000 events acquired on a Becton-Dickinson LSRII. Clusters of events differing from normal B-cell maturation were defined as MRD by B.L. Wood, and quantified relative to total mononuclear cells and total nucleated cells. Data were analyzed using Woodlist software version 2.7 (in-house software written by B.L. Wood; equivalent software is commercially available; ref. 14).

### HTS

**Sequencing CDR3 regions.** *IGH* CDR3 regions were amplified and sequenced from 400 ng of pretreatment and 6 μg of day 29, post-treatment DNA samples, or in a subset of cases using all available extracted DNA. Amplification and sequencing of *IGH* CDR3 regions was carried out using the clonoSEQ platform (Adaptive Biotechnologies), in which a set of multiplexed forward primers matching V and D gene segment sequences are combined with a set of reverse primers matching J gene segment sequences to amplify both mature VDJ and immature DJ *IGH* rearrangements. Sequencing was performed starting from the J gene segment and extending 87 bp into the CDR3 region. Sequences for *IGH* CDR3 regions were delineated according to criteria established by the International ImmunoGenetics collaboration with a standard algorithm to identify V, D, and J gene segments (15, 16). Rearranged CDR3 sequences were classified as nonproductive if insertions or deletions were identified that resulted in frameshifts or premature stop codons.

**Identifying CDR3 sequences and defining clonality.** DNA from bone marrow isolated from 9 healthy individuals was used to amplify *IGH* sequences using the same protocol as the day 29, post-treatment B-ALL samples. The frequency of the most common *IGH* gene rearrangement from the marrow aspirate of these individuals averaged 0.08% ± 0.04%

of total nucleated cells (mean  $\pm$  SD; Supplementary Fig. S1). For this study, a very conservative definition of a neoplastic clone was chosen as representing a minimum of 10% of nucleated cells. For samples in which the two most common *IGH* sequences were of comparable frequency, biallelic rearrangement was assumed with both sequences considered to represent the lymphoblast clone.

In day 29 post-treatment samples, MRD was identified by searching for CDR3 sequences that identically matched the clonal sequence defined in pretreatment samples, requiring a complete (87 bp) match. Both the presence and the frequency of the MRD clone relative to the total *IGH* repertoire and total nucleated cells were determined. To determine if the clonal CDR3 sequences identified were specific, all post-treatment samples were screened for the presence and frequency of all identified pretreatment *IGH* CDR3 clones (17).

**Use of a synthetic template to minimize bias in multiplexed PCR.** Because accurate quantification of lymphoblast clones for MRD detection is critical, we developed an approach to ensure minimal bias in multiplex PCR (17). Briefly, each potential VDJ rearrangement of the *IGH* locus contains one of nine J segments, one of 27 D segments, and one of 124 V segments, many of which have disparate nucleotide sequences. To amplify all possible VDJ combinations, we used a single tube, multiplex PCR assay with 84 V and 15 D forward and 9 J reverse primers. To remove potential PCR bias, every possible V–J and D–J pair was chemically synthesized as a template with specific barcodes (17). These templates were engineered so as to be recognizable as nonbiologic and have universal 3' and 5' ends to permit amplification with universal primers and subsequent quantification by HTS. This synthetic immune system can then be used to calibrate the multiplex PCR assay. Iteratively, the multiplex pool of templates is amplified and sequenced with our *IGH* V/D- and J-specific primers, and the primer concentrations are adjusted to rebalance PCR amplification (Supplementary Fig. S2). Once the multiplex primer mixture amplifies each V and J template nearly equivalently, residual bias is removed computationally.

**Quantifying the fraction of B cells in bone marrow or blood.** The clonoSEQ assay amplifies and sequences rearranged *IGH* molecules. We quantify the amount of DNA input into the assay and convert this to the equivalent total number of nucleated cells, assuming approximately 6.4 pg genomic DNA per diploid cell. clonoSEQ then amplifies and sequences the molecules with rearranged *IGH*. Because the clonoSEQ assay includes analysis of DJ rearrangements, most B cells produce two sequences, one of which is a complete VDJ rearrangement and the other either a VDJ or an incomplete DJ rearrangement. To estimate the number of starting templates with *IGH* rearrangements that were in the sample, the average number of sequence reads for each starting template was measured. Synthetic control templates are also spiked at limiting dilution into each sample such that each template may be present at most as a single copy. One then can compute the average number of reads for each sequenced spiked synthetic template. The

total number of B cells sequenced is then derived as the total number of sequencing reads divided by this computed average reads per template (fold-coverage), further divided by two, because there are two alleles per B cell. The total fraction of B cells in the mixture is this number of B cells divided by the total number of input cells as determined by DNA input quantity. This method is precise across a large range of initial B cell fractions (Supplementary Fig. S3).

**Identifying evolved *IGH* clonotypes.** To detect ongoing recombination at the *IGH* locus (so-called clonal evolution), we applied the algorithm proposed by Gawad and colleagues (12). Briefly, this approach defines evolved clonotypes of *IGH* as those that (i) share an identical  $J_H$  sequence with the index clone, (ii) share at least six bases of identical NDN sequence with the index clone (starting from the J-segment boundary, with D-segment matches collapsed to two effective NDN bases), and (iii) are annotated with a different  $V_H$  gene than the index clone (12). We verified the specificity of this approach using cross-patient comparisons and find that among clones that share a  $J_H$  sequence, only 0.005% were considered "evolved" in other patients, as compared with 60.9% of  $J_H$ -matching clones within the same patient, confirming that this algorithm has a low false-positive rate (12).

## Results

Using unselected, consecutive residual material derived from 99 paired pre- and day 29 post-treatment B-ALL samples from patients enrolled in the COG AALL0932, we sequenced the complementarity-determining region 3 (CDR3) of *IGH* using the equivalent of approximately 300,000 genomes for pretreatment samples and 1,000,000 genomes for day 29 post-treatment samples. One case unexpectedly failed at the outset with inadequate DNA from the extraction of the pretreatment sample and was excluded from further consideration. The pretreatment sequencing analysis permitted us to define for 91 of 98 patients the unique, recombined *IGH* gene sequences (V–DJ complete rearrangements > 10%), representing the patient's clonal, neoplastic B lymphoblasts (Table 1, Fig. 1A). The remaining 7 patients without a B lymphoblast clone with a complete *IGH* rearrangement of 10% or higher of total cells in the bone marrow were studied individually. One patient had a large B lymphoblast clone with two D–J rearrangements, but no complete V–DJ *IGH* rearrangements. A second patient had a clone comprising approximately 4% of nucleated cells in the bone marrow, which still represents a statistically significant expansion compared with healthy donors (Supplementary Fig. S1), and this value agreed with the corresponding flow cytometry result. The remaining 5 patient samples did not have an identifiable clonal *IGH* sequence at diagnosis, defined as that comprising more than 10% among total nucleated cells of a complete (V–J) or incomplete D–J *IGH* sequence, and were not considered further herein. By flow cytometry, these 5 cases did not have a distinguishing or common pretreatment immunophenotype (Supplementary Fig. S4,

**Table 1.** Summary of 99 consecutive patient cases

Failed initial DNA extraction	1
Adequate DNA	98
Pretreatment sequencing	
Pretreatment clonal <i>IGH</i> (V–J)	91
Pretreatment clonal <i>IGH</i> (D–J) only	1
Pretreatment biallelic <i>IGH</i>	26 <sup>a</sup>
Post-treatment MRD	
HTS+/mpFC+ post-treatment MRD	23
HTS+/mpFC– post-treatment MRD	28
HTS–/mpFC– post-treatment MRD	40
Eight additional mpFC-negative cases, triple flow cytometry–sorted for HTS	
Evidence of clonal, pretreatment <i>IGH</i> in sorted mature B-cell fraction	1
No evidence of clonal, pretreatment <i>IGH</i> in sorted mature B-cell fraction in samples with adequate DNA	7

<sup>a</sup>Adequate DNA was only available to test for *IGH* D–J rearrangements (and therefore biallelic samples) in 84 of the 98 cases. Samples with three or more highly expanded *IGH* sequences were also not considered biallelic.

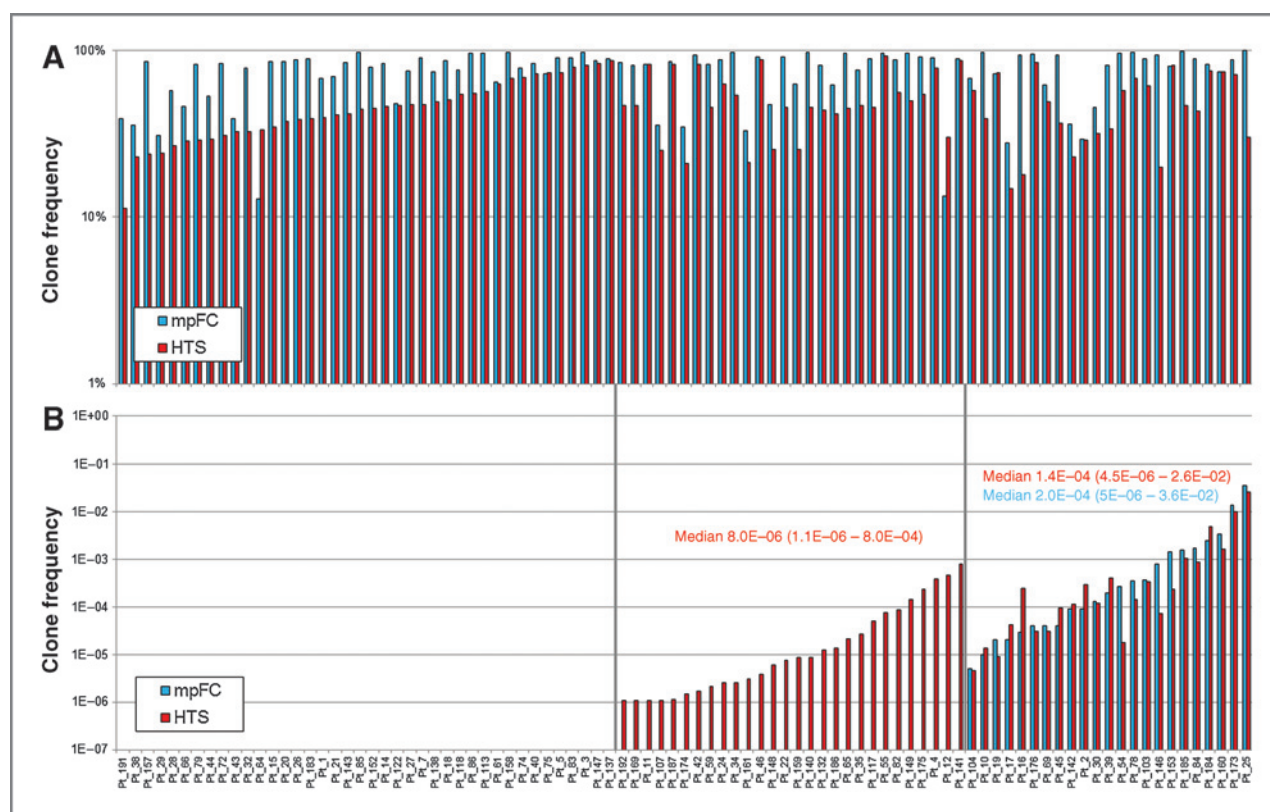
Supplementary Table S1). Thus, of 98 unselected samples, 92 cases at diagnosis had a clonal *IGH* complete or incomplete DJ-rearrangement meeting our definition of a clonal B-cell population comprising more than 10% of total cells, with one additional case having a clonal *IGH* rearrangement at 4%, matching flow cytometry.

A total of eighty-four samples were examined with an assay designed to amplify V–DJ and D–J rearrangements. Fifty-eight of these samples had at least two *IGH* sequences with frequencies higher than 10%: 26 had two clonal *IGH* above 10% and the third most frequent clone at least 10-fold smaller than the second most frequent, providing the potential to improve specificity for this assay by post-treatment tracking of both alleles. The use of a synthetic *IGH* immune system in which templates of all V<sub>H</sub> and J<sub>H</sub> primer pairs are used to adjust primer concentrations minimizes multiplexed PCR bias. Among these 84 patients, incomplete D–J rearrangements with a frequency of at least 10% were observed in 22 samples, one of which showed no evidence of any V(D)J clonal expansion (thus making the 92 samples in which we find a clonal *IGH* rearrangement; Table 1).

With knowledge of the index *IGH* CDR3 sequences of the patient's clonal population in the pretreatment samples (Fig. 1A), we examined the potential for HTS to identify the same sequence at day 29 after treatment and compared these findings with results obtained by multiparametric flow cytometry (mpFC) performed as part of the COG trial (Fig. 1B). In those patients with biallelic *IGH* rearrangements, quantified MRD for the two alleles was highly correlated (Fig. 2). HTS to detect the patient's original clonal *IGH* sequences in day 29 post-treatment samples revealed three subgroups of patients in this cohort: (i) those for whom MRD was not detected by either HTS or mpFC, 40 cases; (ii) those for whom MRD was detected only by HTS but not mpFC, 28 cases; and (iii) those in which MRD was detected by both HTS and mpFC, 23 cases (Fig. 1B). Importantly, there were no cases (false-negative results) for which

MRD was detected only by mpFC, but not by HTS of *IGH*. For the 28 cases for which HTS did and mpFC did not detect MRD, the MRD was, on average, 10- to 100-fold lower than for the 23 cases for which both mpFC and HTS detected MRD (median,  $8.0 \times 10^{-6}$  vs.  $1.4 \times 10^{-4}$ ;  $P = 7.0 \times 10^{-5}$  by two-tailed *U* test; Fig. 1B). As a conservative estimate, there was approximately a 10-fold increase in the lower detection limit for MRD detection by HTS as compared with mpFC. Tracking of *IGH* clones could be improved by following both rearranged alleles in tumors that had evidence of biallelic rearrangement (Fig. 2). However, for the purposes of subsequent comparisons between flow cytometry and sequencing, tracking of only a single dominant sequence was performed.

As previously reported (11, 12), we also find widespread evidence of ongoing clonal evolution of *IGH* in these B-ALL samples, both in the pre- and MRD-positive post-treatment (Table 2). In post-treatment samples, the predominant clone in pretreatment samples was typically the most frequent clone. Clones consistent with V<sub>H</sub> replacement were found in 19 patients. In 1 patient, the only MRD detected was a single clone consistent with V<sub>H</sub> replacement at a level of approximately 1 in 1,000,000. The clinical significance of this low-level MRD is unclear. In the other 18 post-treatment MRD-positive cases with evidence of V<sub>H</sub> replacement, the dominant clone identified before treatment was also dominant after treatment. On average, 3.5% of total *IGH* rearrangements matched the dominant clone after treatment, whereas only 0.036% of *IGH* rearrangements were consistent with V<sub>H</sub> replacement of the major clone. Among pretreatment samples in which V<sub>H</sub>-replaced clones were detected, all V<sub>H</sub>-replaced clones together were 0.4% as large as the dominant clone on average (median). Among post-treatment samples, V<sub>H</sub>-replaced clones were on average 0.6% as large as the dominant clone. These trends are illustrated for a particular patient with MRD and a large number of V<sub>H</sub>-replaced



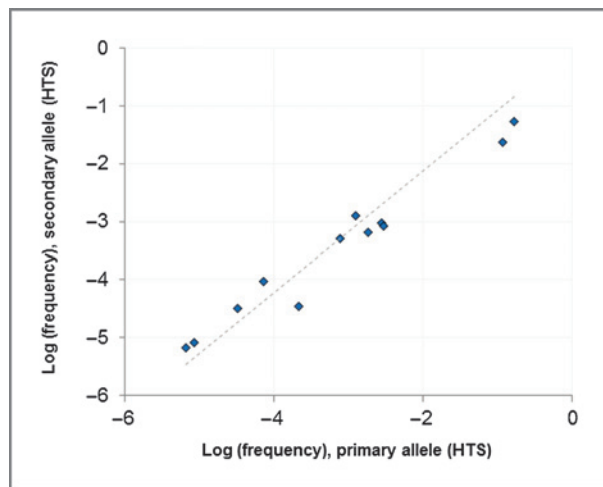
**Figure 1.** Pre- and day 29 post-treatment B lymphoblast frequencies by HTS versus mpFC pretreatment and day 29 post-treatment, clonal B lymphoblasts were identified by HTS (red) or by mpFC (blue;  $N = 91$ ). HTS data are reported as the frequency of the clonal sequence of total rearranged *IGH* sequences; mpFC data are reported as the percentage of total nucleated cells by mpFC. Cases without a complete *IGH* gene rearrangement in pretreatment samples ( $n = 7$ ) are not shown. A, HTS identifies pretreatment, clonal *IGH* sequences in 91 of 98 patients. B, HTS of *IGH* identifies post-treatment MRD detected by flow cytometry and in an additional subset of patients for whom flow cytometry analysis was negative. Day 29, post-treatment samples ( $n = 91$ ) include only those cases with complete V-J rearrangement and exclude 1 case with only a D-J rearrangement and 5 cases for which no clonal *IGH* rearrangement was seen. Three subsets of cases are identified in post-treatment samples (left to right): (i) MRD not detected by either HTS or mpFC, (ii) MRD detected by HTS, but not by mpFC, and (iii) MRD detected by both HTS and mpFC.

clonotypes in Supplementary Fig. S5. Taken together, these data indicate that generally there is little change in the relative proportions of the dominant clone and  $V_H$ -replaced subclones through induction chemotherapy.

To evaluate the specificity of HTS for assessment of MRD, we evaluated the frequency of the specific clonal *IGH* sequence from each patient being present in the other 97 patient post-treatment samples at day 29. This estimate provides an assessment of the relative likelihood after treatment that B cells may by chance have the exact same clonal index *IGH* sequence. Prior analysis has reported that nonspecific amplification of *IGH* rearrangements in B-ALL may be dependent on the time point after therapy and also the gene segment and therefore the target rearrangement being considered (18). In our cohort, at the level of 1 cell in 100,000, nine shared sequences of 8,918 *IGH* comparisons (0.10%) in cross-patient comparisons were detected. This rate is slightly higher (0.72%) when clones at a frequency of 1 cell in 1,000,000 are considered. Of note, the level of these coincidental clones was lower by approximately one order of magnitude on average as compared with true

MRD positives (i.e., index sequences found in the corresponding post-treatment samples, and not in cross-patient samples).

Of note then, 52 of 91 unselected patients had MRD clones at the level of 1 cell in a million or higher that was detectable by HTS of *IGH* alone, compared with only 23 cases of MRD positivity by flow cytometry (Fig. 1B). As many of the samples that were MRD negative by flow cytometry and MRD positive by HTS had levels of MRD within the expected range of detection of flow cytometry ( $\sim 1$  in 10,000 to 100,000), it is possible that flow cytometry may be missing relatively high-level MRD in a subset of patients. This could be due to post-treatment changes in the antigen expression resulting in immunophenotypic normalization (19). We, therefore, sought to determine if the detection of clonal sequences matching the original diagnostic samples could be derived from the mature B-cell fraction of samples. To test this hypothesis, we analyzed 10 additional, paired consecutive pre- and post-treatment B-ALL MRD samples for which residual material was available, and for which there was no evidence of MRD as determined by flow cytometry. This sample size was chosen



**Figure 2.** Comparison of clonal frequency of MRD in cases with presumed biallelic rearrangement of *IGH* versus mpFC. In samples with biallelic rearrangement, tracking of both alleles provides enhanced specificity for quantification of MRD. A total of 26 diagnostic samples generated clear evidence for biallelic rearrangements (exactly two dominant *IGH* sequences): in these samples, the frequency of each allele was tracked in post-treatment samples. In 10 samples, neither allele was observed after treatment. In 4 samples, only one allele was observed after treatment (at an average frequency of  $1.2 \times 10^{-5}$ ). In the remaining 12 samples, both alleles were observed after treatment. The frequencies of the dominant and secondary alleles in post-treatment samples are charted. Post-treatment allele frequencies in biallelic samples are highly correlated ( $r^2 = 0.97$ ).

because approximately 15% to 20% of post-treatment samples were MRD negative by flow cytometry, yet had MRD detected by HTS within the sensitivity of flow cytometry (Fig. 1B). We triple flow cytometry–sorted the mature B-cell fraction from the post-treatment marrow aspirate samples to isolate "mature" B cells and then sequenced these fractions to define the B-cell repertoire in these post-treatment samples, while subsequently sequencing the paired, triple-sorted pretreatment samples to minimize the possibility of contamination of the post-treatment samples by the pretreatment ones. Eight samples had sufficient DNA after flow cytometry sorting for subsequent DNA sequencing. Whereas 7 of 8 post-treatment samples showed no evidence of clonal *IGH* sequence overlap between the post-treatment, flow cytometry–sorted mature B-cell fractions, and the pretreatment diagnostic samples, there was one case in which the pretreatment *IGH* V–J sequence was found within the mature B-cell compartment, consistent with the concept of immunophenotypic normalization. The level of this sequence was 0.01% of total cells. By flow cytometry, this leukemia clearly had an abnormal antigenic phenotype at diagnosis (Supplementary Fig. S6A), with aberrant immunophenotypic expression of CD10 (increased), CD38 (decreased to absent), CD45 (decreased), and CD58 (slightly increased). This aberrant immunophenotype was not evident in the post-treatment, day 29 sample (Supplementary Fig. S6B), from which the mature B-cell fraction was sorted.

## Discussion

Accurate risk stratification is important for the personalized treatment of patients with B-lineage acute lymphoblastic leukemia (2–5). Recent clinical studies have confirmed the importance of MRD in guiding patient care in both pediatric and adult B-ALL (9, 20). However, widespread implementation of diagnostic assays to accurately stratify patient risk in the clinical laboratory has been challenging. Each existing method of analyzing MRD, such as flow cytometry or qPCR, has its own drawbacks and technical challenges. Here, using a cohort of patient samples submitted to our institution for flow cytometric analysis of MRD, we confirm the broad potential of sequencing of *IGH* to permit highly specific molecular detection of clonality in nearly all-comers (91 of 98, ~93%) without the laborious requirement of individualized PCR or allele-specific oligonucleotide design, implicit in current molecular-based approaches for monitoring MRD (8, 21).

Although conventional practice has focused on identifying and following at least two clonal molecular markers for MRD detection (22), recent data from the AIEOP-BFM ALL 2000 study of pediatric and adolescent B-ALL suggests that patients with only one marker can be similarly stratified as they have outcome results quite similar to those patients followed by two markers (20). Specifically, of 3,184 patients, 454 had only one marker and event-free survival (EFS) results in this subset of patients was 5-year EFS of 94.1% for standard-risk (vs. 92.3% EFS for two markers) or 83.3% for intermediate-risk (77.6% for 2 markers; ref. 20). Thus, the ability to detect clonal *IGH* rearrangements in a large proportion of patients in our unselected cohort of B-ALL patients suggests that HTS of *IGH* should be, at the minimum, a viable strategy for MRD detection in many patients with B-ALL. Moreover, our data further support the analysis for both complete (VDJ) and incomplete (DJ) *IGH* rearrangements in B-ALL for MRD assessment, and highlight the potential utility of this approach to improve accuracy of quantification of MRD (Fig. 2). In addition, in patients with biallelic *IGH* rearrangements, tracking of both alleles can help to confirm the detection of MRD.

Prior work by Gawad and colleagues using next-generation sequencing has identified remarkable diversity in pretreatment diagnostic samples due to ongoing clonal evolution of *IGH*, with some tumors being composed of thousands of apparent subclones with respect to *IGH* and that seem to be derived from an ancestral clone due to a  $V_H$  replacement model (11, 12) or alternately, ongoing V(D)J recombination of a clone with incomplete DJ recombination. We similarly identify herein the evidence of ongoing recombination of *IGH* in pretreatment samples. However, in contrast with analysis by Gawad and colleagues, who did not evaluate the impact of this on MRD detection (12), we show here that clonal diversity as defined by evolved *IGH* clonotypes does not substantively change in day 29 post-treatment samples. Furthermore, in a MRD-positive, post-treatment sample, the dominant clone found at relapse by sequencing was identical to the dominant clone seen at

**Table 2.** Summary evolved clonotypes in pretreatment clonal *IGH* CDR3 sequences

	<b>N (%)</b>	<b>Mean, median (range)</b>
No VDJ clone	7/98 (7.1%)	—
Pretreatment VDJ clone identified	91/98 (92.9%)	—
No evidence of evolved clonotypes	6/91 (6.6%)	—
Evidence of evolved clonotypes	85/91 (93.4%)	—
Number of evolved clonotypes		Mean, 195; median, 22 (range, 1–1,953)
Proportion of evolved clonotypes of total clone		Mean, 3.4%; median, 0.4%; (range, 0.0010%–59.8%)

NOTE: Clonal *IGH* CDR3 sequences were identified in nearly all patients as either complete V–DJ or incomplete D–J rearrangements. Among the 91 patients in which a complete V–DJ rearrangement was observed, the number of clones due to ongoing *IGH* evolution by  $V_H$  replacement at diagnosis is indicated, as well as the proportion of total clone size (major clone plus all evolved clonotypes) accounted for by these  $V_H$ -replaced clones. We used the definition of an evolved *IGH* clonotype initially proposed by Gawad *et al.* (12).

diagnosis, and the relative proportions of the dominant clone versus the subclones were similar in pre- and post-treatment samples. These findings together suggest that within this early time frame, ongoing rearrangement at the *IGH* locus does not substantially alter the fitness of lymphoblast clones during induction chemotherapy. Additional studies at subsequent time points will need to be performed to further document how *IGH* repertoire may change over time, as several studies have shown that late relapsed clones can often be retrospectively identified in pretreatment samples (23). Nevertheless, our data provide the first picture of clonal *IGH* evolution through induction chemotherapy.

Similar to our findings in T-ALL (10), HTS can identify MRD in a subset of cases for which flow cytometry evaluation is negative. Indeed, in this cohort, 5 cases had MRD detected by HTS at a level of >1 cell in 10,000 that should have been detected by flow cytometry (Fig. 1B). To consider the possibility that these detected *IGH* sequences may be derived from cells with an altered immunophenotype, we examined an additional subset of eight cases that had detectable MRD by next-generation sequencing, but were negative by flow cytometry. Of these, 1 of the 8 patients had a clonal MRD sequence identified in the post-treatment sample that matched the patient's index clone and was found within a triple flow cytometry–sorted B-cell fraction that appears to be immunophenotypically mature. Our finding of rare index *IGH* sequences within the mature B-cell fraction raises the possibility that in some patients, the clonal lymphoblast population detected by sequencing may undergo substantial antigenic maturation during therapy. We do not believe that this finding is artifact due to contamination or detection of cell-free *IGH* sequences in plasma as shown for some mature aggressive B-cell lymphomas (24) or residual DNA associated with dead B lymphoblasts as shown in rare instances in which PCR detects MRD but not flow cytometry (25), as we minimized these possibilities by performing triple flow cytometry sorting and sequencing of the post-treatment sample before analysis of the pretreatment sample in all cases. Furthermore, we do not believe this to be coincidental identification of a clonally unrelated,

reactive B-cell population with identical VDJ recombination of *IGH*, because in our analysis of post-treatment samples in our cohort, we did not detect coincidental clonal *IGH* sequences at the level that was seen in this patient at approximately 0.01%, even among the very rare examples of shared sequences. Our finding of antigenic shift is similar to that reported by Gawad and colleagues who showed in their diagnostic pediatric B-ALL samples that a portion of evolved clones may have substantial antigenic differences as compared with the index clone (12). Given that the malignant potential of a clone such as this with antigenic maturation is not known, its clinical import is undetermined despite its relatively high frequency in this patient.

The patient samples considered in this study were derived from the COG AALL0932 trial, from patients who were regarded as being generally low- to standard-risk for relapse. However, as shown in our findings, (Fig. 1B), many of these cases (51 of 92) have clonal sequences detectable by HTS but are flow cytometry negative. Although the clinical significance of these MRD levels below current diagnostic threshold of <0.01% is outside the scope of this study, the significance of these low-level sequences requires further study. It is likely that MRD positivity by sequencing will be clinically important for a subset of patients. Several studies support this claim. First, Paganin and colleagues show that detectable, but not quantifiable MRD below the level of  $<10^{-4}$  had an intermediate EFS of 45% at 3 years versus patients who had MRD results that were either outright negative or outright positive at a level  $\geq 10^{-4}$  (26). The EFS for these patients were 73% and 19% for negative versus positive MRD, respectively. Second, Stow and colleagues showed in a study involving 455 clinical samples and using PCR analysis of MRD at one time point (day 46, at end of remission induction) that levels below the conventional clinical threshold of 0.01% were important for predicting relapse (27). In this work, patients in whom PCR was able to detect very low levels of MRD (0.001% to <0.01%) had a 12.7% ( $\pm 5.1\%$ ; SE) cumulative risk of relapse at 5 years compared with 5% ( $\pm 1.5\%$ ) for those below the MRD level of 0.01% ( $P < 0.047$ ). Stow and colleagues (27) conclude that MRD positivity below the standard threshold of 0.01%

has prognostic significance for some patients, and that patients with low-level positivity by PCR should be closely monitored. Third, Pulsipher and colleagues (28) reported data showing that patients with pretransplant detectable MRD by deep sequencing of *IGH* had a significantly increased cumulative incidence of relapse when compared with those patients with no evidence of disease by sequencing (57% versus 4.4%;  $P = 0.008$ ) in a cohort of 64 COG patients who underwent total body irradiation-based myeloablative transplant for high-risk ALL in CR1 or CR2. Furthermore, although patients from this same cohort who were MRD negative based on flow cytometry had a cumulative risk of relapse of nearly 25%, by comparison, patients who were MRD negative by deep sequencing of *IGH* using the identical platform/assay described herein had a cumulative risk of relapse of less than 5%. Finally, recent work comparing quantitative PCR-based methods versus flow cytometry for MRD detection in a cohort of 1,547 children with acute lymphoblastic leukemia enrolled into the AIEOP-BFM ALL 2000 trial has suggested that the outcome of patients with discordant MRD results between PCR-based methods and flow cytometry is likely to be different than for those cases for which MRD results are concordant between the two approaches (25). Therefore, low-level MRD detected by sequencing is likely to be meaningful for a subset of patients and suggests that these patients, at the very least, should be closely monitored.

Indeed, the increased sensitivity of MRD determination by HTS offers the opportunity to clinically assess for trends in MRD over time at time points when the level of disease may be beneath the detection limit of flow cytometry. Prior work has suggested that in patients with B-ALL, there is an inverse correlation between the level of MRD detected versus time to late relapse (29). Data from the AIEOP-BFM ALL 2000 study further support these findings, as patients with high MRD levels at day 33, but no MRD detected by molecular analysis at day 78 had a decreased 5-year cumulative relapse incidence of 20.7% versus 40.7% in patients who were positive for MRD at day 78, but had persistent MRD at a level less than  $10^{-3}$ . Additional work suggests that oftentimes, the clone that represents late relapse may be identified retrospectively in the index sample (20, 23, 29). Our studies demonstrate robust analytic ability to quantify *IGH* rearrangements (Supplementary Fig. S3).

In summary, we demonstrate the utility of HTS of *IGH* genes for the detection of MRD in B-ALL. Despite ongoing recombination of *IGH*, the analysis of *IGH* gene rearrangement permits robust identification of the neoplastic lymphoblast population in a high proportion of unselected cases. For cases in which no clonal *IGH* gene rearrangement was identified, evaluation of other loci, such as *IGK*, *TRB*, *TRG*, and *TRD* genes may be helpful, as previously suggested by others and Faham and colleagues (11). Because HTS could greatly reduce the complexity associated with patient-specific molecular analysis of MRD and improve on observer variation in flow cytometry, it offers the potential to improve clinical prognostication in B-ALL. Further evalua-

tion of sequencing-based MRD detection, however, must be confirmed in subsequent studies involving independent laboratories. However, the synthetic *IGH* templates that we use herein to ensure accurate and unbiased, multiplex PCR could be one approach to ensure standardized assay performance. Clinical laboratories adopting sequencing for MRD could use this synthetic immune system to confirm consistent test performance, permitting broad implementation of this technology.

In addition, it will be important to understand the clinical implications of cases in which relatively higher level of MRD was missed by flow cytometry but detected by sequencing. Although a limited observation, we show that clonal *IGH* index sequences in some patients can be identified within the immunophenotypically apparent mature B-cell subset, suggesting that some B lymphoblast clones may undergo antigenic drift and/or maturation. The clinical significance of this finding merits further study and is underway.

#### Disclosure of Potential Conflicts of Interest

D. Wu reports receiving a commercial research grant from Adaptive Biotechnologies. R.O. Emerson, A. Sherwood, B. Howie, and I. Kirsch are employees of and have ownership interests (including patents) in Adaptive Biotechnologies. M. Rieder has ownership interest (including patents) in Adaptive Biotechnologies. C. Carlson and H. Robins have ownership interests (including patents) and are consultants/advisory board members for Adaptive Biotechnologies. No potential conflicts of interest were disclosed by the other authors.

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