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Brief Articles

Next-Generation Sequencing in Adult B Cell Acute Lymphoblastic Leukemia Patients

Olga Sala Torra [1,*], Megan Othus [2,3], David W. Williamson [4], Brent Wood [5], Ilan Kirsch [4], Harlan Robins [2], Lan Beppu [1], Margaret R. O'Donnell [6], Stephen J. Forman [6], Frederick R. Appelbaum [1], Jerald P. Radich [1]

[1] *Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, Washington*
[2] *Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington*
[3] *SWOG Statistical Center, Seattle, Washington*
[4] *Adaptive Biotechnologies, Seattle, Washington*
[5] *Department of Laboratory Medicine, University of Washington, Seattle, Washington*
[6] *Department of Hematology & Hematopoietic Cell Transplantation, City of Hope Medical Center, Duarte, California*

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We used next-generation sequencing (NGS) of the immunoglobulin genes to evaluate residual disease in 153 specimens from 32 patients with adult B cell acute lymphoblastic leukemia enrolled in a single multicenter study. The sequencing results were compared with multiparameter flow cytometry (MFC) data in 66 specimens (25 patients) analyzed by both methods. There was a strong concordance (82%) between the methods in the qualitative determination of the presence of disease. However, in 17% of cases, leukemia was detected by sequencing but not by MFC. In 54 bone marrow (BM) and peripheral blood (PB) paired specimens, the burden of leukemia detected by NGS was lower in PB than in BM, although it was still detectable in 68% of the 28 paired specimens with positive BM. Lastly, patients without disease detected by NGS or MFC had a 5-year relapse free survival of > 80%. The results suggest that residual disease detection by immunoglobulin gene sequencing is an extremely sensitive technique and may identify patients that might benefit from transplantation. Moreover, the increased sensitivity of the method may allow frequent peripheral blood testing to supplement marrow sampling to measure disease response.

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INTRODUCTION

The detection of measurable residual disease (MRD) is an important marker of an increased risk of relapse in pediatric and adult acute lymphoblastic leukemia (ALL). MRD is associated with higher relapse rate and worse event- or relapse-free survival after conventional therapy or allogeneic transplantation [1-7]. United States' and European pediatric studies perform risk stratification based on MRD level to modify therapy in cases with elevated MRD and/or decrease therapy in the absence of MRD [2-4]. The use of MRD in adult ALL has followed pediatric studies and similar clinical studies are being conducted in adult patients with ALL [1,4,8,9].

MRD in ALL is generally measured either by multiparametric flow cytometry (MFC), polymerase chain reaction (PCR) of the IgH VDJ and/or TCR gene rearrangements or leukemia-specific fusion transcripts (eg, BCR-ABL in Philadelphia chromosome–positive ALL). MFC assesses the expression of multiple antigens at a single-cell level using the enumeration of a discrete population with an aberrant immunophenotype as the readout of the assay. The sensitivity of MFC is approximately 10^{-4} (that is, 1 ALL blast in 10^4 normal cells). The main advantage of MFC is speed; relative ease and cost-effectiveness of the procedure and its broad applicability are also advantages. Disadvantages of MFC include a current lack of standardization across testing sites and different intersample sensitivity, depending on the specific abnormal immunophenotype of the leukemic population and the number of normal cells of similar type in the sample. Quantitative PCR of allele-specific IgH VDJ rearrangements is a highly sensitive method of detection of MRD (~10^{-4} to 10^{-5}). It is, however, labor intensive and costly, as the MRD

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* Correspondence and reprint requests: Olga Sala Torra, MD, Clinical Research Division, Fred Hutchinson Cancer Research Center, D4-100 1100 Fairview Ave N, Seattle, WA 98109
E-mail address: osala@fredhutch.org (O. Sala Torra).

assay requires the characterization of leukemia-specific Ig/TCR gene rearrangement for each patient, as well as specific assay design and optimization for each patient. MRD levels between molecular and immuno-phenotypic approaches are highly correlated [10-14].

In chronic myeloid leukemia, standardized molecular monitoring of BCR-ABL chimeric mRNA has revolutionized study design, as several clinical trials use MRD as a surrogate endpoint of outcome in pivotal drug trials [15,16]. In ALL, MRD could play a similar role in radically changing treatment strategies and allowing for rapid drug development. This will require reproducible, sensitive, and scalable (ie, available in many settings) MRD detection methods. Recently, methods that combine multiplex PCR of the IgH VDJ sequences with "next-generation" sequencing (NGS) to quantitatively and sensitively measure MRD in lymphoid malignancies have been developed [17-20]. In this study we compare MFC and NGS in adult ALL patients studied in a single clinical trial.

METHODS

Patients and Specimens

Cryopreserved diagnostic and follow-up peripheral blood (PB, n = 108) and bone marrow samples (BM, n = 89) from 32 adult patients with ALL treated on SWOG S0333 were used for this study. Forty-four specimens were collected at study registration and 153 were collected for MRD analysis per protocol schedule or suspicion of relapse. The median number of follow-up specimen time points per patient was 3 (range, 1 to 5). Flow cytometry data were available for 66 of the follow-up specimens.

S0333 was a phase II study of double-induction combination chemotherapy to treat newly diagnosed adult ALL. Total accrual for the study was 78 patients, and the median age 42 years (range, 18 to 64 years). The median age of the patients included was 34 years (range, 18 to 64 years) and the median WBC 22.6×10^9/L (range, 1.1×10^9/L to 219.1×10^9/L). All patients gave written informed consent to the banking of specimens and their use in research protocols.

NGS and Data Analysis

Pretreatment specimens were analyzed to identify the clonal leukemic sequence of the VDJ or DJ fragment(s). Clonal sequences representing more than 5% of the total reads were considered leukemic (following guidelines for conventional diagnostic techniques), also considering the distribution of sequence frequencies across the entire repertoire of IgH molecules profiled in the sample. Follow-up specimens were subsequently sequenced and the presence of the leukemic marker sequence(s) previously identified in the diagnostic sample was searched for explicitly.

DNA was extracted from cryopreserved WBC, using the Qiagen DNeasy kit (Hilden, Germany). Amplification and sequencing of the IgH complementarity determining region 3 was performed using the clonoSEQ assay (Adaptive Biotechnologies, Seattle, WA, www.clonoSeq.com), starting with 6 μg to 7 μg of input DNA, yielding limit of detection of approximately 1 leukemic cell in a background of 1 million nucleated cells. Calculation of the frequency of clonal sequences and the level of MRD were as previously described [17,21,22]. Sequencing analysis was performed blinded to the MFC result.

MFC

MFC was performed on fresh specimens as described previously, using the antibody combination of CD20 FITC, CD10 PE, CD34 PerCP-Cy5.5, CD38 A594, CD19 PE-Cy7, CD58 APC, and CD45 APC-H7. Cells with surface marker patterns that differed from normal B cell maturation were defined and quantified as MRD, as previously described [23].

Statistical Methods

Overall survival (OS) and relapse-free survival (RFS) were measured from the date of registration for second induction until death from any cause or date of first relapse or death respectively, with patients last known to be alive (and in complete remission for RFS) censored at the date of last contact. Concordance between MRD-positive/negative status was tabulated and, between quantitative measurements, was visually summarized with scatterplots. OS and RFS were estimated using the Kaplan-Meier method and *P* values were calculated using log-rank tests.

RESULTS

NGS Marker Identification

At least 1 Ig clonal sequence was identified in pretreatment specimens from 29 of 32 (91%) of cases analyzed. The 3 patients lacking a detectable clonal sequence in pretreatment material were excluded from further analysis (in all 3 cases only PB specimen at diagnostic was available). In the remaining 29 cases, the leukemic clonal sequence was a complete VDJ rearrangement in 17 of 29 patients (59%), an incomplete DJ rearrangement in 8 of 29 patients (28%), and in 3 of 29 (10%) cases, both VDJ and DJ sequences were present. One patient had a light chain rearrangement (kappa). Seventeen of 29 (59%) cases contained more than 1 IgH clonal rearrangement at diagnosis (median, 2; range, 1 to 4).

Comparison of Disease Detection Determined by NGS and MFC

A total of 66 follow-up specimens (61 BM and 5 PB) had data from both MFC and NGS. The determination of the presence (or absence) of leukemia was concordant in 54 of 66 (82%) of samples. MRD was detected by sequencing in 11 specimens that were negative by MFC and 1 sample was positive by MFC, but no MRD was detected by NGS (flow level of MRD, .002%). Figure 1A shows the correlation of leukemic burden detected by NGS and MFC in BM specimens.

Comparison of Disease Detection by NGS in Matching BM and PB Specimens

Leukemia was detected in 33 of 68 (49%) of the BM samples and in 30 of 69 (43%) of the PB samples. There were 54 paired samples of BM and PB (Figure 2). Twenty-five pairs (46%) showed no detectable MRD in either specimen and 19 (35%) had leukemia detected in both specimens. In the concordant-positive pairs, the leukemic clone in BM was 6-fold higher than in PB (range, .39 to 821-fold; Figure 1B).

Nine pairs of samples (17%) had disease detectable in BM but not in PB by NGS. In 6 of them, BM MRD by MFC was negative, and for the 3 remaining pairs, MRD was detected at low levels by MFC (.003%, .004%, and .013%). One pair had disease detectable only in PB. In this case, MRD was not detected by either MFC or NGS of the marrow sample.

A graphic view of the correlation between the levels of disease in BM and PB is presented in Figure 1B, where the subset of 24 BM specimens with positive disease detected by NGS and coexisting MFC data are represented by shapes, according to the presence or absence of disease in the corresponding PB specimen.

MRD Levels and Outcome

OS and RFS of patients from second induction therapy (a protocol specified time-point for sample collection) was analyzed (Figure 3A,B). Twenty-one patients had specimens (BM or PB) available for analysis and all of them had less than 5% blasts by morphology. The median time from registration at first induction to registration at second induction was 41 days (range, 35 to 75 days).

Patients who were MRD negative by NGS and MFC had excellent OS and RFS of above 80%, whereas patients positive by both NGS and MFC have the poorest outcome ($P = .0049$ and $P = .003$ for OS and RFS, respectively). Patients who were NGS positive but MFC negative had an intermediate outcome ($P = .028$ and $P = .04$ for OS and RFS, respectively, when compared with patients with MRD positive by both MFC and NGS).

Only 1 of 7 (14%) patients with MRD negative by NGS relapsed, in contrast to 5 of 12 (42%) patients who were MRD

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