Molecular Measurable Residual Disease Testing of Blood During AML Cytotoxic Therapy for Early Prediction of Clinical Response

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Measurable residual disease (MRD) testing after initial chemotherapy treatment can predict relapse and survival in acute myeloid leukemia (AML). However, it has not been established if repeat molecular or genetic testing during chemotherapy can offer information regarding the chemotherapy sensitivity of the leukemic clone. Blood from 45 adult AML patients at day 1 and 4 of induction (n = 35) or salvage (n = 10) cytotoxic chemotherapy was collected for both quantitative real-time PCR (qPCR) assessment (WT1) and next generation sequencing (>500 × depth) of 49 gene regions recurrently mutated in MDS/AML. The median age of subjects was 62 (23–78); 42% achieved a complete response. WT1 was overexpressed in most patients tested but was uninformative for very early MRD assessment. A median of 4 non-synonymous variants (range 0–7) were detected by DNA sequencing of blood on day 1 of therapy [median variant allele frequency (VAF): 29%]. Only two patients had no variants detectable. All mutations remained detectable in blood on day 4 of intensive chemotherapy and remarkably the ratio of mutated to wild-type sequence was often maintained. This phenomenon was not limited to variants in DNMT3A, TET2, and ASXL1. The kinetics of NPM1 and TP53 variant burden early during chemotherapy appeared to be exceptions and exhibited consistent trends in this cohort. In summary, molecular testing of blood on day 4 of chemotherapy is not predictive of clinical response to cytotoxic induction therapy in AML. The observed stability in variant allele frequency suggests that cytotoxic therapy may have a limited therapeutic index for clones circulating in blood containing these mutations. Further validation is required to confirm the utility of monitoring NPM1 and TP53 kinetics in blood during cytotoxic therapy.

Keywords: acute myeloid leukemia (AML), MRD, remission, measurable residual disease (MRD), WT1 = Wilms tumor 1, somatic mutations in cancer, Next Gen Sequencing (NGS)
INTRODUCTION

The use of high sensitivity techniques to measure residual leukemic burden in patients achieving a complete remission by cytomorphological criteria is increasingly considered part of the standard of care for acute myeloid leukemia (AML) (1–4). While testing for measurable residual disease (MRD) in AML is typically performed using multi-parameter flow cytometry (MPFC) or real-time quantitative PCR (qPCR) there is increasing recent research interest in the potential of sequencing-based approaches (3–14). The results of MRD testing in AML appear prognostic when measured at key landmark timepoints following initial therapy, typically after 1–2 cycles of induction therapy or before allogeneic transplantation (15–21). It is currently not known if testing changes in residual leukemic burden at earlier timepoints, for example during initial induction therapy, would have clinical utility.

We used two independent molecular techniques for AML MRD quantification, WT1 expression by qPCR and targeted DNA sequencing for common MDS/AML variants. The Wilms tumor gene WT1 is expressed in approximately 90% of cases of AML and has been extensively tested and standardized as a method of MRD detection in AML (22, 23). The utility of WT1 testing is limited to a subset of AML MRD cases and more recently the quantitative assessment by DNA sequencing of variants in genes known to be recurrently mutated in myeloid malignancies has been proposed as a more broadly applicable measure of AML MRD (6, 9, 14). We used both these molecular techniques to determine if early assessment of blood from AML patients during the first 4 days of intensive cytotoxic therapy can predict subsequent clinical response.

METHODS

Patients and Sample Collection

Blood was collected daily from day 1 through at least day 5 of intensive cytotoxic therapy from 45 adult AML patients with a median age of 62 years-old (range: 23–78) following informed consent on IRB-approved protocols (Figure 1 and Table 1, Tables S1, S2).

Ten patients (median age: 52, range: 23–66) had relapsed or refractory AML (RR-AML) and were recruited to the National Institutes of Health to receive salvage chemotherapy (NCT02527447). Nine of these patients were treated with National Heart, Lung, and Blood Institute (NHLBI) at the National Institutes of Health and high dose cytarabine (Table S2). While testing for measurable residual disease (MRD) in AML is typically performed using multi-parameter flow cytometry (MPFC) or real-time quantitative PCR (qPCR) there is increasing recent research interest in the potential of sequencing-based approaches (3–14). The results of MRD testing in AML appear prognostic when measured at key landmark timepoints following initial therapy, typically after 1–2 cycles of induction therapy or before allogeneic transplantation (15–21). It is currently not known if testing changes in residual leukemic burden at earlier timepoints, for example during initial induction therapy, would have clinical utility.

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Blood was also collected from healthy adult donor subjects following informed consent on an IRB-approved protocol.

Nucleic Acid Extraction From Blood and Marrow

Samples were processed with NucleoSpin Blood QuickPure kits (Machery-Nagel) and NucleoSpin RNA Blood kits (Machery-Nagel), as per manufacturer's instructions (NIH) or stored in PAXgene Blood/Marrow RNA tubes (PreAnalytiX) at Duke and shipped frozen to the NIH. Upon thawing PAXgene tubes, 2 mL for gDNA isolation were pelleted and resuspended in PBS then processed with the Qiagen Blood QuickPure kits (Machery-Nagel) and NucleoSpin RNA Blood kits (Machery-Nagel). It is currently not known if testing changes in residual leukemic burden at earlier timepoints, for example during initial induction therapy, would have clinical utility.

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Real-Time Quantitative PCR (qPCR)

RNA (130–260 ng) was reverse transcribed using the RT² First Strand kit (330404, QIAGEN). When necessary, RNA was concentrated with a Savant SVC-100H centrifugal evaporator. Resultant cDNA was loaded into a Custom RT² Profiler PCR Array containing lyophilized qPCR primers for WT1 and ABL1 using the QIAGen (QIAGEN) qPCR was performed (hold 2 min at 50°C, hold 10 min at 95°C, then 50 cycles of 15 s at 95°C and 60 s at 60°C) on the Rotor-Gene Q Platform (QIAGEN) and Ct values were collected with a threshold of 0.06. Healthy levels of WT1 expression were established based on upper limit observed in blood of 34 healthy donors.

DNA Sequencing Using a Myeloid Panel During Chemotherapy

A total of 49 gene or gene regions recurrently mutated in MDS and/or AML were sequenced using amplicon-based targeted DNA sequencing (RainDance, Billerica, MA). This
included genes with functions in methylation, chromatin-cohesin, signaling, transcription as well as TP53, NPM1, and others (see Table S3). Libraries were prepared from 100 ng of gDNA and paired-end 300 bp sequencing was performed on the MiSeq instrument (Illumina), as per manufacturer’s instructions. Results were analyzed using the NextGENe v2.4.2.1 software (SoftGenetics, PA). Sequences were aligned to human genome build v37 (hg19). Non-coding and synonymous variants, along with known sequencing artifacts and regions with <500-fold coverage were removed. Remaining variants (i.e., missense, in-frame, frameshift, and non-sense mutations) with variant allele frequencies (VAFs) above 5% at either day 1 or 4 were considered in subsequent analyses.

**FIGURE 1** | Cohort of 45 patients. Our patient cohort consisted of 10 patients who received salvage chemotherapy at NHLBI, NIH (NCT02527447) and 35 newly diagnosed patients who received induction chemotherapy at Duke University School of Medicine. Variants refers to the number of targets identified by DNA sequencing.

**FIGURE 2** | Kinetics of WT1 expression during chemotherapy is uninformative for clinical response. (A) Thresholds for WT1 overexpression in blood for this assay were established based on the upper limit observed by qPCR in 34 healthy donors and were consistent with previous reports (24). AML patient blood samples showed overexpressed WT1 levels in 31 (91%) on Day 1 and 23 (68%) at Day 4. (B) Patients achieving a complete remission after therapy had at least a 4-fold reduction of WT1 expression during therapy in 7 of 13 cases evaluated by qPCR (54%); 5/13 (38%) had <4-fold reduction; 1/13 (8%) had undetectable WT1 levels. (C) Non-responding patients had at least a 4-fold reduction of WT1 expression in 7 of 21 evaluated by qPCR (33%) NR; 14/21 (67%) had less than a 4-fold change. Two patients initially had WT1 levels that were not overexpressed, but became so by treatment day 4. qPCR, quantitative real-time PCR; Green, at least 4-fold decrease; Black, <4-fold change; Gray, undetectable; Gray box indicates healthy donor range.
Custom DNA Sequencing for MRD Tracking After Chemotherapy

In order to track variants in longitudinal blood samples in 2 patients, a custom DNA sequencing assay was designed (DB0188, VariantPlex, ArcherDX). Libraries were prepared from 400 ng gDNA; using paired-end 150 bp sequencing on MiSeq instrument (Illumina), as per manufacturer's instructions. Archer Analysis software version 5.1.3 was used for analysis.

Statistical Analysis

All statistical analyses were performed using Prism v7.02 (GraphPad Software, CA). The Wilcoxon signed-rank test was performed on paired D1/D4 VAFs. Additive (D4–D1, with denominator of D1 or D4) and multiplicative (D4/D1) differences between D1 and D4 were calculated, then the Wilcoxon rank-sum test was performed on these differences between CR/NR groups. The Chi-square test was performed based on whether the additive difference was positive or negative between CR and NR groups. The unpaired t-test was performed on relative expression levels of WT1. For all statistical tests, \( P < 0.05 \) was considered significant.

RESULTS

Patients and Sample Collection

Full demographics, risk classification, treatment and responses are listed in Table 1, Tables S1,S2. Overall, 19 of 45 patients achieved a complete remission (CR) after intensive therapy (42%). Average white blood count was 10 K/ul (range: 0.3–60) on day 1 decreasing to 2.3 K/ul (range: 0.2–26) by treatment day 4. Obtaining sufficient quantities of nucleic acid from blood is the limiting factor for molecular testing during these early time points of cytotoxic chemotherapy in AML. All 45 patients had sufficient DNA for sequencing but only 34/45 patients had enough RNA from paired day 1 and 4 samples for qPCR analysis. There was insufficient RNA and DNA yields from blood beyond day 4 in most patients (Figure S1).

WT1 Expression Level on Day 4 Is an Uninformative Biomarker of Clinical Response

WT1 gene expression (normalized to ABL1 expression) was determined in 34 patients with sufficient RNA for qPCR analysis isolated from blood on days 1 and 4 of treatment. Compared with the upper limit of expression observed in healthy donors (Figure 2A) 31 of 34 patients overexpressed WT1 on Day 1 (91%) consistent with prior reports (22, 23). By day 4 of induction therapy 23 patients had WT1 over-expression. 7 of 13 (54%) patients achieving a CR had at least a 4-fold reduction in WT1 expression, although notably 6 of these 7 patients remained overexpressed compared with healthy donors on day 4 (Figure 2B). Seven of 21 (33%) patients who were non-responders (NR) also had at least a 4-fold reduction in WT1
Two NR patients with undetectable WT1 on day 1 had low level expression on day 4 (Figure 2C). Overall, changes in WT1 expression in blood between day 1 and 4 of intensive cytotoxic chemotherapy for AML appear uninformative as a biomarker for clinical response in this cohort.

DNA Sequencing for Variants Associated With Myeloid Malignancy Pre-treatment

All patients had targeted DNA sequencing (Table S3) of blood taken on day 1 and 4 of chemotherapy. An average of 4 coding variants (range 0–7) were identified per patient, and only two patients had no variants suitable for disease tracking available (Figure 3). A total of 163 variants were found in 43 patients and some patients had multiple variants found within a single gene (considering multiple variants in one gene in the same patient as single event results in a total of 140 mutated genes). The most frequently mutated gene regions were consistent with those previously reported (25, 26) (see Figure 3, Figures 4A, B). There was no difference in the number of coding variants detectable at baseline in CR vs. NR patients (Figure 3). Variant allele frequencies (VAF) on day 1 were a median of 29% (range: 0.2–71%) with 75% having a VAF <40% (Figure 4C) on day 1. Consistent with current prognostic risk classifications (1), the TP53 and NPM1 mutation classes had predictive significance, with 9 of 9 patients with TP53 mutations not achieving remission while all 5 of 5 patients with NPM1 mutations achieved CR.

detectable in blood on day 1 of chemotherapy remained detectable in blood on day 4.

Variant allele frequencies (VAFs) on day 1 and 4 were compared based on the hypothesis that changes in detectable mutation burden in blood very early during intensive cytotoxic treatment may correlate with clinical response as later assessed by morphological examination of bone marrow at count recovery (i.e., approximately 30 days later). Day 1 and 4 VAFs, at both the genetic functional class level and individual gene level, were compared using the Wilcoxon Sign Test, for all patients and also for the subgroups achieving either CR or NR (Figures 5, 6). TP53 mutations were only detected in NR patients and showed a significant difference in VAF between days 1 and 4 ($P < 0.05$) with a mean increase of 34% ($n = 9$; range: −2 to 94%). NPM1 mutations were observed only in CR patients with a mean decrease of −44% ($n = 5$; range: −2 to −98%) ($P = 0.0625$). Furthermore, additional testing for 2-sample statistical significance of the additive and multiplicative differences was assessed between Day 1 and 4 VAFs at the genetic functional class level between the CR and NR patient groups, all of which were non-significant.

Somatic mutations in DNMT3A, TET2, and ASXL1 (referred to as “DTA” mutations) are commonly found in AML patients but are also seen in clinically asymptomatic individuals with increased prevalence with aging (27–30). Mutations in these genes are known to not be useful in measuring residual disease in AML (6, 12). Patients with and without DTA mutations were therefore analyzed separately (Figure 7). Overall, 27 patients (60%) had a DTA mutation, and this observation was consistent between the NHLBI relapsed/refractory (median age: 52) and Duke newly diagnosed (median age: 63) AML cohorts. DTA patients expressed significantly lower levels of WT1 than non-DTA patients (median dCT of 4.8 compared to 3.1 $P < 0.05$) but had greater decrease in WT1 levels by day 4 (median dCT 7.6 vs. 3.6, $P < 0.05$). 48% of DTA patients achieved CR compared
FIGURE 5 | Changes in variant frequency during chemotherapy. All variants identified by targeted DNA sequencing in blood from day 1 of treatment (median VAF of 29%, range: 0.2–71%) were also detectable in blood from day 4 (median VAF 27%, range: 0.1–86%). Heatmap shows statistically significant changes in VAF between day 1 and 4 by either gene functional class or by individual gene/gene region, for all patients or just those with complete remission (CR) or non-responder (NR). Variants were only counted once per gene region per patients. Mutated TP53, detected only in NR patients, was significantly different (red, \( P < 0.05 \)) and mutated NPM1, detected only in CR patients, demonstrated a consistent trend (blue, \( P = 0.0625 \)). The remaining functional groups and individual genes were either non-significant (blue) or had too few data points for analysis (white). VAF, variant allele frequency.

with 33% of non-DTA patients. NPM1 mutations (\( n = 5 \)) were seen exclusively in DTA patients while TP53 mutations were seen in both DTA (\( n = 5 \)) and non-DTA (\( n = 4 \)) patients. Only the transcription-related gene class, in non-DTA patients, showed significant difference between day 1 and 4 VAF levels (Figure 7).

**Tracking Post-treatment MRD With Targeted DNA Sequencing During Remission**

Given the inability of targeted DNA sequencing of blood early during therapy to predict response to a cycle of intensive chemotherapy, we also investigated the utility of this technique in predicting post-remission relapse. Longitudinal blood samples were available from two patients from this cohort both of whom achieved CR. The first patient had no change in the ratios of wild type to mutated sequence of five genes between day 1 and 4 of therapy, despite decreasing WBC count from 60,000 to 10,000/µl during this period and subsequently achievement of a durable CR. Mutation levels remained negligible however during a durable remission lasting at least two years (Figure 8A). In the second patient, detectable KRAS mutant in blood decreased during the first 4 days of therapy while the DNMT3A mutant remained stable. During remission however both mutations were undetectable, returning at the time of relapse together with the emergence of a second KRAS mutation (Figure 8B).

**DNA Sequencing From Blood vs. Bone Marrow Samples**

In a subset of 22 patients confirmatory sequencing was also performed on pre-treatment bone marrow samples (10/10 NHLBI, 12/35 Duke). Concordance between the number of variants identified and the VAF of each detected variant in blood compared with bone marrow was assessed (Figure 9A). In the 15 patients with a pre-treatment WBC of at least 2,500/µl only a single variant identified from bone marrow was not detected from blood (of 41 variants identified in total, i.e., 98%). Notably, there was good correlation in the VAF determined for each variant from both tissue sources in these patients. Conversely,
five variants were identified only from blood and not from bone marrow.

Interestingly, the presence or absence of mutations in the blood vs. bone marrow was often correlated with WBC. Two patients with high pre-treatment WBC (34,100 and 60,400/µl) accounted for four of the five variants variants observed in the blood but not the bone marrow (Figure 9B). Likewise, for 7 patients with pre-treatment WBC <2,500/µl, only 20 out of a total of 27 variants detected in bone marrow were also detected from blood (74%), with the consequence that 5 of 7 leukopenic AML patients had an incomplete mutational characterization by DNA sequencing when using blood alone (Figure 9C). Those variants found in marrow but not identified in blood could however be identified in the raw sequencing data but were filtered...
FIGURE 7 | WT1 expression and gene variants in DTA and non-DTA patients. Patients were separated into groups based on detection of at least one DTA (DNMT3A, TET2, and ASXL1) mutation. The 34 genes in which variants were identified were classified into eight functional groups and Day 1 vs. 4 VAFs were analyzed with the Wilcoxon signed rank test. 162 coding variants were condensed into 140 by averaging the VAFs of multiple variants in the same gene in the same patient. The DTA group had 100 variants and the non-DTA group had 40 variants. (A) WT1 expression was higher in non-DTA patients compared to DTA patients. Out of the 34 patients with complete WT1 expression data, 19 patients were DTA patients and 15 were non-DTA. Here, non-DTA patients had higher relative WT1 expression than DTA patients at both D1 (median relative expression 3.07 compared to 4.75, \( P < 0.05 \)) and D4 (median relative expression 3.59 compared to 7.60, \( P < 0.05 \)).
out by the 5% VAF threshold for variant calling. For all 20 variants identified in both tissues the VAF was lower in blood than marrow in these leukopenic patients.

DISCUSSION

As we strive to personalize treatments to cancer patients, both in terms of the genetic basis of their cancer and their response to therapy, there is a great interest in earlier assessments of disease burden and characteristics (31, 32). Acute myeloid leukemia (AML) offers a unique opportunity to study the validity of blood-based assessments of residual tumor, as both the primary site of disease (bone marrow) and blood are repeatedly sampled as part of the clinical standard of care. It is increasingly recognized that blood, except in cases of leukopenia or low circulating blast count, may substitute for marrow examination in some circumstances for morphology, cytogenetics, and molecular testing in AML patients (33–35). We show here that blood, even after 3 days of intensive cytotoxic chemotherapy, can be used to identify most of the MDS/AML-associated DNA variants detectable by targeted sequencing in pre-treatment bone marrow aspirate, providing that pre-treatment white blood cell count was within or above normal range.

Complete remission is a necessary, but often insufficient, step toward long-term cure in AML. Given that the outcome of patients with relapsed and refractory AML is generally poor (36, 37) there is great interest in early interim assessments of likely response to optimize therapy in AML. While the role of bone marrow examination on day 14 of induction therapy remains unclear (35, 38–41), there has been considerable interest in kinetics of early blast clearance in blood during induction therapy as a prognostic factor (40, 42–44). Persistence of variants detected by targeted sequencing in AML patients in CR after treatment has significant independent prognostic value for both relapse and survival (6). It was therefore intriguing to consider if such molecular assessments, performed during cytotoxic therapy and prior to any response assessment, could offer a “real-time” evaluation of treatment efficacy and extremely early identification of treatment failure. We show that molecular testing of blood on treatment day 4, by either qPCR assessment of WT1 expression or DNA sequencing for common MDS/AML variants, is not predictive of clinical response to intensive cytotoxic therapy in AML. Surprisingly, all variants found by DNA sequencing in blood on day 1 remained detectable on the fourth day of intensive chemotherapy. Remarkably the ratio of mutated to wild-type sequence was often maintained during this therapy despite considerable reductions in white blood cell count. This finding of stability was not limited to potential germline mutants or to variants in DNMT3A, TET2, and ASXL1. The observed stability in VAF during cytotoxic therapy may suggest a limited therapeutic index for clones circulating in blood containing these mutations, although similar studies in patients receiving highly effective and specific therapy would be needed to prove this
had at least one variant with VAF < 1%. Leukemic clone that may be detectable before and after therapy (49 commonly mutated genes or gene regions in MDS/AML) we used a targeted sequencing panel designed to detect just malignancies with many patterns of genetic etiology whereas at baseline. Secondly, AML represents a wide range of myeloid to, variant kinetic analysis. Additionally, the majority of variants chemotherapy so they would not be informative for, or contribute additional trends during therapy in this cohort and are markers potentially worthy of future study. Ultimately DNA sequencing may have greater utility for tracking of AML MRD post-treatment rather than predicting response during therapy.

This study has several limitations. Firstly, we did not perform germline sequencing to allow categorization of identified variants by DNA sequencing as somatic. This was intentional, given the translational nature of this study, we wished to replicate testing as commonly performed in clinical practice. Importantly germline mutations would not be expected to change during chemotherapy so they would not be informative for, or contribute to, variant kinetic analysis. Additionally, the majority of variants identified had a VAF of <40%, and 42 of 43 assessed patients had at least one variant with VAF <40% detectable in blood at baseline. Secondly, AML represents a wide range of myeloid malignancies with many patterns of genetic etiology whereas we used a targeted sequencing panel designed to detect just 49 commonly mutated genes or gene regions in MDS/AML (Table S3). While more comprehensive approaches have been used to characterize potential genetic variants associated with the leukemic clone that may be detectable before and after therapy (11), we felt assessment of the most currently observed mutated regions was most easily translatable. Finally, we demonstrate consistent observations in two independent cohorts, at two different stages of disease treated with different intensive chemotherapy regimens. Given the genetic heterogeneity of this disease however it is possible that larger cohorts of patients would identify additional trends and will certainly be needed to quantify any benefit associated with tracking the two targets, TP53 and NPM1, we identify as potential candidates for monitoring during intensive therapy.

In conclusion, our study demonstrates that molecular testing of peripheral blood during the first 3 days of AML intensive chemotherapy does not appear to be predictive of clinical response. Indeed, we show that the majority of variants identified prior to treatment are still present and often at similar ratios of mutant to wild-type often despite considerable cytotoxic effect of therapy. Validation in a larger cohort is needed to confirm the utility of monitoring NPM1 and TP53 variant kinetics in blood early during AML treatment in addition to their current use as pre-treatment predictive markers. Consistent with reports using other modalities we show that blood may substitute for bone marrow for targeted DNA sequencing in AML patients, although this approach may be suboptimal in those with leukopenia pre-treatment. Longitudinal assessment of molecular MRD during follow-up time points after completion of initial cytotoxic induction therapy may have greater clinical utility than evaluation of very early time points during AML treatment.

ETHICS STATEMENT

Ten subjects were recruited to clinical protocol (NCT023527447). All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the NHLBI IRB. Samples from thirty-five patients were collected on a Duke IRB approved protocol and transferred to NIH as coded clinically annotated samples via a material transfer agreement judged exempt from NIH IRB by the NIH OHSRP.

AUTHOR CONTRIBUTIONS

HW, AS, and CH, contributed conception and design of the study. HW, KL, SS, GR, MG, MM, JTh, and LD performed laboratory research. AS, SS, DR, NR, JTh, CD, KR, CL, and CH performed clinical research. HW and D-YK performed the statistical analysis. HW wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2018.00669/full#supplementary-material
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