Nanopore sequencing methods detect cell-free DNA associated with minimal residual disease and central nervous system infiltration in pediatric Acute Lymphoblastic Leukemia

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Conflict of Interest Statement:

The authors have declared that no conflict of interest exists.

Abstract

Acute Lymphoblastic Leukemia (ALL) patients that have minimal residual disease (MRD) after therapy or infiltration of ALL into the central nervous system (CNS) are considered high risk. These patients are often given intensified and/or additional rounds of chemotherapy in the hopes of eliminating their disease. Current methods to diagnose MRD and CNS infiltration rely on detecting ALL cells in patient samples using pathology, flow cytometry, or isolation of ALL genomic DNA for next-generation sequencing. However, blasts may be present in the patient but not detectable in a bone marrow biopsy or cerebrospinal (CSF) fluid sample, leading to incorrect or delayed patient diagnosis. We have developed a nanopore sequencing assay to detect B-ALL-associated cell-free DNA in patient blood and CSF samples. Quantitation of B-cell specific VDJ recombination events in cell-free DNA samples defined B-ALL clonal heterogeneity. Monitoring cfDNA in blood and CSF samples allowed us to track the response of individual B-ALL clones throughout each patient's course of treatment. Detection of cell-free DNA predicted the clinical diagnosis of MRD and CNS disease. We also identified patients diagnosed as CNS negative who had low levels of cell-free DNA in their CSF sample. These data suggest that cell-free DNA assays may be useful in detecting the presence of ALL in the patient even when blasts are not in the biofluid sample. In total, nanopore analysis of cell-free DNA is a simple, rapid, and inexpensive assay that can serve as a useful complement to traditional clinical diagnostic approaches in the treatment of ALL.

Introduction

Acute Lymphoblastic Leukemia (ALL) is the most common pediatric malignancy. Tremendous progress has been made in the outcomes of ALL patients; however, despite cure rates approaching 90%, relapsed ALL remains the second leading cause of cancer related death in the pediatric population. Response to therapy as measured by the detection of minimal residual disease (MRD) of >0.01% at the end of induction remains the most powerful prognostic indicator of outcome (1-3). The central nervous system (CNS) is also an important site of involvement in ALL, and the CNS is a common site for ALL relapse (4, 5). The prevalence of CNS disease in ALL is sufficiently high enough that every ALL patient receives prophylactic treatment involving multiple doses of intrathecal chemotherapy, which can have negative and long term side-effects in pediatric patients. Better stratification of patients based on risk status and potential for relapse or CNS disease will rely on part on more precise diagnostics for ALL.

Current standard clinical methods to diagnose MRD and CNS disease in ALL rely on pathology and flow cytometric detection of blasts in patient samples. Development of more sensitive assays that rely on the molecular detection of leukemic blasts via next-generation (NGS) sequencing has proven difficult for this disease. ALL has one of the lowest mutational burdens of any cancer (6, 7), which precludes the design of targeted, cancer specific sequencing probes that are commonly used in NGS assays in other types of cancers. Recently, the ClonoSEQ assay was developed by Adaptive Biotechnologies, which is an NGS assay that sequences VDJ rearrangements within T and B cell receptors instead of cancer specific mutations (8, 9). This strategy is ideal for lymphocytic leukemias, as every T and B cell undergoes VDJ recombination to generate functional B and T cell receptors as part of the cell's maturation, and the specific sequence of the rearrangement in the receptor is unique to an individual B or T cell. ALL results from the abnormal expansion of typically 1-5 T or B cell clones, each of which will have a unique VDJ sequence (10, 11). ClonoSEQ identifies clones based on a major expansion of that particular VDJ sequence within the patient's blood and bone marrow samples at leukemia diagnosis. At the end of

treatment, ClonoSEQ can detect as few as 1 in a million cells within a patient sample that carry that same VDJ rearrangement to sensitively and accurately detect MRD. ClonoSEQ is now in clinical trial in the US and has been implemented in Europe for MRD detection in B cell lymphoid malignancies (12-14)

The major limitation for the ClonoSEQ method is that it relies on the presence of leukemia cells within the patient sample for collection of genomic DNA. A major concern for any cell-based assay is the accurate detection of CNS disease in ALL, for which ClonoSEQ is not currently used. Research using animal models showed that ALL cells often become embedded in the CNS tissue or attached to spinal nerves (15, 16). Autopsies of ALL patients that succumbed to disease demonstrated that 85% had CNS disease present in the brain or spinal cord at autopsy (17), although typically only 11% patients are clinically diagnosed as CNS disease positive based on detection of lymphoblasts in the cerebrospinal fluid (CSF) (4, 5). These findings highlight a critical need for the development of more sensitive assays to complement cell-based pathology, flow cytometry, and ClonoSEQ assays, especially pertaining to ALL spread into the CNS.

The utilization of cell-free DNA as a biomarker for cancer progression is set to transform cancer care. Cell-free DNA is free-floating DNA in patient biofluids that is constitutively released by cancer cells, through unknown mechanisms (18, 19). Tumor specific cfDNA increases as tumors grow, and the half-life of cfDNA is only 15-120 minutes, which has made it a valuable and proven biomarker for in detecting cancer development in healthy individuals and in monitoring tumor response to treatment in cancer patients (20). As of fall 2020, FoundationOne's Liquid CDx cfDNA test has been FDA-approved to detect *EGFR*, *BRCA1/2*, *ALK*, *ATM*, and *PIK3CA* mutations in cfDNA in ovarian, breast, prostate, and lung cancers to stratify patients into treatment regimens (21). Detection of VDJ rearrangements in cfDNA has been used to monitor lymphoid lymphoma progression (22-24), and emerging evidence in myeloid malignancies suggests that cfDNA monitoring will be a useful diagnostic tool for leukemia patients as well. For example, in Acute Myelogenous Leukemia, cfDNA assays detected bone marrow relapse 30 days earlier than the standards flow cytometry-based methods (25). Finally, emerging evidence suggests

that cfDNA may be more useful than cell-based assays in monitoring CNS tumors. For example, in leptomeningeal carcinomatosis, ctDNA was detectable in cases in which microscopy did not reveal malignant cells in the CSF and cfDNA longitudinally fluctuated over time correlating with CNS tumor burden (26). A comprehensive examination of several types of CNS malignancies showed that cfDNA isolated from the CSF was much more reliable than genomic DNA isolated from cells within the CSF in identifying tumor associated mutations (27). In this study, cells could not be routinely obtained from CSF samples, and DNA quality from cells was often not suitable for NGS. These data suggest that cfDNA can identify CNS associated cancers much more accurately than assays that rely the presence of cancer cells within the CSF. To our knowledge, cfDNA has not been used to examine MRD status or CNS disease in ALL.

Our study outlines a method for isolation and detection of leukemia-derived cfDNA in the peripheral blood and CNS of pediatric B-ALL patients. We developed a simple and inexpensive workflow based on Nanopore MinION sequencing of PCR amplified VDJ rearrangements in cfDNA, which allowed us to analyze as little at 25 picograms of cfDNA per patient biofluid sample. We found that cfDNA can provide a more accurate assessment of B-ALL heterogeneity, as cfDNA sequencing could detect clones that were not present in the genomic DNA of bone marrow biopsy samples. Plasma cfDNA samples were used to monitor the response of specific clones to treatment, and identified B-ALL clones that lead to MRD. Additionally, the cfDNA sequencing workflow could detect B-ALL associated cfDNA in the CSF of patients that had been clinically diagnosed with CNS disease and in some patients that were diagnosed as CNS-negative. The clearance of CNS associated clones could be confirmed by loss of cfDNA in the patient CSF sample. In total, our data demonstrate the utility of cfDNA in assessing B-ALL heterogeneity and in monitoring B-ALL progression. The low cost and ease of use make the MinION cfDNA workflow ideal for cfDNA analysis in research settings, and the specificity and sensitivity of the assay in detecting MRD and CNS disease suggests that cfDNA may ultimately be a useful complement to current cell-based clinical assays like ClonoSEQ.

Results and Discussion

Characteristics of B-ALL patients

Pediatric B-cell acute lymphoblastic leukemia (B-ALL) patients were admitted to the Kentucky Children's Hospital at initial diagnosis of B-ALL and enrolled for sample collection. Samples were retrospectively chosen for use in this study based on whether CNS infiltration was observed in the patient at diagnosis, and on the patient's MRD status after initial therapy. In total, our cohort size had 5 patients out of which one patient (AAL-008) was MRD positive at the end of induction. Patient AAL-004 was clinically MRD negative, but some leukemic circulating blasts were observed, although the quantify did not reach the cutoff of clinical threshold for MRD. Patients AAL-010, 009 and 012 were MRD negative at the end of induction, and patients AAL-008 and 010 were positive for CNS disease at the time of diagnosis. The complete characteristics of these patients are summarized in **Table 1**. For these patients, genomic DNA was isolated from bone marrow aspirate collected at diagnosis, and cell-free DNA was isolated from plasma and cerebrospinal fluid (CSF) samples that were collected throughout the course of treatment.

Table 1. Patient Characteristics

Subject ID	*Blast Count at Diagnosis (*10^9/L)*	*WBC Count at Diagnosis (*10^9/L)*	*Number of Days to Clear Circulating Blasts*	*EOI MRD Status*	*CNS Disease Status*	*Number of Days to Clear CSF*
ALL-004	0.57	3.33	9	Negative*	Negative	N/A
ALL-008	1.53	4.79	9	Positive	Positive	8
ALL-009	14.92	19.38	11	Negative	Negative	N/A
ALL-010	1.58	6.32	13	Negative	Positive	9
ALL-012	23.88	31.84	11	Negative	Negative	N/A

*Negative MRD at threshold of 1/10,000 cells but a few leukemic cells noted

WBC, white blood cell; EOI, end of induction; MRD, minimal residual disease; CNS, central nervous system; CSF, cerebrospinal fluid

Identification of B cell clones via MinION sequencing of the recombined IGH gene

We developed a novel experimental workflow that combines PCR of the IGH region with the third-generation Oxford Nanopore MinION sequencer (ONT) (28), to detect B-ALL patient specific IGH clonal rearrangements and to track these clones throughout the patient's treatment, with the goal of applying these methods to detection of B-ALL specific cfDNA. Ultimately, this method can be applied to both genomic DNA isolated from blasts in patient samples or to cfDNA collected from patient biofluids. In the first step, a commercially available PCR kit was used to amplify the IGH sequence between the conserved framework region 1 (FR1) and the conserved Joining (J) region, which allow complete representation of all Variable regions within the IGH gene (29). PCR products were subjected to library preparation to add adapters and barcodes that allow for multiplexing, then samples were run through the benchtop MinION nanopore sequencer. The output files were then analyzed by a simple pipeline comprised of freely available software: i) MinKNOW, which operates the sequencer and automatically performs simultaneous sequencing, demultiplexing and barcode trimming using the built-in Guppy toolkit, ii) Minimap2 to map the merged fastq files to create BAM alignment files, and iii) Featurecount to determine how many reads align with the IGHV region. Step-by-step instructions for analysis using these tools are found in the Supplemental Methods. The entire process from sample collection to data analysis takes less than one working day, costs less than $30 per sample, and can be carried out by users with basic computer skills.

Limit of input DNA detection on MinION workflow

The recommended amount of input DNA for IGHV PCR is 2 ng (30). As our ultimate goal was to adapt our workflow towards detection of cfDNA, we determined the lowest level of input DNA that could be used in IGHV PCR and MinION sequencing. We tested our workflow on healthy tonsil control DNA, ranging from 10-100 pg. The human IGH region is located on the long arm of chromosome 14 (Chr14, **Figure 1A**), and we used the MinION generated reads that mapped to this locus over other locations as a readout of PCR specificity and sensitivity. Libraries of IGHV PCR reactions from all DNA

concentrations tested had reads that mapped to chromosome 14. Predictably, mapping frequency to Chr14 decreased with decreasing input DNA amount, however only a small reduction in total mapped reads was observed after DNA input was lowered to 0.025ng (**Figure 1B**). When mapped reads were distributed across all chromosomes, it became evident that non-specific mapping increased inversely with template amount and that the signal to noise was indistinguishable at concentrations below 0.025ng, as reads mapping to Chr14 dropped below the levels of mapping to other chromosomes (**Figure 1C**). Yet even with the lowest input DNA amount, we could detect reads mapping to the same IGHV clones as with the highest DNA input amount (**Figure 1D**), suggesting that sensitivity with nanopore sequencing can be maintained even when specificity decreases. This aspect of nanopore sequencing is essential when working with precious patient samples, such as cfDNA, that are too low to quantify by standard means.

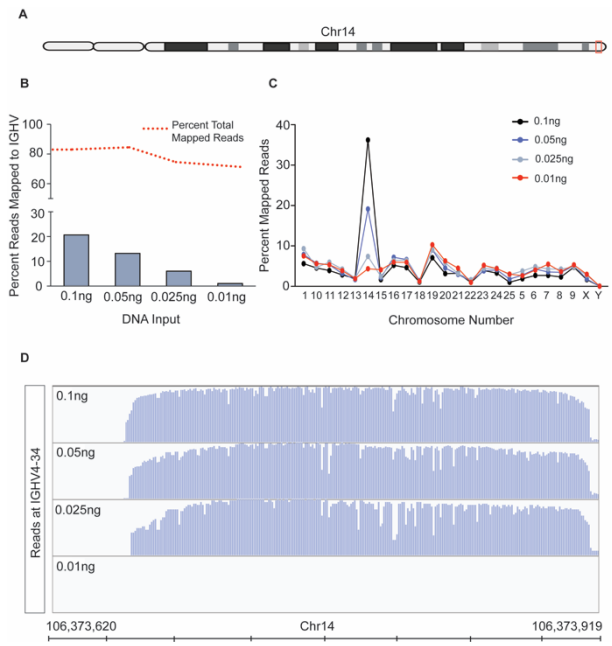

Figure 1. The MinION sequencing workflow can reliably detect IGHV reads in as little as 25 picograms of DNA. **A**) Schematic of chromosome 14. The red box denotes the region containing the IGHV sequence that all MinION reads were aligned to. **B**) The IGHV region was PCR amplified from the input amount of normal tonsil DNA indicated. Approximately 4,000 reads were obtained from the resulting libraries using nanopore MinION sequencing. The red dashed line shows the percent of all reads that mapped to the human genome, and the bars denote the percentage of those reads that mapped to an IGHV sequence. **C**) The percent of all reads mapped from the samples in (B) are plotted across all chromosomes. **D**) Alignment plots to a selected IGHV, V4-34, shows accurate alignment of reads when input DNA is as little as 0.025ng.

cfDNA is detectable by the MinION sequencing workflow

cfDNA is emerging as a powerful prognostic biomarker that can be used to assess the tumor heterogeneity as well as tumor burden (31). Additionally, because cfDNA is localized to patient biofluids, sample collection is much less invasive than bone marrow biopsy. cfDNA has not previously been assessed in

ALL—bioanalyzer traces of cfDNA samples isolated from the plasma of patient AAL-004 and AAL-010 matched expected cfDNA sizes of ~150 base pairs (**Supplemental Figure 1**). PCR and MinION sequencing of IGVH were applied to both the genomic DNA (gDNA) isolated from bone marrow aspirate and cfDNA isolated from the plasma at diagnosis. The total number of reads that were generated from these samples and the percentage of the reads mapped to the chromosome 14 in the are provided in **Supplemental Table 1**. Only sequences that had ≥ 80% match to the IGHV region of chr14 were considered B cell "clones" and included for further analyses. Clones that comprised ≥ 5% of the total mapped reads were considered major clones in the sample. We reasoned that a ≥ 5% representation of a particular IGHV sequence in the sample would most likely be due to clonal expansion of the B-ALL. Additionally, the clonal distribution of the both the gDNA and cfDNA samples that was identified by MinION sequencing was confirmed using MiSeq analysis of the same PCR libraries, both in terms of the clone identity as well as the clonal abundance (**Supplemental Figure 2**). These data indicate that the MinION sequencing workflow can be used to assess the IGH clonal repertoire in B-ALL and can identify those same clones in B-ALL patient cfDNA, and that this method is comparable to the more time consuming and costly next-generation sequencing methodologies that are routinely used in the clinical setting.

MinION sequencing of cfDNA identifies B-ALL clones that are not cleared in patients with MRD

The half-life of cfDNA is predicted to be 15-120 minutes (32), which makes it an excellent biomarker for assessing leukemia burden and analyzing the response of individual clones to therapy. We analyzed both the gDNA from bone marrow biopsy and the cfDNA from plasma collected from 4 patients at diagnosis to identify the expanded clones comprising the B-ALL, as described above. These clones were then tracked in subsequent plasma cfDNA samples to assess the changes in clonal distribution throughout treatment. Samples were taken ~ 1 week into induction chemotherapy (denoted as Early-I in **Figure 2**), ~2 weeks into induction therapy (denoted mid-I), at the end of induction chemotherapy (denoted EOI),

and in some cases, at the end of consolidation (EOC) therapy, which can occur several months after diagnosis.

Two patients, AAL-010 and AAL-012, were clinically diagnosed as MRD negative at the end of induction therapy. At their initial disease diagnosis, both patients had heterogenous B-ALL, with 6 (AAL-010) or 4 (AAL-012) distinct IGHV sequences present as ≥ 5% of the total mapped reads. In patient AAL-010, we observed a clonal population, IGHV3-35, that was present in the cfDNA at diagnosis and early induction that was not in the gDNA biopsy sample. All clones were eliminated after treatment, consistent with the clinical diagnosis of MRD-free (**Figure 2A**). However, we did observe a sharp increase in reads mapping to chr14 in the end of induction cfDNA sample for this patient, although all of the previously identified clones were cleared (red dashed line, **Figure 2A**). This spike may be attributed to a clonal expansion of a B cell caused by an illness or other antibody response in the patient. Just as with all other types of DNA sequencing used for ALL clonality detection, the major caveat of our MinION workflow is that it cannot distinguish between B-ALL and normal B cells.

Similar to AAL-010, patient AAL-012 had a clone, IGHV4-39, that was not present in the diagnosis gDNA bone marrow biopsy sample but was identified in the diagnosis cfDNA plasma sample (**Figure 2B**). Because every B-ALL clone releases cfDNA into the plasma, cfDNA may ultimately be more accurate in detecting the true clonal composition of the B-ALL than the bone marrow biopsy, which can only detect clones present at the site of biopsy. Additionally, early into both patient AAL-10's and AAL-012's treatment, we observed a sharp increase in cfDNA associated with these clones, which likely indicates leukemia cell death and release of DNA into the blood and may indicate a good response to treatment.

Two additional patients, AAL-004 and AAL-008, had blasts present in their clinical samples at the end of induction therapy. Patient AAL-004 was not formally diagnosed with MRD, as the blast count fell below the 1 blast per 10,000 cell threshold for clinical MRD. Patient AAL-008 was MRD positive. In both patients, we detected heterogenous B-ALL that was comprised of several distinct IGHV sequences

in both the gDNA from bone marrow biopsy and the plasma cfDNA isolated at diagnosis. Patient AAL-004 appeared to clear their clones during induction therapy, as we were not able to detect reads linked with chr14 in the cfDNA samples. However, we observed clone IGHV3-74, a major clone that was present at diagnosis, re-emerge in cfDNA samples collected at the end of consolidation therapy (**Figure 2C**). Patient AAL-008 unfortunately never cleared the clones that were detected in the biopsy gDNA and cfDNA sample at diagnosis. Interestingly, we observed that clone IGHV3-30, a minor clone in the cfDNA at diagnosis, was the predominate clone at the end of consolidation therapy (**Figure 2D**). Together, these data highlight the utility of cfDNA analysis as a method to track B-ALL clonal dynamics during patient treatment. This workflow may be useful in identifying patients whose B-ALL does not respond well to therapy, such as patient AAL-008. Since cfDNA is isolated from blood samples, it can be used as a non-invasive method to assess patients for clone re-emergence after treatment, as shown in patient AAL-004.

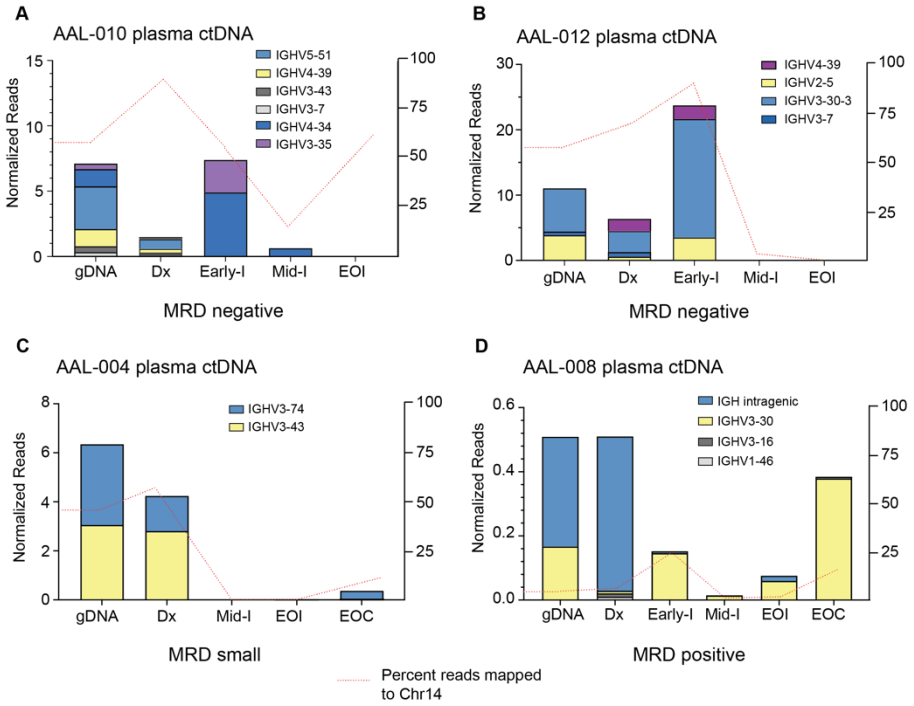

Figure 2. IGHV sequencing of cfDNA can be used to determine B-ALL heterogeneity and to track clonal response throughout the course of treatment. cfDNA was extracted from blood samples of four patients, IGHV regions were PCR amplified, and resultant libraries were sequenced using MinION. Data were analyzed using Galaxy. Clones in the genomic DNA (gDNA) and diagnosis cell-free DNA (Dx) samples that comprised at least 5% of total reads were tracked across all timepoints. Clone abundance is normalized to total mapped reads per library. Percent reads aligned to Chr14 (red line) is a relative indicator of leukocyte abundance at each timepoint/sample. Patients in (**A**) and (**B**) were clinically diagnosed as MRD negative. Patient (**C**) had blasts present in the sample but did not reach the more than 1 blast per 10,000 cell threshold for MRD diagnosis. (**D**) was diagnosed as MRD positive.

cfDNA can be detected in the CSF of B-ALL patients using the MinION sequencing workflow

Approximately 4% of B-ALL patients will have infiltration of ALL blasts into their CNS at the diagnosis, and 30-40% of the patients will have pronounced CNS disease at relapse (4). CNS infiltration is a significant negative prognostic indicator in B-ALL, but the only way to diagnose it is through detection of blasts in the CSF sample. However, B-ALL cells can be present in the brain or associated with spinal neurons, and not be freely floating and detectable in the CSF, meaning patients might have CNS disease but present clinically as negative for CNS infiltration. For this reason, every ALL patient is given intrathecal chemotherapy. We reasoned that since cfDNA is released by B-ALL cells into patient biofluids, it might be detectable in CSF samples even if the blasts themselves are not. We applied our MinION sequencing workflow to cfDNA isolated from CSF samples taken from patients that were diagnosed as CNS positive (AAL-008 and 10) and CNS negative (AAL-009 and 012). As expected, we were able to detect IGHV reads in the cfDNA from CNS positive B-ALL patients (**Figure 3A-B**), and these clones were cleared by the end of induction therapy. Interestingly, we detected very low levels of IGHV sequence in the diagnosis CSF cfDNA sample from patient AAL-009 (**Figure 3C**), even though this patient was diagnosed as CNS negative. Finally, patient AAL-012 was CNS negative at diagnosis, but we observed an IGHV read in the cfDNA from the CSF of this patient at diagnosis that significantly expanded by the end of induction (**Figure 3D**). CSF isn't drawn from patients after induction therapy if they are diagnosed as CNS negative, so we were unable to follow up on the IGHV in the cfDNA in CSF sample of AAL-012, although their plasma cfDNA remained clear of IGHV reads throughout consolidation. Finally, across all patients, we observed that some of the IGHV reads in the cfDNA from the plasma that were also present in the cfDNA in the CSF, indicating B-ALL clones were present at both locations. In other instances, clones were exclusively present in one location or the other, suggesting that cfDNA analysis may be useful in research focused on ALL homing to the CNS.

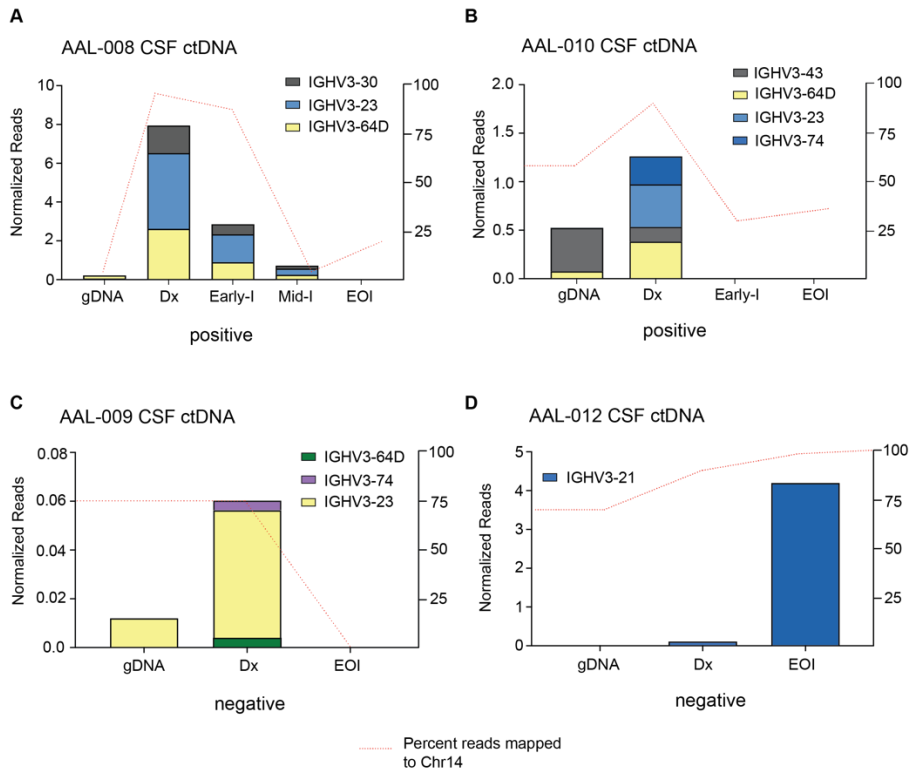

Figure 3. IGHV sequencing can detect cfDNA in the cerebrospinal fluid of B-ALL patients. cfDNA was extracted from CSF samples of four patients, IGHV regions were PCR amplified, and resultant libraries were sequenced using MinION. Data were analyzed using Galaxy. Clones in the genomic DNA diagnosis cell-free DNA (Dx) CSF samples that comprised at least 5% of total reads were tracked across all timepoints, and in genomic DNA isolated from bone marrow biopsy. Clone abundance is normalized to total mapped reads per library. Percent reads aligned to Chr14 (red line) is a relative indicator of leukocyte abundance at each timepoint/sample. Patients in (**A**) and (**B**) were clinically diagnosed as CNS positive, indicating infiltration of B-ALL cells into the CNS. Patient (**C**) and (**D**) were diagnosed as CNS negative, although IGHV reads were detectable in the CNS sample.

Taken together, our study demonstrates the utility of a MinION sequencing workflow as a simple, rapid, and low-cost method to assess clonal VDJ rearrangements in B-ALL. This workflow will be useful to research laboratories interested in lymphocytic clonality and similar MinION sequencing strategies could be developed and applied to detect tumor associated mutations in cfDNA in any cancer type. We found that quantitation of IGHV reads from cfDNA samples provided insights into B-ALL heterogeneity and could be used to assess the dynamics of B-ALL clones in response to treatment. While additional studies are needed with expanded patient cohorts, our data suggest that cfDNA monitoring could be a non-invasive method to routinely monitor ALL burden in patients undergoing treatment, which may

allow for earlier diagnosis of MRD and more accurate risk stratification. Analysis of cfDNA may also ultimately prove to be a clinically useful complement to the current cell-based ClonoSEQ methodology, in the diagnosis of CNS disease, in which blasts may not be physically present in the CSF sample but are continuously releasing cfDNA.

Methods

Further information can be found in the Supplemental Methods.

Study approval. All patient samples were collected after obtaining informed consent according to protocol 44672, approved by the University of Kentucky's Institutional Review Board.

Author Contributions

SS, YC, MGH, TCB and JSB designed experiments; SS and YC conducted experiments and acquired data; SS, YC, IAS analyzed data; TJT, BLF, TCB consented patients and collected samples; MGH, LHM, AHC, IAS processed samples; SS, YC, MGH, and IAS drafted the manuscript; JSB edited the manuscript; JSB provided supervision and funding.

Acknowledgements

Funding for this research was provided by a Kentucky Pediatric Cancer Research Trust Fund research grant to JSB and TCB, NIH grants DP2CA228043 and R37CA227656 to JSB, as well as T32CA165990 to MGH.

References

1. Hunger SP, and Mullighan CG. Acute Lymphoblastic Leukemia in Children. *N Engl J Med.* 2015;373(16):1541-52.
2. Kato M, and Manabe A. Treatment and biology of pediatric acute lymphoblastic leukemia. *Pediatr Int.* 2018;60(1):4-12.

3. Coustan-Smith E, Mullighan CG, Onciu M, Behm FG, Raimondi SC, Pei D, et al. Early T-cell precursor leukaemia: a subtype of very high-risk acute lymphoblastic leukaemia. *Lancet Oncol.* 2009;10(2):147-56.
4. Lenk L, Alsadeq A, and Schewe DM. Involvement of the central nervous system in acute lymphoblastic leukemia: opinions on molecular mechanisms and clinical implications based on recent data. *Cancer Metastasis Rev.* 2020;39(1):173-87.
5. Ranta S, Nilsson F, Harila-Saari A, Saft L, Tani E, Söderhäll S, et al. Detection of central nervous system involvement in childhood acute lymphoblastic leukemia by cytomorphology and flow cytometry of the cerebrospinal fluid. *Pediatr Blood Cancer.* 2015;62(6):951-6.
6. Waanders E, Gu Z, Dobson SM, Antić Ž, Crawford JC, Ma X, et al. Mutational landscape and patterns of clonal evolution in relapsed pediatric acute lymphoblastic leukemia. *Blood Cancer Discov.* 2020;1(1):96-111.
7. Wu C, and Li W. Genomics and pharmacogenomics of pediatric acute lymphoblastic leukemia. *Crit Rev Oncol Hematol.* 2018;126:100-11.
8. Ching T, Duncan ME, Newman-Eerkes T, McWhorter MME, Tracy JM, Steen MS, et al. Analytical evaluation of the clonoSEQ Assay for establishing measurable (minimal) residual disease in acute lymphoblastic leukemia, chronic lymphocytic leukemia, and multiple myeloma. *BMC Cancer.* 2020;20(1):612.
9. Monter A, and Nomdedéu JF. ClonoSEQ assay for the detection of lymphoid malignancies. *Expert Rev Mol Diagn.* 2019;19(7):571-8.
10. Mullighan CG, Phillips LA, Su X, Ma J, Miller CB, Shurtleff SA, et al. Genomic analysis of the clonal origins of relapsed acute lymphoblastic leukemia. *Science.* 2008;322(5906):1377-80.
11. Fries C, Adlowitz DG, Spence JM, Spence JP, Rock PJ, and Burack WR. Acute lymphoblastic leukemia clonal distribution between bone marrow and peripheral blood. *Pediatr Blood Cancer.* 2020;67(6):e28280.
12. Bal S, and Costa LJ. Best practices for the assessment of measurable residual disease (MRD) in multiple myeloma. *Clin Adv Hematol Oncol.* 2020;18 Suppl 1(1):1-20.
13. Hussaini MO, Srivastava J, Lee LW, Nishihori T, Shah BD, Alsina M, et al. Assessment of Clonotypic Rearrangements and Minimal Residual Disease in Lymphoid Malignancies: A Large Cancer Center Experience Using clonoSEQ. *Arch Pathol Lab Med.* 2021.
14. ClonoSEQ Cleared for Residual Cancer Testing. *Cancer Discov.* 2018;8(12):Of6.
15. Münch V, Trentin L, Herzig J, Demir S, Seyfried F, Kraus JM, et al. Central nervous system involvement in acute lymphoblastic leukemia is mediated by vascular endothelial growth factor. *Blood.* 2017;130(5):643-54.
16. Yao H, Price TT, Cantelli G, Ngo B, Warner MJ, Olivere L, et al. Leukaemia hijacks a neural mechanism to invade the central nervous system. *Nature.* 2018;560(7716):55-60.
17. Bojsen-Møller M, and Nielsen JL. CNS involvement in leukaemia. An autopsy study of 100 consecutive patients. *Acta Pathol Microbiol Immunol Scand A.* 1983;91(4):209-16.
18. Wan JCM, Massie C, Garcia-Corbacho J, Mouliere F, Brenton JD, Caldas C, et al. Liquid biopsies come of age: towards implementation of circulating tumour DNA. *Nat Rev Cancer.* 2017;17(4):223-38.
19. Volik S, Alcaide M, Morin RD, and Collins C. Cell-free DNA (cfDNA): Clinical Significance and Utility in Cancer Shaped By Emerging Technologies. *Mol Cancer Res.* 2016;14(10):898-908.
20. Pessoa LS, Heringer M, and Ferrer VP. ctDNA as a cancer biomarker: A broad overview. *Crit Rev Oncol Hematol.* 2020;155:103109.
21. Woodhouse R, Li M, Hughes J, Delfosse D, Skoletsky J, Ma P, et al. Clinical and analytical validation of FoundationOne Liquid CDx, a novel 324-Gene cfDNA-based comprehensive genomic profiling assay for cancers of solid tumor origin. *PLoS One.* 2020;15(9):e0237802.
22. Sarkozy C, Huet S, Carlton VE, Fabiani B, Delmer A, Jardin F, et al. The prognostic value of clonal heterogeneity and quantitative assessment of plasma circulating clonal IG-VDJ sequences at diagnosis in patients with follicular lymphoma. *Oncotarget.* 2017;8(5):8765-74.

23. Kwok M, Wu SP, Mo C, Summers T, and Roschewski M. Circulating Tumor DNA to Monitor Therapy for Aggressive B-Cell Lymphomas. *Curr Treat Options Oncol.* 2016;17(9):47.
24. Kubaczkova V, Vrabel D, Sedlarikova L, Besse L, and Sevcikova S. Cell-free DNA - Minimally invasive marker of hematological malignancies. *Eur J Haematol.* 2017;99(4):291-9.
25. Zhong L, Chen J, Huang X, Li Y, and Jiang T. Monitoring immunoglobulin heavy chain and T-cell receptor gene rearrangement in cfDNA as minimal residual disease detection for patients with acute myeloid leukemia. *Oncol Lett.* 2018;16(2):2279-88.
26. De Mattos-Arruda L, Mayor R, Ng CKY, Weigelt B, Martínez-Ricarte F, Torrejon D, et al. Cerebrospinal fluid-derived circulating tumour DNA better represents the genomic alterations of brain tumours than plasma. *Nat Commun.* 2015;6:8839.
27. Shah M, Takayasu T, Zorofchian Moghadamtousi S, Arevalo O, Chen M, Lan C, et al. Evaluation of the Oncomine Pan-Cancer Cell-Free Assay for Analyzing Circulating Tumor DNA in the Cerebrospinal Fluid in Patients with Central Nervous System Malignancies. *J Mol Diagn.* 2021;23(2):171-80.
28. Jain M, Olsen HE, Paten B, and Akeson M. The Oxford Nanopore MinION: delivery of nanopore sequencing to the genomics community. *Genome Biol.* 2016;17(1):239.
29. Bruggemann M, Kotrova M, Knecht H, Bartram J, Boudjogrha M, Bystry V, et al. Standardized next-generation sequencing of immunoglobulin and T-cell receptor gene recombinations for MRD marker identification in acute lymphoblastic leukaemia; a EuroClonality-NGS validation study. *Leukemia.* 2019;33(9):2241-53.
30. Vergani S, Korsunsky I, Mazzarello AN, Ferrer G, Chiorazzi N, and Bagnara D. Novel Method for High-Throughput Full-Length IGHV-D-J Sequencing of the Immune Repertoire from Bulk B-Cells with Single-Cell Resolution. *Front Immunol.* 2017;8:1157.
31. Heitzer E, Haque IS, Roberts CES, and Speicher MR. Current and future perspectives of liquid biopsies in genomics-driven oncology. *Nat Rev Genet.* 2019;20(2):71-88.
32. Khier S, and Lohan L. Kinetics of circulating cell-free DNA for biomedical applications: critical appraisal of the literature. *Future Sci OA.* 2018;4(4):Fso295.