A Pilot Study of Plasma Microbial Cell-Free DNA Following Chimeric Antigen Receptor T Cell Therapy in Pediatric Patients with Relapsed/Refractory Leukemia

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September 28, 2023

Abstract

Chimeric antigen receptor (CAR) T cell therapy is a promising treatment for pediatric patients with relapsed or refractory B cell acute lymphoblastic leukemia (R/R B ALL). Cytokine release syndrome (CRS) is a common toxicity after CAR T cell therapy and fever is often the first symptom. Differentiating CRS from infection after CAR T cell therapy can be challenging. Plasma microbial cell free DNA (mcfDNA) is a novel diagnostic tool which allows for qualitative and quantitative assessment of over 1,000 organisms. This pilot study sought to characterize mcfDNA results in pediatric patients with R/R B ALL in the first two months after CAR T cell therapy.

Introduction

Chimeric antigen receptor (CAR) T cell therapy is a promising treatment for pediatric patients with relapsed or refractory B cell acute lymphoblastic leukemia (R/R B ALL).¹,² Cytokine release syndrome (CRS) is a common CAR-mediated toxicity characterized by fever and hemodynamic instability due to pro-inflammatory cytokines. This syndrome can mimic sepsis and can range from mild reversible symptoms requiring supportive care alone to a severe, life-threatening syndrome. Approximately 90% of patients experience some degree of CRS with fever as the most common symptom.³ Treatment varies depending on severity of the CRS but often includes immunosuppressive therapy.

In addition to risk of CRS, patients receiving CAR T cell therapy are at high risk of infection.⁴,⁵ Due to multiple prior intensive chemotherapy regimens, patients are often severely immunocompromised and neutropenic. Vora et al. described rates of infectious complications following CAR T cell therapy in 83 pediatric, adolescent, and young adult patients with R/R B ALL. Infections were most common in the first month following CAR T cell infusion with 40% of patients experiencing an infection in the first 28 days.⁴ Moskop et al. reported similar data with 40% of patients experiencing an infection after CAR T cell infusion with 50%, 37%, and 7% due to bacterial, viral, and fungal infections, respectively. Infection contributed to death in 5.4% of patients. Patients who experienced an infection had significantly lower survival.⁵

Because both infection and CRS commonly present with fever and signs of inflammation, they can be difficult
to distinguish. The two can also occur concurrently. Vora et al. reported that higher severity of CRS was associated with increased risk of infection after CAR T cell infusion.\textsuperscript{4} The immunosuppressive nature of CRS-targeted treatment puts patients at further risk of infection. Certain pathogens (especially fungal and viral) are difficult to detect via routine blood culture, and PCR-based detection is only available for a limited number.

The Karius Test, developed and validated in Karlus’s CLIA-certified/CAP-accreditted lab (Redwood City, CA), detects microbial cell-free DNA (mcfDNA) in plasma.\textsuperscript{6} Following DNA extraction, metagenomic sequencing is performed, and DNA sequences are aligned against a curated database of more than 20,000 genome sequences reporting on the presence and quantitation across more than 1,000 microorganisms. Microbial cell-free DNA of microorganisms present above background, at statistical threshold are reported and quantified in molecules per microliter (MPM). KT has been used in a variety of immunocompromised populations.\textsuperscript{7-17}

Preliminary data in pediatric cancer patients from St Jude suggests that KT may detect mcfDNA in the plasma before clinical symptoms emerge.\textsuperscript{9} This could potentially lead to preemptive therapy and prevent the morbidity and mortality associated with infections in this heavily immunocompromised population. Due to the quantitative nature of KT, results could also be used to document clearance of an infection and decrease antimicrobial exposure.\textsuperscript{8,18}

While surveillance for mcfDNA in patients receiving CAR T therapy has been performed outside the US, to the best of our knowledge, surveillance for plasma mcfDNA following CAR T therapy in US pediatric patients has not been described.\textsuperscript{19,20} Here we describe detection of plasma mcfDNA via KT in pediatric and young adult patients with R/R B ALL in the first 2 months post CAR T cell therapy and assess the association of plasma mcfDNA results with clinical and laboratory markers of inflammation as well as the concordance of mcfDNA results with conventional diagnostic measures including blood culture, viral and fungal PCR, and radiographic studies. We demonstrate the feasibility of following mcfDNA in this severely immunocompromised population and its utility in early identification of treatable pathogens.

A better understanding of the quantitative nature of plasma mcfDNA and how that correlates with the clinical presentation in this population will serve as preliminary data for a multi-institutional, prospective study and ultimately inform management decisions.

Methods

We identified patients who had received CAR T cell therapy for R/R B ALL at Lucile Packard Children’s Hospital at Stanford between November 2017 and March 2021 with banked plasma samples available for KT. Patients received CARs targeting CD19, CD22, or CD19-CD22 bispecific. We tested for the presence of mcfDNA in previously banked plasma samples at the following time points: prior to CAR T infusion, week 1, week 2, week 3 and week 4 or later after CAR T infusion. Not all patients had banked plasma samples available for all time points. Data was analyzed at the level of CAR T cell infusion. The samples were analyzed on Karius 3.10.

For defining pathogenicity of mcfDNA results, we decided \textit{a priori} two different definitions of a positive plasma mcfDNA result. Definition 1 was designed to capture organisms that are known pathogens in pediatric patients with cancer. This included any mcfDNA detected in the plasma that is identified in >1% of central line associated bloodstream infections in children with cancer.\textsuperscript{9} Because this definition only includes bacterial and fungal infections, we expanded it to include any organism detected on at least 2 contiguous time points. This allowed for capture of potential viral mcfDNA. Definition 2 was broad and included any detection of mcfDNA in the plasma.

Demographic and baseline characteristics, clinical and laboratory markers of inflammation, microbiologic results, and radiographic findings were abstracted from the medical record and entered into a secure RedCap\textsuperscript{®} database hosted at Stanford University.\textsuperscript{21} Clinical markers of inflammation included temperature and CRS score. Laboratory markers of inflammation included ALT, AST, ferritin, LDH and CRP. Microbiologic
results included blood and urine cultures, viral and fungal PCR, and Aspergillus galactomannan. Organism detected by plasma mcfDNA as well as level in MPM were also entered into RedCap®. This study received Stanford institutional review board approval.

We described baseline characteristics of patients and plasma mcfDNA results. We then assessed the relationship between clinical and laboratory markers of inflammation and detectable plasma mcfDNA using generalized linear mixed effect models with a random subject-specific intercept to account for correlation of errors associated with plasma mcfDNA measurements within the same patient over time. We performed this analysis using both definitions of a positive plasma mcfDNA result as described above.

To assess concordance between plasma mcfDNA results and conventional measures of infection, we evaluated blood and urine culture results, viral PCR, fungal PCR, exam findings, and imaging findings concerning for infection. Microbiologic and clinical adjudication was performed by the primary author (CA). If concordance was not clear, the primary author sought input from KD and DB. Microbiologic evidence of infection was defined as a positive blood culture, urine culture, viral PCR, or fungal PCR. Clinical evidence of infection was defined as imaging or exam findings concerning for infection. For example, pulmonary nodules in a patient with febrile neutropenia or molluscum on exam. Concordance results were reported descriptively. Results were considered “clinically consistent” if a) microbiologic results matched plasma mcfDNA results, b) clinical evidence of infection matched plasma mcfDNA results, or c) microbiologic testing and plasma mcfDNA results were both negative. Results were considered “clinically inconsistent” if a) microbiologic or clinical evidence of infection with negative mcfDNA results, b) no microbiologic or clinical evidence of infection with positive mcfDNA results, c) microbiologic or clinical evidence of infection that did not match mcfDNA results, or d) microbiologic evidence of RNA virus (KT only detects DNA viruses).

**Results**

Between November 2017 and March 2021, 39 pediatric, adolescent, and young adult patients received CAR T cell therapy for R/R B ALL at Lucile Packard Children’s Hospital at Stanford. Of those, 25 patients had at least one banked plasma sample available for mcfDNA testing at Karius. Two patients received CAR T cell therapy twice during the study time frame for a total of 27 available courses for analysis (Figure 1). An overview of patient demographics is shown in Table 1. Four of 27 courses were complicated by bacteremia (Supplemental Table 1).

A total of 112 samples were sent to Karius for analysis (Figure 1). Of those, 10 (8.9%) had insufficient volume for testing, 72 (64.3%) had no mcfDNA detected and 30 (26.8%) had at least one organism detected. Of those with an organism detected, 19 (63.3%) had 1 organism detected, 3 (10%) had 2 organisms detected, 3 (10%) had 3 organisms detected, and 5 (16.7%) had more than 3 organisms detected. The majority of the 62 organisms detected were bacterial (36; 58.1%) followed by viruses (19; 30.6%) and fungi (7; 11.3%). Overall, *human herpesvirus 6B* was the most commonly detected organism. Using Definition 1, 21.2% of results were positive compared with 29.3% of results using Definition 2.

Table 2 summarizes microbiologic and clinical concordance with plasma mcfDNA results. In approximately two thirds of evaluations, all measures of infection were negative. Details of four evaluations with discordant positive results are shown in Table 3. Patients 3 and 20 had positive mcfDNA results that were also detected by blood culture. *Staphylococcus epidermidis* was detected by mcfDNA between 2 and 9 days prior to detection by blood culture. The MPM for the mcfDNA results that were found by blood culture were higher than MPM for other organisms. Patient 20 had a complicated clinical course which is shown in Figure 2. In addition to the *Staphylococcus epidermidis* detected by mcfDNA and on blood culture, this patient had an additional mcfDNA detection of *Mycoplasma hominis* which was discordant with results found on autopsy. Of note, this patient had sequential increases in the MPM for *Mycoplasma hominis* on consecutive plasma samples (Figure 2). Patients 36 and 38 had mcfDNA results that were concordant with clinically adjudicated findings of molluscum contagiosum on exam and pneumatoasis intestinalis on radiograph.

Approximately one third of the plasma mcfDNA detections were clinically inconsistent with microbiologic or clinically adjudicated results (Table 4). In three of the patients, the discrepancy was related to RNA viruses.
However, most of the discrepancy arose from detectable plasma mcfDNA in patients with clinical symptoms. For example, patient 20 had low MPM levels of *Lactobacillus fermentum*, *Prevotella melaninogenica* and *Veillonella parvula* detected by mcfDNA in the setting of mucositis with negative microbiologic studies. We hypothesized *a priori* that discordant results like this could represent low levels of translocation in the setting of increased inflammation after CAR T infusion. Such low levels might not be associated with clinical signs or symptoms of illness. Therefore, second objective, we evaluated whether measures of inflammation including temperature, CRS, ALT, AST, ferritin, LDH and CRP correlated with mcfDNA results using either definition for positivity (Supplemental Table 2). There was no correlation between the detection of plasma mcfDNA and inflammatory markers.

Nine patients had detectable mcfDNA with no microbiologic or clinical signs of infection (Table 4). Most of these patients had mcfDNA results which detected viruses that are not routinely assessed in our clinical lab, such as human herpesvirus 5. Three patients (patients 2, 15, and 20) had infection detected by conventional methods with no mcfDNA reported by KT from samples sent within 7 days of conventional testing. Patient 15 had 10,000 colonies of *Escherichia coli* detected in his urine in the setting of a neurogenic bladder which could represent colonization rather than true infection. Of note, he was treated with two days of ceftriaxone due to bladder pain. Patent 2 had plasma mcfDNA testing after he had been on antimicrobial treatment. On day 11 after CAR T cell infusion, Patient 2 became febrile, and blood cultures grew *Streptococcus mitis* from two of two bottles. He was started on cefepime and remained persistently febrile with negative blood cultures on day 12 and 13 post infusion. Plasma mcfDNA collected on day 14, three days after initiation of antibiotics and subsequent negative blood cultures, detected *Escherichia coli* (11,560 MPM). No blood culture was collected the day that *Escherichia coli* mcfDNA was detected. The patient remained on cefepime and subsequently defervesced. Finally, patient 20 had HSV detected from the eyelid with negative mcfDNA suggesting that it may have been a localized infection.

**Discussion**

This retrospective cohort study evaluated plasma mcfDNA results in 25 pediatric and young adult patients with R/R B ALL who underwent 27 CAR T cell infusions at a single institution. We are aware of only two groups that have evaluated mcfDNA testing in patients receiving CAR T cell therapy. Both studies were outside the United States. Nie et al. sent plasma mcfDNA from 102 patients who experienced a fever in the first 30 days after CAR T cell infusion. Sixty-nine patients (67.6%) had detectable mcfDNA, and 51 patients (50%) had infection detected by conventional methods. Viral organisms were most commonly detected followed by bacterial and then fungal. Shen et al. performed plasma mcfDNA testing in 51 adult patients who experienced a fever in the first 30 days after CAR T cell infusion. Twenty seven patients (52.9%) had detectable mcfDNA, and 5 patients (9.8%) had infection detected by conventional methods. Bacterial organisms were most commonly detected followed by viral and then fungal. In both studies, mcfDNA results were considered “positive” if the organism detected had a coverage rate scored 10-fold greater (bacteria and viruses) or 5-fold greater (fungi) than that of any other microbes. The patients were primarily adult and received CAR T cell therapy for a variety of malignancies including ALL, multiple myeloma, acute myeloid leukemia, lymphoma, chronic myeloid leukemia, and chronic lymphocytic leukemia.

As with other studies in patients with R/R B ALL after CAR T cell therapy, we found a high rate of clinical and microbiologic infection in this small pediatric CAR T cohort with 4 of 27 infusions (14.8%) complicated by bacteremia. We did not find that inflammation as assessed by ALT, AST, ferritin, LDH and CRP and CRS score was associated with increased level of plasma mcfDNA. We hypothesize that the detection of low level mcfDNA may be related to translocation from mucosal barrier injury rather than increased inflammation after CAR T infusion in this patient population.

Plasma mcfDNA can be especially helpful in detecting fastidious organisms or those not typically detected by conventional testing. This was true of the detection of *Mycoplasma hominis* in patient 20. *Mycoplasma hominis* is a fastidious organism which does not routinely grow in blood cultures. In patient 20, *Mycoplasma hominis* likely contributed to her death and was only detected at autopsy. In another example of DNA viral detection, HHV-6 was the most commonly detected organism by mcfDNA. Our hospital does not routinely...
test for this virus. Rebechi et al. described two cases of HHV-6 encephalitis in adults after CAR T cell therapy. In one of these patients, diagnosis was significantly delayed because the symptoms of encephalitis were initially thought to be secondary to neurotoxicity after CAR T cell therapy rather than HHV-6.22 In our study, many of our asymptomatic patients only had viruses detected by mcfDNA.

We observed that a higher mcfDNA MPM was generally associated with microbiologic concordance. Several other groups have noted an association between higher mcfDNA MPM and increased likelihood of concordance with conventional diagnostic testing. For example, Lee et al. retrospectively reviewed the clinical utility of mcfDNA at a pediatric institution over a 2 year period.23 They classified mcfDNA results as “clinically relevant” if a) the organism matched an organism diagnosed through conventional testing or b) the organism matched the clinical scenario. The median MPM for clinically relevant organisms was significantly higher than for “clinically irrelevant” organisms.

To avoid potential bias, we established definitions of concordance a priori. We found this task unexpectedly challenging. For example, patient 10 had 10,000 colonies of *Escherichia coli* detected in his urine but a negative mcfDNA result. This was in the setting of a neurogenic bladder so may have represented colonization and not true infection. However, since we had not considered this in our definition of concordance a priori, we classified these results as discordant. The difficulty in defining concordance and determining whether an organism is pathogenic is evident in the widely variable definitions across the literature.6,8,11,19,20 Establishment of standardized definitions would aid in interpretation and clinical applicability of plasma mcfDNA going forward.

KT reported mcfDNA from more than one organism in 36.7% of our samples. Others have reported mcfDNA from more than one organism in 47-61% of samples.8,11,12 These polymicrobial results could represent transient mucosal barrier injury, translocation, colonization, or contamination. To help elucidate this, others have incorporated negative controls. For example, Blauwkamp et al. performed mcfDNA in 350 patients with a “sepsis alert” and 166 control samples with no concerns for sepsis.6 The test reported detection in 62 of 166 control samples. They noted lower MPM in control samples suggesting that lower levels of mcfDNA may represent colonization rather than infection. Others have also detected mcfDNA in control samples.9,18

It is important to note that negative plasma mcfDNA results do not guarantee absence of infection. Absence of mcfDNA detection can be secondary to a localized infection, prior antimicrobial treatment, obtaining the sample late in the infection, a microbe not currently in the database, or a plasma sample that fails sequencing depth for a variety of reasons. For example, in a group of six pediatric oncology patients with proven invasive fungal disease, mcfDNA detected the same organism in only 4 of 6 cases.7 In the case of a 15 month old with ALL admitted for febrile neutropenia with an eschar on her cheek, mcfDNA was negative, but Rhizopus was identified on 18s rDNA and culture from a skin biopsy. These situations may represent localized infections with lack of dissemination, similar to our patient 20 who had a localized HSV infection of an eyelid with negative plasma mcfDNA. Eichenberger et al. reported on plasma mcfDNA results from patients with culture-confirmed bloodstream infection collected within 24 hours of the index positive blood culture. In 15 of 140 samples, mcfDNA did not detect the organism from the positive blood culture.18 As with our patient 2 with *S. mitis* bacteremia, it is possible that the plasma mcfDNA was obtained too late after the blood culture was obtained to detect *S. mitis*. Although mcfDNA can be detected for longer than organisms detected by blood culture, there is a decline in mcfDNA over time.18

To our knowledge, this pilot study represents the first report of plasma mcfDNA surveillance in pediatric and young adult patients with R/R B ALL in the first two months following CAR T cell infusion. Strengths of this study include application of a novel technology to a uniform population of patients, availability of samples across multiple time points, and concurrent clinical and microbiologic data. This study has several limitations including a small sample size and plasma mcfDNA testing on previously frozen samples which could increase the risk of environmental contamination. Furthermore, plasma was banked at prespecified time points that did not necessarily align with clinical events and not all patients had banked samples from all time points. Despite these limitations, this study provides the foundation for a future larger study investigating plasma mcfDNA in patients after CAR T cell therapy. As with any other diagnostic test, clinicians must
interpret mcfDNA results within the clinical context in patients with high pre-test probability for infections. Current microbiologic testing has significant pitfalls, and unbiased plasma mcfDNA may be especially useful for early detection of fastidious organisms and to guide diagnosis and treatment in persistently febrile or clinically unstable patients after CAR T cell therapy.

Acknowledgements

We would like to acknowledge the funding provided by the Stanford Maternal and Child Health Research Institute which helped support this project.

Conflict of Interest Statement

Drs. Aftandilian, Davis and Bito have no conflicts of interest to disclose.

Dr. Duttagupta was previously employed by Karius.

Dr. Berman is currently employed by Karius.

References


Legend

Figure 1. Summary of mcfDNA samples analyzed

Figure 2. Clinical history, conventional testing results and mcfDNA results in patient 20

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