Editorial Commentary

A big STEP (Simultaneous Tumor and Effector Profiling) forward in the battle against chimeric antigen receptor (CAR) T-cell therapy resistance

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Comment on: Sworder BJ, Kurtz DM, Alig SK, *et al.* Determinants of resistance to engineered T cell therapies targeting CD19 in large B cell lymphomas. Cancer Cell 2023;41:210-25.e5.

Keywords: Chimeric antigen receptor (CAR); CD19 CAR T-cell; lymphoma; resistance

Submitted Jul 17, 2023. Accepted for publication Sep 22, 2023. Published online Oct 10, 2023.
doi: 10.21037/tcr-23-1247
View this article at: https://dx.doi.org/10.21037/tcr-23-1247

Chimeric antigen receptor (CAR) T-cell therapy is an effective treatment that has emerged as a game-changing approach in the treatment of hematological malignancies. This has brought about significant advances in the management of acute lymphoblastic leukemia, multiple myeloma, and diffuse large B-cell lymphoma (DLBCL) as well as in solid tumor neuroblastoma (1). CAR-T cell therapy combines the targeting capabilities of monoclonal antibodies with the persistence and cytotoxicity of T lymphocyte mediated responses. To implement this approach, a patient's autologous T-cells are harvested and genetically engineered to incorporate the CAR—a synthetic surface protein (2). After *ex vivo* expansion, the genetically engineered T-cells are reinfused and binding to the epitope on malignant target cells leads to the activation and proliferation of the CAR T-cells. This activation subsequently initiates the process of elimination of tumor cells by the CAR T-cells.

Around one-third of large B-cell lymphoma (LBCL) patients relapse after first-line chemoimmunotherapy and require subsequent therapy (3). The use of anti-CD19 CAR-T cell therapy has become the standard second-line therapy for relapsed LBCL after the Zuma-7 trial showed improved event-free survival (EFS) and responses with axicabtagene ciloleucel (axi-cel) when compared to standard treatment. At a follow-up of 24.9 months, the median EFS was 8.3 months for the axi-cel group and 2.0 months for the standard-care group (4). Axi-cel was initially approved for use in LBCL after data from the ZUMA-1 trial demonstrated efficacy in achieving responses in LBCL after failure of standard therapy (5,6). Another study done with brentuximab vedotin, a CD30-directed antibody-drug conjugate (ADC), of a five-year follow-up for patients with stage III or IV Hodgkin's lymphoma showed progression-free survival (PFS) benefits for those treated with brentuximab vedotin plus doxorubicin, vinblastine, and dacarbazine (A + AVD), as compared to the above with bleomycin (ABVD) (7). The overall survival (OS) percentages were 93.9% for A + AVD and 89.4% for ABVD, while PFS was shown to be longer with A + AVD than in the ABVD group (7). These pivotal trials have paved the way for future clinical trials to assist in choosing the right CAR T-cell product for patients with LBCL and stratify patient response with molecular tumor

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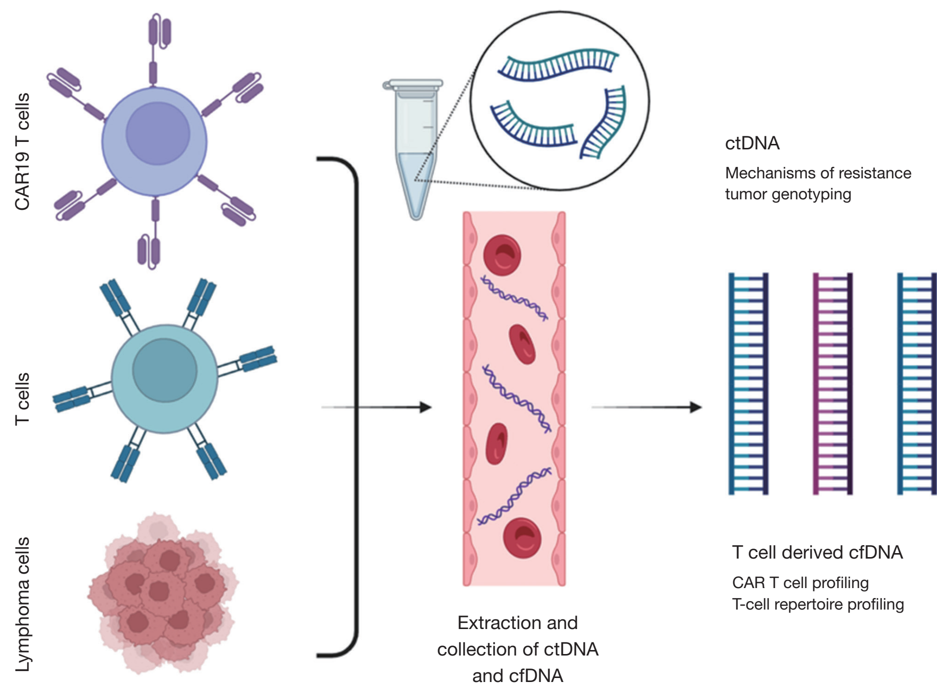

Figure 1 A visual representation demonstrates the approach employed by the STEP platform, which enables the simultaneous profiling of ctDNA, cfCAR19, and cfTCR from a plasma sample. CAR19, anti-CD19 chimeric antigen receptor; ctDNA, circulating tumor derived DNA; cfDNA, cell-free DNA; STEP, Simultaneous Tumor and Effector Profiling; cfCAR19, CAR19-derived cell free DNA; cfTCR, cell free T-cell receptor rearrangements.

characteristics (8). Despite the efficacy of anti-CD19 CAR T-cell (CAR19) therapy in LBCL, the mechanisms of resistance to CAR T-cell therapy need to be better understood in order to determine which patients may be at high risk of resistance and how to design future trials to circumvent resistance. Certain tumor genomic factors, such as BCL2 translocations as well as apolipoprotein B mRNA editing enzyme catalytic polypeptide-like (APOBEC) protein mutational signatures, are linked to failure of treatment in CAR T-cell therapy (9,10). Recently, techniques for detection of cell-free DNA (cfDNA) derived from tumors, known as circulating tumor derived DNA (ctDNA), have provided valuable opportunities for studying tumor biology noninvasively. These methods involve analyzing ctDNA present in the plasma, offering insights into tumor dynamics and response to therapy without the need for invasive procedures. Targeted sequencing methods like cancer personalized profiling by deep sequencing (CAPP-seq) have proven effective in identifying various genetic alterations derived from tumors using ctDNA. These alterations include somatic copy number alterations (SCNAs), single-nucleotide variants (SNVs), gene fusions, and small insertions/deletions (indels). By utilizing ctDNA, these techniques offer a means of molecular profiling without relying on tumor tissue samples (11).

Although ctDNA profiling has shown promise in assessing tumors in LBCL, the comprehensive evaluation of both tumor cells and non-tumor cells, including both engineered and native T-cells has not been addressed during active therapy. To address this gap, Sworder *et al.* extended the CAPP-seq technique by implementing a hybrid capture strategy (12). This strategy enables the concurrent assessment of tumor-derived ctDNA and CAR19-derived cell free DNA (cfCAR19) originating from specific recombinant retroviral sequences. They applied this combined approach to patients receiving axi-cel treatment and by doing so, the authors could simultaneously analyze the molecular response, identify alterations in genes associated with failure of treatment, and profile CAR19 activity. This platform that the authors called Simultaneous Tumor and Effector Profiling (STEP) facilitated a comprehensive understanding of how these factors interact and contribute to CAR19 resistance, enabling integrated analyses of their combined impact (*Figure 1*).

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In this study, the authors conducted profiling on a comprehensive set of 708 blood and tissue samples obtained from two distinct cohorts: a discovery cohort (consisting of 65 subjects) and a validation cohort (consisting of 73 subjects). These individuals were undergoing axi-cel therapy for relapsed or refractory LBCL at Stanford University. Blood samples, including peripheral blood mononuclear cells (PBMCs) were collected before lymphodepletion therapy and prior to CAR T-cell therapy infusion. Serial blood samples were collected longitudinally after treatment, allowing the authors to track changes over time. Additionally, samples were obtained at the time of relapse to assess the disease progression.

By utilizing the STEP platform, the authors measured the levels of ctDNA, cfCAR19, and cell-free T cell receptor rearrangements (cfTCR) before CAR19 infusion and at numerous time points following cell therapy in the discovery cohort.

A study has suggested that higher levels of ctDNA before and after treatment occur in patients undergoing CAR19 therapy (13). In line with these studies, their observations revealed that patients who eventually experienced disease progression had significantly higher levels of ctDNA prior to treatment initiation. The median ctDNA levels 1 week post infusion decreased in the ongoing responders and progressors as well, and higher ctDNA levels were exhibited in the progressors during therapy. In particular, the analysis demonstrated that elevated levels of ctDNA were correlated with disease progression both one week and four weeks after CAR19 T cell infusion. This suggests that higher ctDNA levels at these time points may serve as indicators of unfavorable treatment outcomes and advanced disease.

Using the discovery cohort, Sworder *et al.* aimed to identify the optimal level of ctDNA that could be used to stratify patients based on EFS following axi-cel therapy. Surprisingly, they found that the same thresholds for ctDNA levels, which have previously been validated to stratify outcomes in treatment-naive LBCL patients undergoing chemoimmunotherapy, were also applicable in this context. Notably, on the day of CAR T-cell therapy infusion, they observed a strong correlation between high ctDNA levels and shorter EFS. This suggests that elevated ctDNA levels can serve as a robust predictor of poorer outcomes in terms of EFS following CAR T cell therapy.

After establishing the molecular thresholds for ctDNA levels at baseline and early stages of therapy in the discovery cohort, the authors proceeded to validate their significance in an independent validation cohort. Significantly, they found that both the pretreatment ctDNA threshold on day 0 ($P=0.003$) and the major molecular response (MMR) threshold at week 4 ($P=0.028$) successfully stratified EFS outcomes in the validation cohort. This validation reinforces the importance of these ctDNA thresholds as predictive markers for assessing treatment response and prognosis in patients receiving axi-cel therapy. To quantify cfCAR19, Sworder *et al.* utilized a method that involved aligning sequenced reads to an expanded human genome that included the retroviral genome construct specific to axi-cel. It's worth noting that the concentration of cfCAR19 per milliliter of blood, as measured by CAPP-seq, was approximately 1,000 times lower than the levels of CAR19-positive PBMCs measured by CAR19 fluorescence-activated cell sorting (CARFACS). Despite this disparity in concentrations, there was a significant correlation between these two measurements of CAR19 T cells, with a Spearman correlation coefficient of 0.69 and a P value less than 0.001.

The correlation between cfCAR19 and cellular CAR19 measurements persisted at both early (week 1: Spearman $r=0.60$, $P<0.001$) and later (week 4: Spearman $r=0.63$, $P<0.001$) time points. This correlation was consistently observed in the validation cohort as well (Spearman $r=0.67$, $P<0.001$). Notably, these findings suggest that the expansion and persistence of CAR19 T cells can be quantified accurately using cfDNA. Interestingly, the levels of cfCAR19 were found to be similar between patients who experienced ongoing response to the treatment and those who had treatment failure. There was no significant difference in cfCAR19 levels between these two groups, indicating that the quantification of cfCAR19 may not be predictive of treatment outcomes in terms of response or failure.

Consistent with previous research findings, the authors observed a characteristic difference in fragment length profiles between mutant ctDNA molecules and wildtype cfDNA counterparts. Additionally, they found that retroviral cfCAR19 DNA fragments were shorter in length compared to wildtype non-tumor-derived human cfCAR19 DNA fragments. The size profile of cfCAR19 fragments resembled that of mutant ctDNA fragments derived from lymphoma, suggesting that a subset of shorter cfCAR19 molecules originates from CAR T cells present within the tumor microenvironment and exposed to tissue nucleases. Conversely, bigger cfCAR19 fragments may better represent circulating CAR T cells. These fragment length differences provide insights into the dynamics and sources of cfCAR19 DNA molecules in the context of CAR T cell therapy.

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The proportion of "long" cfCAR19 molecules (>310 bp) was found to be significantly correlated with the levels of circulating CAR19 T cells measured by CARFACS (Spearman r=0.36, P<0.001). However, such correlation was not observed for "short" cfCAR19 molecules (<150 bp). Interestingly, the authors also observed that blood samples with high levels of circulating CAR19 T cells, as determined by CARFACS measurements above the median of all post-treatment measurements, had significantly longer cfCAR19 fragments compared to samples with lower CAR19 T cell levels. Additionally, CAR19 higher measurements of CAR19 are predictive of significantly higher EFS. These findings suggest that the length of cfCAR19 fragments may provide valuable information about the abundance and activity of CAR19 T cells in circulation.

Sworder *et al.* set out to establish a genomic map from both pretreatment tumor and plasma samples in patients with relapsed LBCL and treatment naïve LBCL. The reported quantity of mutations was similar in both cohorts. However, when comparing the somatic mutational profile in pre-CAR19 DLBCL (excluding other LBCL histologic subtypes or transformed low-grade lymphomas), the relapsed DLBCL cases had more frequent alterations in tumor suppressor gene *TP53*, proto-oncogene *MYC* and transcription activator *EP300* than treatment naïve DLBCL. The cell-of-origin (COO) algorithm uses gene expression profiling to categorize the molecular heterogeneous DLBCL into germinal center B-cell-like (GCB) and activated B-cell-like (ABC) subgroups (14). Here, COO distributions were leaning to a predominant GCB classification in the relapsed cohort. Use of the LymphGen algorithm, a tool in precision medicine that converts DLBCL next generation sequencing data into 7 molecular subtypes, identified an enriched distribution of EZB (based on EZH2 mutations and BCL2 translocations) and A53 (characterized by TP53 mutations and deletions) subtypes in the relapsed/refractory cohort, and MCD (gain of function mutations in MYD88 and CD79B) tumors in treatment naïve DLBCL (15). Interestingly, A53 enriched tumors frequently carry mutations that provide mechanisms of immune surveillance escape, by deleting or mutationally inactivating β2-microglobulin (*B2M*). This is something to keep in mind when observing response to CAR19 in the relapsed DLBCL cohort.

Defining a catalog of genetic alterations that arise as a result of CAR-T cell therapy is another benefit provided by the STEP platform that could in the future help stratify patients who would benefit from this treatment. A series of *de novo* mutations following CAR19 T cell treatment was associated with inferior EFS in both cohorts (n=138). Among these, mutations in *TMEM30A*, which plays a role in immune microenvironment modulation, were identified only in patients who progressed after therapy. *TMEM30A* regulates the signal that promotes phagocytosis by macrophages and has the potential to predict the therapeutic response to macrophage checkpoint inhibitors. The prognostic significance of all gene mutations in a comprehensive analysis revealed that the *TMEM30A* mutation, especially when bi-allelic alterations are present, is a strong favorable prognostic factor in R-CHOP therapy for aggressive B-cell lymphomas (16). The function of the *TMEM30A* mutations identified by the STEP analysis remains to be elucidated at this time, but nevertheless should be explored in patients after CAR-T cell treatment failure. Other significant identified CAR19 resistance-associated alterations include *IRF8*, critical for B-lymphocyte development and shown to reduce DLBCL proliferation in knockdown models (17), and *TP53*. Sworder *et al.* were able to further categorize those genomic alterations that were under positive selective pressure from CAR19 T cells, and found alterations in *CD19*, *PPM1D*, *TP53*, and *PAX5* among progressing patients.

The authors evaluated tumor genotype and plasma cfCAR19 levels from both pre- and post-CAR19 time points to assess how tumor intrinsic factors can influence therapy outcomes. Target antigen loss and checkpoint gene copy number alterations were among the novel mechanisms of immune evasion identified at time of relapse. A case of a patient that relapsed post-CAR19 showed that plasma ctDNA level increase was correlated with increasing allele frequency of a *CD19* nonsense mutation and associated with CAR19 cell re-expansion. This further supports the notion that ctDNA and cfCAR19 provide insight to intrinsic tumorigenic mechanisms. CAR19 re-expansion should not be interpreted as a favorable outcome, as persistence of CAR19 cells permits immune evasion through genetic or epigenetic tumor *CD19* loss and thus treatment failure.

A CAR19 expansion index was established in each patient by integrating cfCAR19 and flow cytometry measurements to inspect the relationship of the tumor genotype and CAR19 expansion. Patients with an initial low expansion had more mutations in *TNFRSF14* and *BCL2*, while patients with high expansion had more *IRF4* mutations. Deconvolution of the tumor immune microenvironment in pre-CAR19 relapsed LBCL revealed that tumors harnessing the *TNFRSF14* mutation had increased levels of resting

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memory CD4 T cells and T follicular helper cells. Further description of tumor genotypes and the evolution of their respective tumor immune microenvironment after CAR19 infusion is imperative to better understand the influence these factors have on CAR19 expansion. Perhaps another useful correlation found by Sworder *et al.* via CAPP-seq quantification is that of CAR19 levels in tumors after relapse and patients' plasma cfCAR19. This is because high intratumoral CAR19 levels at relapse are associated with T-cell exhaustion and thus ineffective CAR19 therapy.

An important limitation in this study is the absence of a mutational signature common for the majority of the relapsed LBCL cases that progress after CAR19. Although this means a standard next step in treatment for all cases might not be available, we can still use plasma cell free DNA findings to recognize when a patient will benefit from either continued CAR-T cell therapy or from discontinuation and use of therapeutic alternatives. To support this, hazard regression models were used to define the relationship between ctDNA and cfCAR19 plasma levels with EFS and OS in the patient cohort. A good prognostic indicator includes the finding that increased cfCAR19 in week 1 following CAR19 therapy was associated with a significantly improved EFS. Furthermore, higher ctDNA levels at all time points were associated with decreased EFS and OS.

Overall, the study by Sworder *et al.* represents a big first STEP in better understanding CAR T-cell therapy resistance mechanisms and highlighting areas in which we still need further investigation. Through their method of simultaneous profiling of ctDNA, cfCAR19, and cfTCR, a major finding of theirs is that alterations in numerous gene classes are associated with resistance. Further, somatic tumor alterations affect CAR19 T cell expansion and persistence. Lastly, CAR19 T cells shape tumor phenotype and genotype in a reciprocal manner. These findings will improve CAR-T personalized therapeutics.

Acknowledgments

Funding: The study was supported by the National Cancer Institute (NCI)/the National Institutes of Health (NIH) (No. K08CA230319 to JT), the Doris Duke Charitable Foundation (to JT) and the Edward P. Evans Foundation (to JT).

Footnote

Provenance and Peer Review: This article was commissioned by the editorial office, *Translational Cancer Research*. The article has undergone external peer review.

Peer Review File: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-1247/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-1247/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Cite this article as: Sondhi AK, Risso CB, Taylor J. A big STEP (Simultaneous Tumor and Effector Profiling) forward in the battle against chimeric antigen receptor (CAR) T-cell therapy resistance. Transl Cancer Res 2023;12(10):2442-2447. doi: 10.21037/tcr-23-1247

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