P #858

Rigorous Assessment of Off-Target Editing by CRISPR/Cas9 in VOR33, an Engineered Hematopoietic Stem Cell Transplant for the Treatment of Acute Myeloid Leukemia

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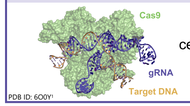
INTRODUCTION

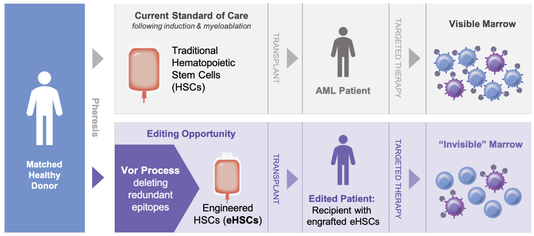
- VOR33 is an engineered allogeneic hematopoietic stem cell (HSC) transplant for treatment of acute myeloid leukemia (AML) in which the CD33 surface antigen is removed by CRISPR/Cas9 gene editing (**Figure 1**).[1]
- This removal enables post-engraftment immunotherapeutic targeting of leukemic cells that display CD33 while sparing the *CD33* gene-edited graft (**Figure 2**).[2-4]
- To ensure safety of gene-edited CD34+ hematopoietic stem and progenitor cells (HSPCs) and engrafted progeny, a well-defined analyses of unintended and off-target editing is necessary. However, paradigms for off-target analyses of gene-edited *ex vivo* therapies are not well established.

Figure 1. VOR33 Engineering



CRISPR/Cas9 knockout of biologically dispensable CD33 cell surface antigen in CD34+ HSPCs to create transplantable engineered HSCs (eHSCs) invisible to CD33-targeted immunotherapies

Figure 2. VOR33 Clinical Process



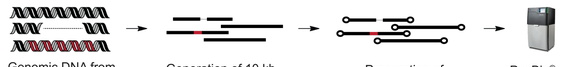
OBJECTIVE

- To enable rigorous assessment of unintended and off-target editing events by CRISPR/Cas9 in VOR33 using an ensemble of sensitive genomic assays and approaches.

OFF-TARGET STRATEGY

Potential Off-Target Concerns	Analytic Approach
Unintended on-target structural variation (SV)	**Long-range PCR and long-read DNA sequencing (Figure 3)**
Off-target sites with high homology to *CD33* on-target site	***In silico* prediction of possible genomic sites ≤5 mismatches (Figure 4)**
Off-target sites with moderate/poor homology to *CD33* on-target site	**Unbiased identification by GUIDE-seq (Figure 5)**
Quantifying off-target indel frequencies	**Hybrid capture-based next generation sequencing (NGS) (Figure 6)**
Gross genomic instability	**G-banded karyotyping (Figure 7)**

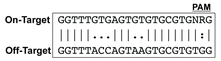
Figure 3. Unintended On-Target SV Detection: Long-Range PCR and Long-Read Sequencing of On-Target *CD33* site



PacBio® SMRT long-read sequencing facilitates detection of on-target *CD33* structural variants, such as large DNA deletions, insertions, and inversions >50 bp.

Figure 4. *In Silico* Off-Target Prediction

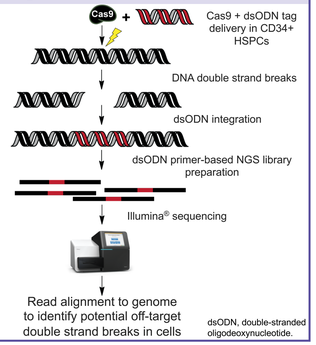
A deep query of genomic sites containing a 5'-NRG-3' PAM and (i) ≤5 mismatches with no indels, or (ii) ≤3 mismatches and a one-base insertion, or (iii) ≤3 mismatches and a one-base deletion relative to the on-target protospacer was performed with an internal pipeline based on Cas-OFFinder.[5]



Illustrative example of on-target and off-target with 5 bp mismatch

PAM, protospacer adjacent motif.

Figure 5. Unbiased Identification of Off-Target Sites: GUIDE-seq[6]



dsODN, double-stranded oligodeoxynucleotide.

Figure 6. Homology-Dependent Off-Target Editing: Hybrid Capture-Based NGS[7]

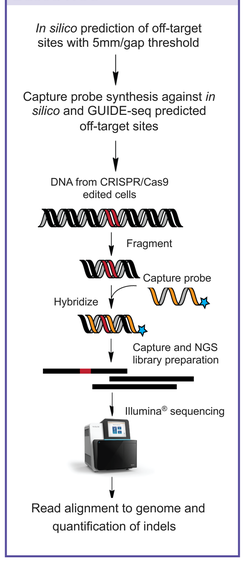
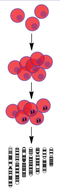


Figure 7. Genomic Stability Detection: G-banded Karyotyping



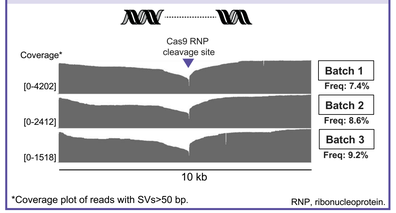
Evaluation of potential gross chromosomal aberrations such as translocations or aneuploidy.

RESULTS

- Analysis of on-target SVs by long-read sequencing revealed total frequencies of 7%–9% across 3 VOR33 research-scale batches. Large deletions account for the majority of SVs. Large on-target inversions and insertions were detected at very low frequencies, similar to those previously reported for CRISPR/Cas9 (**Figure 8**).[6] Fine mapping of the SVs suggests no perceivable impact on the safety or efficacy of VOR33, as the primary mechanism of action (MOA) by CD33 disruption is preserved.
- By conducting GUIDE-seq analysis on 4 research-scale batches, a total of 29 sites were identified, with 10 showing high homology to the on-target site (≤5 mismatch/gaps) (**Table 1**). The remaining 19 sites had moderate/poor homology (≥7 mismatch/gaps).
- In 4 research scale VOR33 batches, indel frequencies were assessed by hybrid capture-based NGS at >2300 *in silico* predicted sites (**Table 2**). In 7 VOR33 batches manufactured at clinical scale, indel frequencies were assessed by NGS at 2369 *in silico* and GUIDE-seq identified sites (reads ≥500) (**Table 3**). Across batches, no significant and reproducible off-target sites were observed.
- Lastly, karyotyping revealed no detectable abnormalities across multiple research and clinical scale batches, indicating that VOR33 displays preserved genomic stability (**Figure 9, Figure 10**).

Unintended On-Target Structural Variant Characterization

Figure 8. Deletions



*Coverage plot of reads with SVs>50 bp.

RNP, ribonucleoprotein.

Insertions/Inversions

Inversions and large insertions were detected at very low frequencies, between 0.02%–0.04% for insertions and 0.13% –0.18% for inversions across the 3 research scale batches.

Unbiased Prediction of Off-Target Sites

Table 1. GUIDE-seq Predicted Off-Target Sites From 4 Research Scale Batches With 2 dsODN Concentrations (8 Independent Samples)

Mismatches/gaps	Counts
3	8
4	1
5	1
6	-
7 and above	19

Homology Dependent Assessment of Off-Target Editing

Table 2. Research Scale Hybrid Capture NGS (4 batches)

Prediction	*In silico*
Batches	4 batches with matched unedited controls
Gender	2 male, 2 female
Sites tested	>2300
Sites with significant and reproducible indel frequencies above control threshold	0
Sites with reproducible indel frequency difference >0.2%[9]	0

- No reproducible and significantly edited off-target site was observed across 4 batches and >2300 tested sites. In total, >24,000 individual site assessments were performed across edited and control research scale samples.

Table 3. Comprehensive Clinical Scale Hybrid Capture NGS (7 batches)

Prediction	*In silico* and GUIDE-seq
Batches	7 batches with matched unedited controls
Gender	6 male, 1 female
Sites tested	>2300
Sites with significant and reproducible indel frequencies above control threshold	0
Sites with reproducible indel frequency difference >0.2%[9]	0

- No reproducible and significantly edited off-target site was observed across all 7 batches and >2300 tested sites. In total, >33,000 individual site assessments were performed across edited and control clinical scale samples.

Evaluation of Gross Chromosomal Stability

Figure 9. Research Scale (4 batches)

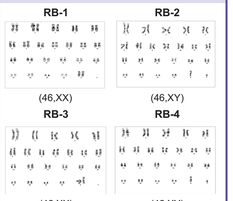
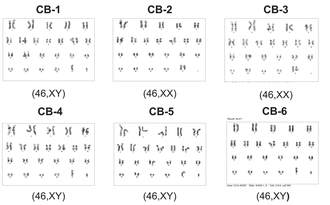


Figure 10. Clinical Scale (6 batches)



- Karyotyping across 10 VOR33 batches (20 examined cells/batch) revealed no detectable chromosomal abnormalities, indicating that the VOR33 process preserves genomic stability.

CONCLUSIONS

- The VOR33 engineering process is robust and reproducible, with no discernable differences in off-target frequencies or patterns in multiple independent cell batches generated with various guide RNA lots, manufacturing scales, and delivery methods.
- An expansive appraisal of off-target editing across multiple VOR33 products was achieved through long-read sequencing, GUIDE-seq, quantification of indel frequencies at >2300 genomic sites, and karyotyping.
- This assessment of off-target editing establishes a rigorous and clinically translatable safety framework to evaluate genotoxicity in CD34+ HSPC-based cell therapies for the treatment of relapsed/refractory AML.

References

1. Zhu X, et al. *Nat Struct Mol Biol.* 2019;26(8):679-685. **2.** Borot F, et al. *Proc Natl Acad Sci U S A.* 2019;116(24):11978-11987. **3.** Humbert O, et al. *Sci Transl Med.* 2019;11(503):eaaw3768. **4.** Kim MY, et al. *Cell.* 2018;173(6):1439-1453.e19. **5.** Bae S, et al. *Bioinformatics.* 2014;30(10):1473-1475. **6.** Tsai SQ, et al. *Nat Biotechnol.* 2015;33:187-197. **7.** Gnirke A, et al. *Nat Biotechnol.* 2009;27:182-189. **8.** Kosicki M, et al. *Nat Biotechnol.* 2018;36(8):765-771. **9.** Chaudhari HG, et al. *CRISPR J.* 2020;3(6):440-453.

Certain illustrations created with BioRender.com.

Acknowledgments

We thank Eric Anderson, Tobias Brambrink, Kerry Chios, Pete Cotter, Alejandra Falla, Juliana Xavier Ferrucio, Mark Jones, John King, Matthew Li, Michelle Lin, Jessica Lisle, Chong Luo, Ankit Mehta, Nipul Patel, Suzanne Pavluk, Taylor Perkins, Mike Pettiglio, Tania Philipp, Robert Pietrusko, Jeff Pimental, Bruce Ricart, Kienan Salvadore, Julian Scherer, and Roberta Toporovski.

Presented at

ASCGT 24th Annual Meeting, May 11–14, 2021.

See the companion oral presentation: 7: *VOR33: A Clinic-Ready CRISPR/Cas9 Engineered Hematopoietic Stem Cell Transplant for the Treatment of Acute Myeloid Leukemia*