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In Depth 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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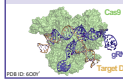
INTRODUCTION

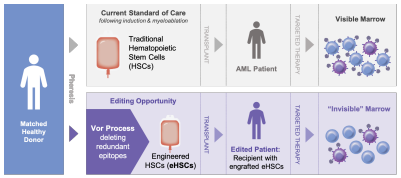
- VOR33 is an engineered allogeneic hematopoietic stem cell (HSC) transplant for treatment of acute myeloid leukemia (AML) in which the CD33 surface antigen is removed by CRISPR/Cas9 gene editing (**Figure 1**).[1]
- This removal enables post-engraftment immunotherapeutic targeting of leukemic cells 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



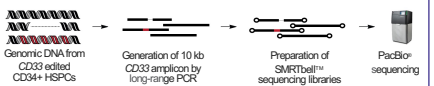
OBJECTIVE

- 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**)

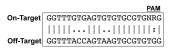
Figure 3. Unintended On-Target SV Detection: Long-Range PCR and Long-Read Sequencing of On-Target *CD33* site



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]

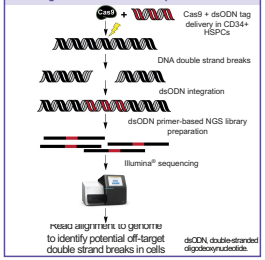


Figure 6. Homology-Dependent Off-Target Editing: Hybrid Capture-Based NGS[7]

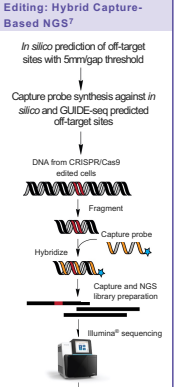
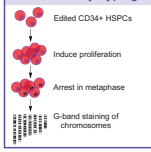


Figure 7. Genomic Stability Detection: G-banded Karyotyping



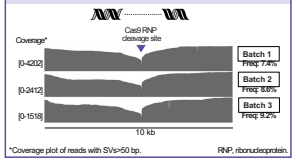
Evaluation of potential gross chromosomal aberrations such as translocations or aneuploidy.

RESULTS

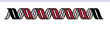
- Analysis of on-target SVs by long-read sequencing revealed total frequencies of 7%–9% across 3 VOR33 research-scale batches. Large deletions account for the majority of SVs. Large on-target inversions and insertions were detected at very low frequencies, similar to those previously reported for CRISPR/Cas9 (**Figure 8**).[8] 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



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)

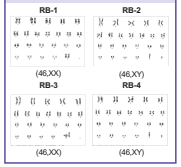
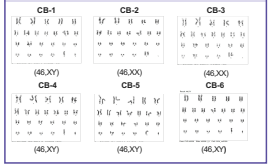


Figure 10. Clinical Scale (6 batches)



- 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 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.

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See the companion presentation: *Pre-clinical evaluation, including genomic off-target analysis, of VOR33: a clinic-ready CRISPR/Cas9 engineered hematopoietic stem cell transplant for the treatment of acute myeloid leukemia* (OR36).

VOR