Assessing Genetic Heterogeneity in the Context of Genome Editing Off-Targets in Gene Therapy Products: An FDA Public Workshop

The following is a summary of the public workshop and does not reflect conclusions from the FDA on the topic.

According to the Food and Drug Administration (FDA), gene therapy mediates effects by transcription or translation of transferred genetic material or by specifically altering host (human) genetic sequences (FDA 2020). Genome editing technologies, such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)-Cas-associated nucleases, including base editors, have provided a variety of tools to modify genomes with great precision (Li et al. 2020). These genetic editing technologies have vastly accelerated the pace of fundamental research in genome editing (Doudna 2020) and the creation of therapeutic products. Although these genome-editing modalities hold great promise for highly specific genetic engineering, it is crucial to critically examine potential off-target effects to refine the techniques and optimize their safety and efficacy. The potential impact of unintended changes, also referred to as off-targets or off-target editing, is a key consideration for the safety of genome editing as a therapeutic strategy. Unintended changes to the genome could be caused by modifying DNA at sites other than those being deliberately targeted (National Academies of Sciences 2017).

Off-target editing can potentially lead to unwanted mutagenesis and chromosomal rearrangements, such as translocations, deletions, and duplications, producing genomic instability. The potential for these off-target events is concerning given the causal function of certain known chromosomal rearrangements in human cancers (Taki and Taniwaki 2006) such as lung cancer (Maddalo et al. 2014) specifically human non-small cell lung cancers (Soda et al. 2007). The likelihood of off-target editing is partially affected by chromatin states, attributes of the editing modality, and the choice of target sites with low homology to other sequences in the genome — all factors that have been investigated (Kim et al. 2019). However, an emerging area of concern is for the potential impact of genetic heterogeneity, also known as genetic variation, on off-target editing.

According to the National Cancer Institute (NCI) at the National Institutes of Health, genetic heterogeneity is defined as “different genetic mechanisms that produce the same or similar phenotypes” (NCI 2023). NCI identifies two types of genetic heterogeneity: allelic heterogeneity, where different variants in a gene locus lead to the same or similar phenotypes, and locus heterogeneity, where variants of different gene loci lead to the same or similar phenotypes. Four types of genetic heterogeneity that could lead to human disease are defined as: 1) individually rare mutations working together, 2) single genes with numerous distinct rare severe mutations affecting unrelated individuals, 3) an identical mutation causing different phenotypes in separate persons, 4) mutations in separate genes from the same or related pathways causing identical disease (McClellan and King 2010). An analysis was performed on the extent of genetic heterogeneity in cancers and the effect of such genetic heterogeneity, in particular single-base substitutions, small insertions and deletions, in tumorigenesis and development of therapies (Reiter et al. 2019). Given the presence of genetic heterogeneity and its potential role in disease, considerations for the effects of genetic heterogeneity on gene therapies developed with genome editing technologies were warranted because genetic heterogeneity may alter the

off-target editing profile in these products on a patient-level basis. This is supported by recent scientific publications, which have concluded that genetic variants can affect or alter the frequency of off-target editing (Scott and Zhang 2017; Canver et al. 2018; Westermann et al. 2022).

While genome-editing products hold promise for the treatment of many diseases with unmet medical need, the technologies used to assess the safety of genome-editing products with respect to off-target editing are continuously evolving. Specifically, it is currently unclear how genetic heterogeneity should be factored in when performing validation of off-target editing. This is problematic given the possible effect genetic heterogeneity may have on producing or altering off-target editing.

On December 16, 2022, an FDA workshop was held to discuss the impact of individual genetic heterogeneity on genome editing, and best practices for validating off-target editing events in the context of human genetic heterogeneity. This workshop was focused on genome-edited gene therapy products and included academia, industry, and other stakeholders involved in research, development, and regulation of genome-edited gene therapy products. Throughout the day-long workshop, there were eight individual presentations, answers to audience questions, and a final panel discussion. Presenters offered their perspectives on best practices for identifying and validating off-target editing and genetic variation, as well as the impact of genetic heterogeneity on the identification of off-target editing. The workshop content was educational in nature and was not intended to officially convey new policies or processes involved in the development of gene therapy products. A meeting transcript and webcast link are also posted to the FDA website.

Presenters offered overviews of various experimental and computational methods for detecting, analyzing, and validating potential off-target effects, including strengths and weaknesses of each approach, characterization of genomic heterogeneity, and how some tools were explicitly designed to incorporate identification of genomic variation. The importance of evaluating the impact of genomic variation on off-target validation was a recurring theme throughout the workshop, which was hosted by the Office of Therapeutic Products (formerly the Office of Tissues and Advanced Therapies) of FDA's Center for Biologics Evaluation and Research (CBER). Summaries from each presentation and the panel are discussed below.

Presentations

Opening remarks from the FDA: CBER Director Dr. Peter Marks delivered opening remarks highlighting the importance of developing best practices for validating off-target editing events in genome editing in the context of genetic heterogeneity. Dr. Marks stated that sequencing the whole human genome has demonstrated that more than 99.9% of DNA sequences are shared between individuals. Therefore, single nucleotide polymorphisms (SNPs) can create significant effects on an individual's response to external factors, as well as their propensity to develop particular diseases. Understanding off-target effects of genome editing is crucial to maximize patient safety. As new technologies develop, a discussion of best practices for identifying and validating off-target editing events is a positive step forward in the field.

Session 1: Progress in Genome Editing Technologies

Dr. Matthew Porteus (Stanford University) highlighted the difference between ex vivo genome editing, wherein the edited cells are the drug, and in vivo genome editing, where the genome-editing vector acts as the drug. CRISPR/Cas9 has transformed genome editing technology by introducing double-strand breaks (DSBs) at specific locations based on a guide RNA (gRNA)-defined target sequence. Dr. Porteus stated this technology could vastly improve outcomes for the 6,000-10,000 individuals with monogenic diseases.

The crucial issues to assess are the frequency of a genetic variant creating a bona fide off-target effect, and the frequency of that off-target effect causing a clinical adverse event (AE). In this presentation, Dr. Porteus emphasized that the intrinsic bias of methods that detect off-target events necessitates a focus on streamlining the development of genomic medicines for rare diseases rather than creating more barriers. Ideas to achieve this are: revamping the orphan disease priority review voucher; creating a different regulatory path for rare diseases; permitting good non-GMP reagents; and establishing mechanisms to improve cost, efficiency, and safety in manufacturing.

Dr. Porteus also suggested that within a single healthy human there is at least 3-log-fold more de novo, natural genetic variation within the HSPC compartment than what a genome editing process creates. While the toolkit for genome editing continues to diversify, there are issues that need to be addressed to improve outcomes for patients. The tools for analyzing molecular genotoxicity are evolving and being refined, but they are not always standardized and made technically accessible. And as new tools are being developed, there can be a ripple effect of increasing the barriers to moving a genome editing strategy from the lab into clinical trials and then from clinical trials into an approved drug. Additionally, tools for functional toxicity/tumorgenicity to predict human results have not been validated. Further, if individualized SNPs show clinical evidence of causing AEs, scientific/evidence-based approaches should be used to uncover solutions. Overall, the message from this presentation was that some level of tolerance for AEs is necessary to develop crucial therapies for patients, and genome-editing-based therapies should be held to the same risk-benefit standard as other medicines.

Session 2: Human Genetic Variation

Dr. Gilean McVean (University of Oxford) played a leading role in the HapMap and 1000 Genomes Projects. He explained that the Human Genome Project has provided a reference for describing genetic variation with the HapMap and 1000 Genomes Projects, as well as the Telomere-to-Telomere (T2T) Consortium, which have helped generate more complete views of human genomic diversity.

Variety in genomics stems most commonly from SNPs as well as the roughly 100 inherited germline mutations present in each individual. And while most of the discovered genetic variants are rare or very rare, most variants in any one individual are common, with approximately 95% and 99% of variants in an individual occurring at a frequency of at least 5% and 1%, respectively, in the total population. Overall, genetic variation is not randomly distributed across individuals, within a genome, or with respect to each other. A widely used measure of population differentiation known as the fixation index shows that about 90% of all human genetic variation is shared among human population groups.

Both genetic and epigenetic variation within genomes can be substantial. Apart from duplications and copy number variations (CNV), new mutations are unlikely to generate a similar sequence. Certain CNVs are associated with pathogenic states, such as inherited risk for autism or generating loss-of-heterozygosity mutations in cancer. Therefore, CNV-susceptible sequences should likely be avoided when designing CRISPR-mediated therapies.

One consideration for on- and off-target CRISPR-based safety assessments is the importance of understanding sequence, which estimates where mismatches in gRNA could be. Overall, careful monitoring during trials and post-approval should be undertaken to ensure AEs are not the result of unexpected off-target editing.

Session 3: Human Genetic Variation in the Context of Genomic Medicines

A session from Dr. David Scott (Arbor Biotechnologies; MIT) began with the observation that the growth of human genome sequencing in health and disease is rapidly identifying new opportunities to intervene in disease at the genetic level. Scott explained that there are many different gene editing modalities with the potential to enable such genomic medicines. Genetic variation can potentially alter both the efficacy and safety of a gene editing target, and large-scale genome sequencing potentially enables population-based evaluation of gene editing targets.

Analysis of off-target variation for gene editing targets, spanning a collection of genes of interest to the field, shows that genomic variation can increase the likelihood of off-target editing compared to the reference genome. Off-targets are more likely to exist at high allele frequencies in small patient populations, while for large patient populations, significant off-targets are likely to emerge at low allele frequencies. Within individual genes, clusters of targets along the sequence can vary significantly in the number of predicted off-targets.

While on-target variation is not common, cytosine-phosphate-guanine (CpG) content in targets and protospacer adjacent motifs (PAMs) may increase target variation in patient populations. Few targets contain common variation observable in the Exome Aggregation Consortium dataset, but clusters of targets with cumulatively higher or lower amounts of on-target variation can be found distributed across most genes.

Session 4: Quantifying Unintended Gene Editing Outcomes: In-Silico Approaches and Beyond

Dr. Gang Bao (Rice University) defined off-target editing events as DNA cleavage induced by an engineered nuclease at a site anywhere in the genome other than the intended target site. This can cause mutations, deletions, translocations, inversions, and other chromosomal rearrangements, which may lead to gain or loss of function. Because of this, off-target effects need to be carefully and exhaustively analyzed. Dr. Bao's lab created the in silico prediction tool COSMID to identify and rank the potential off-target sites.

Bao said it is important to further develop machine-learning-based tools using training and test data sets. In silico tools developed for CRISPR/Cas9 systems can be adopted to predict gRNA-dependent off-target sites. In silico tools have been developed for off-target analysis with

varying predictability; therefore, more accurate in silico tools (including machine-learning-based tools) using more and better data are needed for clinical applications.

Recommendations included developing accurate off-target predictors for base editing and prime editing; combining in silico and experimental analyses to assemble a ranked list of potential off-target sites; and extensively studying unintended large gene modifications occurring with high frequencies at the Cas9 cut sites.

Session 5: Defining the Impact of Genetic Variation of Off-Target Activity of Genome Editors With Sensitive and Unbiased Biochemical and Cellular Assays

Dr. Shengdar Tsai (St. Jude Children's Research Hospital; NIH) emphasized that genome editors can have systematic off-target activity and that even low-frequency, off-target mutations may be relevant if they increase cell growth and oncogenic transformation. It is critical to use sensitive and unbiased experimental methods to identify them. Defining the location of potential off-target mutations enables critical safety monitoring, even if functional interpretation of off-target mutations remains challenging.

Tsai noted that in silico methods are simple in practice but that they are, by definition, biased by their initial assumptions. Thus, he has focused on developing sensitive and unbiased methods to define the genome-wide activity of editors, which are divided in two classes: cellular and biochemical methods. Cell-based methods like GUIDE-seq are what he called "unbiased" in that they do not require a priori assumptions about the sequence of off-target sites before starting experimental discovery. Discover-seq, another cellular off-target discovery method, detects DNA DSBs that are in the process of being repaired and bound by DNA repair factor MRE11.

However, since cellular off-target discovery methods are limited in sensitivity, biochemical methods — like CHANGE-seq and Digenome-seq, which are not dependent on transfection/transduction or DNA repair and have the potential for high sensitivity and may also be easier to automate and scale — are being developed. CHANGE-seq is more sensitive than cellular methods like GUIDE-seq so it is possible to identify ideal targets that are highly active and specific. Digenome-seq, which is advantageous in its simplicity, but limited in sensitivity, is based on the principle of whole genome sequencing of genomic DNA treated with Cas9 and a bioinformatic search for DNA with uniform ends that are likely associated with Cas9 cutting.

Sensitive and experimentally unbiased methods are critical for understanding the fundamental genome-wide off-target activity of editors, since small individual genetic differences can significantly affect off-target activity up to 3,000-fold. Novel precise genome editors such as base and prime editors may require fit-for-purpose methods to understand their off-target activity. Experimentally unbiased methods can identify critical "unknown unknowns," such as activity of gRNA contaminants or effects of genetic variation.

Session 6: Development of CRISPR Therapies for In Vivo and Ex Vivo Applications: gRNA Specificity, Off-Target Identification and Validation, and Genotoxicity Assessment

Dr. Laura Sepp-Lorenzino (Intellia Therapeutics) provided an overview of therapeutic approaches with CRISPR/Cas9. She discussed the advantages of employing rationally designed sgRNA candidates to introduce DSBs with high precision. Appropriately identifying

sgRNA candidates helps avoid unintended off-target edits, improving the overall safety profile. Optimal sgRNA identification relies on several available resources as well as company guidelines, tools, and customizable applications.

The process of ruling-out sgRNAs impacted by common or pathogenic SNPs enriches the candidate pool. Steps are taken to eliminate those with the potential to elicit off-target events within the consensus reference genome. Human genetic variation is modeled by incorporating known SNPs and naturally occurring indels to understand their potential impact on off-target activity. Further selection is based on on-target activity measured by experimental approaches.

When evaluating genotoxicity, using orthogonal, in silico, and experimental approaches to discover all potential off-target sites, followed by aggregation of potential sites in a list of overlapping loci to determine bona fide off-targets, is recommended.

Beyond nucleotide-level effects, chromosomal integrity is key to evaluating genome safety. Characterizing potential DNA structural variants can be achieved using multiple technologies. Some options include short-read next-generation sequencing (NGS), which provide high-sensitivity molecular characterization, long-read NGS, which can detect large insertion-deletion events, and Pinpoint FISH that allows for direct visualization of the genome.

Session 7: Understanding the Impact of Somatic Mutations on Molecular Phenotypes in Induced Pluripotent Stem Cells

Dr. Kelly Frazer (University of California, San Diego) focused on evaluating somatic mutations within patient cells that arise in vitro but are not due to off-target editing effects. She explained that prior research indicated that the average somatic mutation rate in induced pluripotent stem cells (iPSCs) was similar to that observed in normal age-matched adult stem cells. Additionally, iPSC lines with a high mutation rate had a large fraction of C>T single nucleotide variants (SNVs) and CC>TT dinucleotide variations, typical of melanoma/UV damage.

The effects of somatic mutations on gene structure and function may vary, with intergenic and intronic mutations presumably having no impact; synonymous, noncoding, 5’ untranslated region (UTR), and 3’ UTR mutations having low impact; missense and splice site mutations having moderate impact; and nonsense and frameshift mutations having the highest impact. Subclonal SNVs are more likely to be low impact and to occur in functional chromatic regions versus clonal SNVs that were in the parental fibroblast cell line and were under evolutionary constraint. Therefore, somatic mutations that are present in the cell line naturally are under natural pressure and present less of a threat compared with newly arising somatic mutations in iPSCs.

There are several factors to consider when editing patient somatic cells in therapeutic programs because all somatic cells harbor somatic mutations. Most have no impact on gene function or safety and subclonal somatic mutations introduced during culturing of patient cells are likely to have greater functional impact. Whole-genome sequencing (WGS) is inexpensive and its use to compare pre- and post-edited CRISPR somatic cells would identify subclonal somatic mutations and enable their evaluation for functional impact.

Session 8: Human Genetic Diversity Alters Therapeutic Gene Editing Off-Target Outcomes

Dr. Luca Pinello (Harvard University) presented CRISPRme (Cancellieri et al. 2023), a web-based tool designed to consider single-nucleotide polymorphism (SNP) and indel genetic variants for the nomination and prioritization of off-target sites in CRISPR-Cas9 experiments. He noted that current guide-design or enumeration tools to assess off-target potential in genome editing are limited.

Putative on- and off-target sites may be missed by currently available methods that use aligners not optimized for scanning sgRNAs. Also, they may not be able to model complex RNA/DNA bulges or account for genetic variants. CRISPRme addresses these shortcomings through its haplotype-aware and exhaustive off-target search, which permits an arbitrary number of mismatches, bulges, and genetic variants. Importantly, CRISPRme analyses showed that in sickle cell disease and β-thalassemia clinical trials using a BCL11A enhancer-targeting gRNA (Frangoul et al. 2021; Fu et al. 2022), a top off-target allele was prevalent in African-ancestry populations; SpCas9 induced allele-specific indels in CD34+ HSPCs at this off-target, but high-fidelity Cas9 mitigated this off-target, emphasizing the importance of considering genetic variants.

He made several suggestions for best practices. The specificity of editing tools (Cas protein variants, gRNAs) should be maximized. Further, in silico and empiric methods for therapeutic genome editing off-target analysis should include, and in silico nomination methods should be aware of, off-target sites influenced by variants present in the target patient population. Risk assessment of any variant of off-targets (modification likelihood, genomic annotations, allele frequency) should be performed; and, if excess risk is identified, consider including genotype among screening inclusion criteria. Finally, when possible (e.g., hematopoietic cell targeting), prospectively monitor for somatic modifications in patient samples to gather information about the frequency and consequence of such events.

Session 9: Panel Discussion and Q&A — Genetic Variation

The workshop concluded with a panel discussion and chatbox Q&A from workshop attendees, moderated by Dr. Jessica Chery (FDA). The panel members included: presenters Dr. Scott, Dr. Pinello, Dr. Frazer, Dr. Sepp-Lorenzino, Dr. Tsai, Dr. Bao, and Dr. Porteus, as well as Dr. Dan Bauer (Boston Children's Hospital), and Dr. Anna Kwilas (FDA).

The panel addressed questions and discussed key issues for moving the field forward. Some key themes from the panel discussion and Q&A are highlighted below.

Databases (gRNA)

The panelists discussed the importance of establishing a database with current data on validated off-target editing for different gRNA sites. Having a global inclusive depository of data would be worthwhile, but it is challenging to document experimental nuances. There was also agreement on the desirability of establishing a gold standard to assess whether off-target sites are valid; however, there was disagreement about the terminology "gold standard." Rather, it

may be preferable to establish boundaries of amplicon sequencing and then allow scientists to move the boundaries to make them more sensitive, more reproducible, and more precise.

Additionally, it was noted that for populations with high genetic diversity, e.g., those with African ancestry, it would be beneficial to gain more sequence knowledge. In these populations, copy number variations and repetitive regions are harder to assess, and if we could gain better information on these, it would give a more complete view of genetic diversity.

Confirming Off-Target Editing, Off-Target Analysis, and Clinical Monitoring

How to distinguish a bona fide off-target was a common question. One panelist reported that in his experience, off-target indel rate threshold is above 0.1%. Another panelist defined it as having a reproducibly higher indel or other mutational activity above a control or background of unedited cells. It was also noted that definitions of off-target editing vary among scientists, e.g., any modification that isn't intended, or "editing something that shouldn't be edited."

Additionally, there was discussion about the appropriate depth at which an individual person, targeted population, and general population should be evaluated for off-target risks. Panelists noted that there are emerging frameworks with multiple phases of evaluation of predicted off-target editing, including the pooled amplicon sequencing method rhAmpSeq, which allows for evaluation of many different off-target sites in parallel, with subsequent follow-up with high-depth sequencing for individual off-target editing of interest.

Vis-à-vis clinical monitoring of off-target edits, connecting an AE to an off-target event could be accomplished by using the hematopoietic system where edited cells remain accessible even after the therapy has been administered. Therefore, if candidate off-target sites are identified, they could be sequenced from the DNA isolated from those cells. Then, one could see if there were any evidence of clonal expansion in cells carrying the off-target and if it were associated with any clinical AEs.

In answering a question about approaches to evaluating off-target editing for allogeneic immunotherapies using iPSCs, panelists noted that clonal iPSC-derived therapies from a single clone are the easiest way to begin. This approach allows for whole-genome sequencing on that clone, which can be monitored over time. Further, functional outcomes will depend on the actual product downstream, i.e., what the differentiated product will be and what the functional outcome will be on a particular cell type.

Workshop Conclusions

In summarizing the key points of the workshop, presenters agreed on the importance of considering how human genetic variation influences the safety profile of a genome-edited product. One concept suggested was that off-target editing can be broken into two parts: how often does it happen and how often does it have a functional consequence. If human genetic variation does have an impact on the safety and efficacy of therapies, use of certain products may be justified if a risk-benefit analysis is done. There are several tools available and in development that can be used to provide data, including clinical data, to help inform the risk-benefit analysis. It is crucial to understand that a therapy shouldn't be rejected just because some patients experience more neurologic AEs, for example, because of their genetic variation.

If a therapy is proven effective for treating a chronic disease, it needs to be understood that genetic variation may affect safety and efficacy for some, but that therapy should not be discarded for everyone.

While individual genetic variation impacts “one-size-fits-all” genome-editing therapies, there is the hope that the future will hold the possibility of treating patient-specific mutations and analyzing patient-specific risk for specific or individual targets.

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