DANGER ANALYSIS: RISK-AVERSE ON/OFF-TARGET ASSESSMENT FOR CRISPR EDITING WITHOUT A REFERENCE GENOME

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ABSTRACT

The CRISPR-Cas9 system has successfully achieved site-specific gene editing in organisms ranging from humans to bacteria. The technology efficiently generates mutants, allowing for phenotypic analysis of the on-target gene. However, some conventional studies did not investigate whether deleterious off-target effects partially affect the phenotype. Herein, we present a novel phenotypic assessment of CRISPR-mediated gene editing: Deleterious and ANticipatable Guides Evaluated by RNA-sequencing (DANGER) analysis. Using RNA-seq data, this bioinformatics pipeline can elucidate genomic on/off-target sites on mRNA-transcribed regions related to expression changes and then quantify phenotypic risk at the gene ontology (GO) term level. We demonstrated the risk-averse on/off-target assessment in RNA-seq data from gene-edited samples of human cells and zebrafish brains. Our DANGER analysis successfully detected off-target sites, and it quantitatively evaluated the potential contribution of deleterious off-targets to the transcriptome phenotypes of the edited mutants. Notably, DANGER analysis harnessed *de novo* transcriptome assembly to perform risk-averse on/off-target assessments without a reference genome. Thus, our resources would help assess genome editing in non-model organisms, individual human genomes, and atypical genomes from diseases and viruses. In conclusion, DANGER analysis facilitates the safer design of genome editing in all organisms with a transcriptome.

INTRODUCTION

The CRISPR-Cas9 system was initially adapted as a bacterial immune system([1,2]). Over the past decade, this system has been developed as a programmable nuclease that enables site-specific modification of the genomes of various organisms, including humans)[3–5]), insects)[6,7]), microalgae([8,9]), and bacteria([10]). Engineered CRISPR-Cas9 undertakes genomic modification using two components: RNA-guided Cas9 nuclease and single-guide RNA (sgRNA)([11,12]). The Cas9-sgRNA complex generates indels near the target site (on-target site), where the 19–20 bases of the 5´ ends of sgRNA (protospacer) and the protospacer adjacent motif (PAM) of Cas9 protein bind([11–14]). Recently, many CRISPR-Cas9 applications, such as Cas9 nickase (Cas9n)([12]), dead Cas9 (dCas9)([15]), base editors([16,17]), and prime editors([18]), have been

developed. Furthermore, CRISPR-Cas9-mediated genome editing was found to be efficient, with the editing efficiency exceeding 50% over time([19]). Thus, CRISPR technology has dramatically facilitated a reverse genetics approach involving phenotypic analysis using CRISPR-Cas9-based mutants of a user-targeted gene([20–23]).

However, genome editing using CRISPR technology presents two challenges that have not been addressed in previous studies. First, phenotypic effects caused by unexpected CRISPR dynamics are not quantitively monitored. CRISPR-Cas9 is well known for unexpected sequence editing (off-target site) with mismatches when compared to protospacers and PAM[5]. Off-target gene editing results in incorrectly edited mRNA, unexpected phenotypes, and decreased expression of unrelated genes. Some reports predicted and detected off-target editing using genomic PCR and DNA sequencing analysis([14,24–26]), but most studies have not assessed the phenotypic effect of the detected off-targets. Second, CRISPR technology generally depends on basic genomic data, including the reference genome. CRISPR technology has potential applications in organisms with incompletely characterized genomes. However, the design of site-specific sgRNAs requires the factual genomic sequence of materials to be treated with CRISPR technology. This hindrance also emerges in the human genome, particularly in the genomes of patients and cancer genomes. These genomes are assumed to be completely distinct from the reference genome([27,28]). The off-target is always "unexpected." Thus, we need a method to observe factual genomic sequences and reduce potential off-target effects.

We devised a method to overcome the two challenges above: phenotypic risk and dependence on a reference genome. Phenotypic risk can be assessed by phenotype analysis using gene ontology (GO) annotation([29,30]). GO has been widely used for several decades([20,23,31–34]). Recently, many RNA sequencing (RNA-seq) data and mapped genes have been annotated with GO terms to characterize the transcriptome phenotype under a specific condition of the organism. This process is known as enrichment analysis([35]). We expected that we could quantitatively assess the phenotypic risk of off-target genes if each off-target gene with decreased expression was annotated with GO terms. Moreover, *de novo* transcriptome assembly technology can address the dependency problem of reference genomes. The *de novo* transcriptome assembly can generate transcriptome sequences

without the reference genome using RNA-seq data([36–40]). We identified factual genomic sequences in mRNA-transcribed regions using *de novo* transcriptome assembly from gene-edited organisms and cells.

In this study, we combined *de novo* transcriptome assembly and GO annotation analysis in CRISPR editing to establish a DNA on/off-target assessment, including phenotype risk analysis without a reference genome. We named it **D**eleterious and **AN**ticipatable **G**uides **E**valuated by **R**NA-sequencing (**DANGER**) analysis (Figure 1) This bioinformatics pipeline can elucidate genomic on/off-target sites based on de novo transcriptome assembly using RNA-seq data. Then, it identifies the *deleterious off-targets*, defined as off-targets on the mRNA-transcribed regions that represent the downregulation of expression in edited samples compared to wild-type (WT) ones. Furthermore, our pipeline can quantify phenotypic risk at the GO term level by calculating a newly defined indicator of phenotypic risk by the deleterious off-targets, named the D-index.

MATERIAL AND METHODS

Implementation of DANGER Analysis

Our pipeline of DANGER analysis is composed of several processes: "Quality control & Adapter trimming," "rRNA Removal," "de novo transcriptome assembly," " Removal of redundancy," "Detection of on/off-target sites," "Expression quantification," "Search for deleterious off-target sites," "Identification of ORFs and Genes," "GO analysis," and "Validation for phenotypic risk" (Figure 2A).

DANGER analysis examines paired-end RNA-seq data derived from wild-type (WT) and edited samples using the processes depicted in Figure 1. The pipeline generates a *de novo* transcriptome assembly, an expression profile of transcripts belonging to on/off-target sites, and an estimation of the phenotypic risk for off-target sites. The script has been uploaded to our GitHub repository (https://github.com/KazukiNakamae/DANGER_analysis). Most analyses were performed on systems with MacOSX 12.6.1, Apple M1 Max, and 64 GB of memory. The *de novo* assembly and subsequent processes, such as the removal of redundancy and

expression quantification using Trinity, were performed on a system with Ubuntu v. 22.04.1, LTS, and 235 GB of memory. Each process is explained in detail below.

Quality Control & Adapter Trimming. Quality control and adapter trimming were performed using Cutadapt v. 1.18([41]), which also removed low-quality reads. The adapter sequences used were "AGATCGGAAGAG."

Ribosomal RNA (rRNA) Removal. The residual rRNA reads were filtered using bbduk v. 38.18 (https://sourceforge.net/projects/bbmap/). Each sample was filtered twice. In the first and second filters, we used SSU and LSU rRNA datasets from SILVA v. 119.1 (https://www.arb-silva.de). The dataset was downloaded from the CRISPRroots (https://rth.dk/resources/crispr/crisprroots/).

de novo Transcriptome Assembly. The *de novo* transcriptome assembly was performed using Trinity v. 2.12.0([38]). The merged read files were composed of RNA-seq data derived from WT samples. Transcriptome completeness was assessed using the BUSCO v. 5.2.2_cv1([42]). The databases used for conserved mammalian BUSCO genes were "mammalia_odb10" and "actinopterygii_odb10" for human and zebrafish assemblies, respectively.

Removal of redundancy. To remove transcripts with low expression levels, the expression of RNA-seq data derived from WT samples was quantified in advance. Quantification was performed with align_and_estimate_abundance.pl of Trinity v. 2.12.0. The removal of transcripts was performed with filter_low_expr_transcripts.pl of Trinity v. 2.12.0.

Detection of on/off-target sites. Detection of on/off-target sites in *de novo* transcriptome assembly was performed with Crisflash v.1.2.0([43]). Our off-target detection focused on up to 11nt mismatches and NRR PAM because the previous off-target reports indicated that off-

target sites with ≥10 nt mismatches and NGG/NAG/NGA/NAA PAM exist[24,44], although not with a high frequency.

The output file of Crisflash has on-/off-target locations, off-target sequences, and mismatch numbers included in the tab-delimited format (Cas-OFFinder format). The on-target locations were extracted using perfect matching with the expected genomic sequence of Cas9-sgRNA binding. The potential off-target sites were classified by the mismatch number in each text file.

Expression quantification. Using filtered *de novo* transcriptome assembly, we quantified the expression of RNA-seq data derived from each sample. Quantification was performed using align_and_estimate_abundance.pl of Trinity v. 2.12.0. The transcripts per million (TPM) dataset was constructed from "RSEM.isoforms.results" of each output directory and was saved as a single CSV file.

The ratio of TPM values on a transcript was calculated with the following formula:

$$(TPM\ ratio) = \frac{(Average\ of\ TPM\ of\ Edited\ samples)}{(Average\ of\ TPM\ of\ WT\ samples)} \qquad (1)$$

Search for deleterious off-target sites. A transcript with a TPM ratio of less than 0.4 was determined as downregulated TPM (dTPM). Our pipeline defined an off-target site where the transcript was annotated with dTPM as a deleterious off-target site. We counted the number of deleterious off-target sites using an in-house Python script, which required the off-target site profile and the TPM ratio described above.

Identification of ORFs and Genes. The open reading frames (ORFs) and genes of the filtered *de novo* transcript were estimated with TransDecoder v. 5.5.0 and ggsearch v. 36.3.8g using a protein database. The process was based on the Systematic Analysis for Quantification of Everything (SAQE) pipeline (https://github.com/bonohu/SAQE)([33]). In particular,

"11TransDecoder.sh, ", "12GetRefProts.sh", "15ggsearch.sh", "15parseggsearch.sh", and "15mkannotbl.pl" were used with the supplemental script "00_prepare_faa_4Fanflow. sh" in the GitHub repository (https://github.com/RyoNozu/Sequence_editor). The referred protein databases were the Ensembl databases of all translations resulting from Ensembl genes in humans and zebrafish. The DANGER analysis database can be manually customized with the organism from which the analyzed RNA-seq data was extracted.

GO enrichment analysis. GO annotations of gene ontologies were performed using an in-house R script using the org.Hs.eg.db and org.Dr.eg.db R packages for humans and zebrafish, respectively. Enrichment analysis followed by GO annotations was performed using the topGO R package against genes whose off-target sites were determined to be deleterious off-target sites. Enrichment analyses were performed per off-target mismatch number. Finally, the enrichment tables were merged into a single table, named the DANGER table, with the mismatch number annotated.

Validation for phenotypic risk. We defined the following value (D-index) to evaluate the phenotypic risk posed by deleterious off-target effects per GO term.

$$(D - index) = \sum_{m=1}^{11} N(m) \times exp(4 - m) \qquad (2)$$

where N(m) represents the total number of genes, including a specific GO term. The D-index considers both the pathotypic risk and the frequency of off-target effects. Based on previous reports, we used the exponential function to express the frequency of off-targets because the frequency of off-targets tends to decrease exponentially as the mismatch number of an off-target site increases([24,45]). Calculations were performed using an in-house Python script.

Differentially Expressed Genes (DEG) analysis

DEG analysis was performed to compare different analysis methods. The read count data were extracted from the "RSEM.isoforms.results" of each output directory in the section "Implementation of DANGER analysis: Expression quantification". The raw count data were normalized by the Tag Count Comparison (TCC) R package([46]) with the parameter "norm.method="tmm," test.method="edger," iteration=30, FDR=0.1, floorPDEG=0.05" to detect DEG between RNA-seq data derived from WT and Edited samples. The MA plot was constructed using an in-house R script. If a DEG transcript had a negative log-ratio of normalized counts (M-value), it was determined to be downregulated DE (dDE).

Datasets

To evaluate our pipeline, two RNA-seq datasets were analyzed. First, we collected paired-end RNA-seq datasets, which had a total of >100 M reads for all WT samples and an average length of >100 nt, to ensure good quality, which indicated a percentage of complete benchmarking universal single-copy orthologs (BUSCO) genes of >70%, of transcriptome completeness after *de novo* transcriptome assembly. The datasets were downloaded from the Sequence Read Archive (SRA) (Table 1).

GRIN2B. The dataset was extracted from human iPSC-derived cortical neurons with or without indels generated by paired Cas9 nickase (Cas9n)-single-guide RNA (sgRNA) (GRIN2B-FW and GRIN2B-REV sgRNAs) on the GRIN2B locus([23]). Previous studies have established clones with indels resulting in loss-of-function (LOF) and reduced dosage (RD). However, we focused only on LOF samples that had been edited and analyzed using our pipeline. Gorodkin and Seemann have previously reported that off-target sites affect the expression profile of LOF samples using reference-based RNA-seq analysis. Our study used the GRIN2B dataset to benchmark *de novo* transcriptome assembly based and reference-based RNA-seq analyses([44]). Moreover, we profiled the on/off-target assessment of GRIN2B-REV sgRNA.

Park7. The dataset was extracted from the zebrafish brain with or without biallelic indels generated by a single Cas9-sgRNA at the park7 locus (which encodes DJ-1)([20]). The analyzed F2 mutants were generated from a cross between two heterozygous F1 mutants. We used the dataset as an *in vivo* example of the DANGER analysis pipeline in a simple CRISPR-Cas9-mediated knock-out experiment.

RESULTS & DISCUSSION

Assessment of CRISPR-Cas9 off-targets using DANGER Analysis for RNA-seq Data from *in vitro* Differentiated Human iPSC

We investigated whether our DANGER analysis pipelines could detect deleterious off-target sites without information on the reference genome. First, we applied DANGER analysis to the GRIN2B dataset, which was extracted from human iPSC-derived cortical neurons with or without frame-in deletions at the GRIN2B locus([23]). The obtained *de novo* transcriptome assembly contained five isoforms in which on-target sequences of sgRNA (GRIN2B-REV) (Figure 2B) were located. The assembly comprised 342,910 contigs and exhibited BUSCO transcriptome completeness of 79.1% (Figure 2C). Previous studies on *de novo* transcriptome assembly using Trinity reported 64.7%, 77.1%, 80%, and 87% complete BUSCO genes in higher animals such as *Homo sapiens*([36]), *Castor fiber* L.([37]), *Mirounga angustirostris*([39]), and *Dromiciops gliroides*([40]), respectively. We successfully obtained a *de novo* transcriptome with standard quality. Consequently, the pipeline performed an exhaustive search, yielding 4,281,249 potential off-target sites with up to 11 mismatches, using transcriptome assembly.

Next, we quantified the transcriptome-wide expression of each of the four RNA-seq samples from the WT and Edited GRIN2B loci. Our pipeline contemplates that the deleterious off-target gene is located in the transcript and results in decreased expression due to incomplete transcript sequences. Thus, the DANGER analysis screens transcripts with lower expression levels in edited samples compared to WT samples. In general, there are several criteria for estimating expression using RNA-seq. We preliminarily tested two criteria, "downregulated different expression (dDE)" and “dTPM," for the detection of downregulated transcripts (Figure 3A; two callouts). Our dDE criterion uses DEG analysis with TCC normalization (see Materials and Methods). In the case of DEs in a transcript with a negative

M-value in the MA plot (Figure 3A; right callout), we defined the transcript as "dDE." On the other hand, the "TPM" criterion uses the normalized value, named TPM, as first defined by ([47]) and calculates the ratio of TPM between WT and the edited samples. When the ratio of a transcript is < 1/2.5 = 0.4, we defined the transcript as "dTPM" (Figure 3A; left callout). We confirmed the number of transcripts with dDE or dTPM annotation that contained off-target sites (Figure 3A; Venn diagram). There were 7,825 transcripts with dDE off-targets. A total of 158,733 transcripts with dTPM off-targets were detected, which was approximately 20-fold more than those with dDE off-targets. This result suggested that the dTPM approach could detect deleterious off-target effects. Therefore, we employed the dTPM criterion as the standard detection method for expression estimation in the DANGER analysis pipeline.

The Gorodkin and Seemann group previously established the on/off-target assessment pipeline (CRISPRroots)([44]). They then used STAR([48]) to perform expression analysis on the same RNA-seq data of GRIN2B using reference-based mapping. They suggested that two off-target sites (ALK: gcAgaCTGGtTGGAAGCaCCNGG, GBA2: cccTTCcGGccGGAAGCGCCNGG) were binding with the Cas9-GRIN2B-REV sgRNA (AGATTCTGGGTGGAAGCGCCNGG)-DNA seed and were linked to downregulated expression (namely, "RISK: CRITICAL"). The definition of off-targets in their study was similar to our idea of deleterious off-targets. Thus, we compared our list of deleterious off-targets in dTPM and dDE with the two off-targets detected using CRISPRroots. As a result, in the DANGER analysis, the off-target of CRISPRroots in the ALK locus was detected by both dTPM and dDE criteria, whereas the off-target of CRISPRroots in GBA2 was not detected by any criteria (Figure 3B). The absence of GBA2 was understandable because the off-target site on the GBA2 locus was in the promoter region rather than the GBA2 transcript. Our *de novo* transcriptome approach focuses solely on the potential off-target genes in the transcribed region of the genome. Additionally, unlike traditional reference-based RNA-seq analysis, this approach can provide novel insights. For example, DANGER analysis using dTPM detected a transcript with a deleterious off-target downstream GALR2 locus (Figure 3C). The genome database annotation was not the off-target site of the transcribed region (XM_047436984.1). However, the *de novo* transcriptome assembly included the site in the transcript (TRINITY_DN86617_c0_g1_i1). This result indicates that our DANGER analysis pipeline can detect *bona fide* transcripts, which have never been annotated in the reference genome database because of cell-specific

transcription, personal genomic variants, and inadequate genomic locus study. The detection range of our DANGER analysis is limited to the transcribed region. However, our pipeline detected 417,406 off-target sites in identified and unidentified transcripts, as well as 28,060 gene-annotated off-target sites with three to 11 mismatches. In contrast, genome-wide and reference-based RNA-seq analysis (CRISPRroots) features only two off-target sites in genes with six mismatches. Thus, we demonstrated that the DANGER analysis could generate a comprehensive and factual record of off-target sites (Figure 3D).

Finally, our pipeline evaluated the phenotypic risk of deleterious off-targets. Various studies have used Gene Ontology (GO) analysis to assess phenotypic effects([20,23,31–34]). However, this study also considered off-target frequency because deleterious off-targets with many mismatches were expected to occur([5,45]). Thus, our pipeline performed enrichment analysis per mismatch number (Figure 4A) and then combined the results with the mismatch effect by calculating the D-index per GO term (Figure 4B; see Materials and methods). The total number of D-indices in the GO terms associated with "biological processes" was 11,356 (Figure 4C, Supplementary Table S1) in the GRIN2B dataset. The DANGER analysis was used to evaluate the phenotypic risk at the GO level using RNA-seq of the human GRIN2B dataset data without any reference genomes.

Assessment of CRISPR-Cas9 on/off-target using DANGER analysis for RNA-seq data from *in vivo* tissue of zebrafish

We performed DANGER analysis using the RNA-seq data from human cells edited by one of the Cas9n-sgRNAs for benchmarking with the previous method (CRISPRroots) in the last section. Next, we investigated whether DANGER analysis could be used to analyze the RNA-seq data from the non-human tissue that had been *in vivo* edited with a single Cas9 nuclease-sgRNA, a more common experimental design for genome editing. Thus, we downloaded and analyzed RNA-seq data from the park7 dataset derived from the zebrafish brain, with and without indels at the park7 locus([20]). Our DANGER analysis successfully built a *de novo* transcriptome assembly with 90.9% complete BUSCO genes and detected on-target sequences in the two transcripts (Figure 5A). Moreover, DANGER analysis revealed a significant downregulation of the transcript with Cas9-sgRNA on-target sequence in the

expression quantification (Figure 5B). The original report on the park7 dataset reported downregulated park7 mRNA in the park7 mutant using RNA-seq analysis([20]). This result implied that *de novo* transcriptome assembly and the following expression quantification of our DANGER analysis could generate reliable data consistent with the outcome of standard RNA-seq analysis using a reference genome. Consequently, 4,232,633 potential off-target sites in all transcripts were detected by DANGER analysis, which were then defined as deleterious off-targets considering the expression profile. There were 603,395 and 3,101 deleterious off-target sites on all and gene-annotated transcripts, respectively (Figure 5C). The park7 result had no deleterious off-target effects with ≤ 4 mismatches, which meant that frequent off-targeting was not expected in mRNA-transcribed regions. Our pipeline estimated the D-index per GO term associated with the "biological process" to quantify phenotypic risk. The total D-index was 224 (Figure 5D, Supplementary Table S2).

Comparison of phenotypic risks in the GRIN2B and Park7 datasets

In this study, we evaluated the phenotypic risks associated with off-target transcripts using the D-index. The total D-index of the GRIN2B result was approximately 51-fold larger than that of the park7 result. Furthermore, the DANGER analysis found three to four mismatches in the GRIN2B dataset, which is common in genome-wide off-target studies such as GUIDE-seq and Digenome-seq([24,26]), and numerous deleterious off-target sites on transcript sequences annotated with genes. Thus, the total D-index is relatively high, which means that Cas9n-GRIN2B-REV sgRNA may have side effects on the phenotype of differentiated human iPSC. A follow-up study is required to assess the edited GRIN2B LOF clones using WGS or alternative genome-wide methods such as GUIDE-seq([24]). Researchers can identify some clarified points in the future using the result table of the D-index (Supplementary Table S1). For example, the GO term "cell differentiation" (GO ID: GO:0030154) recorded a high D-index (top <1%) for the GRIN2B result (Table 2). The GO term "cell population proliferation" (GO ID: GO:0008283) also recorded a higher percent rank of D-index in the GRIN2B dataset (top 1.206%) than that in park7 (top 23.391%). A previous study showed that the genes expression changes belonging to these GO terms resulted from on-target editing of the GRIN2B locus. However, the results of the DANGER analysis suggested that off-target editing

of additional genes belonging to GO:0030154 and GO:0008283 partially contributed to expression changes. Follow-up studies should include off-target gene analysis. Therefore, DANGER analysis would be helpful in reaching a reasonable conclusion in genome editing studies.

Limitations

In this section, we discuss the major limitations of the proposed pipeline. First, our method depends on the quality of *de novo* transcriptome assembly using Trinity. Pair-end RNA-seq data with sufficient length and read number must be used to guarantee high-quality assembly (see Material and Methods). If we fail to build an exemplary assembly, producing reliable data for the following analyses, such as on/off-target analysis and expression profiles, becomes difficult. Second, the annotation analysis step in our pipeline may fail to annotate transcripts adequately due to limited information from databases on genes, transcripts, proteins, and gene function. When researchers apply our pipeline to RNA-seq data from organisms with limited genomic knowledge and evidence, we recommend using a database of a model organism with a strong genomic relationship with the organism being analyzed. DANGER analysis can analyze genome-edited samples without a reference genome, but studies of a related model organism with a well-annotated genome are still required.

DANGER analysis Provides a New Perception of the Conventional Genome Editing Process in Medicine, Agriculture, and Biological Research

We demonstrated our DANGER analysis pipeline, as it allows for (i) the detection of potential DNA on/off-target sites in the mRNA-transcribed region on the genome using RNA-seq data, (ii) evaluation of phenotypic effects by deleterious off-target sites based on the evidence provided by gene expression changes, and (iii) quantification of the phenotypic risk at the GO term level, without a reference genome. Thus, DANGER analysis can be performed on various organisms, personal human genomes, and atypical genomes created by diseases and viruses([28]). We believe that the perception resulting from our DANGER analysis has not been observed in the conventional scheme for genome editing. We illustrate a new scheme using

DANGER analysis in organisms (I) with and (II) without a reference genome (Figure 6). For example, (I) model organisms such as humans have a reference genome whose information has been generally used for potential on/off-target searches and post-analysis. However, the reference genome is a representative genome and not the personal genome of the patient or cell lines. Reference-based genome editing does not consider unique single nucleotide polymorphisms (SNPs) or spontaneous genomic rearrangements. DANGER analysis can supply a personal transcriptome-based on/off-target profile to ensure the phenotypic risk of unexpected off-target mutations. The new workflow of genome editing would be helpful for *ex vivo* gene therapy and cancer research because the genome of a cancer cell is generally characterized by widespread somatic genomic rearrangements([28]). (II) An organism whose genome has never been comprehensively sequenced and well characterized is not considered a reasonable subject for genome editing, as site-specific genome editing is guaranteed without a reference genome. Some groups, however, have used incomplete genomic information to construct mutants of non-model organisms([49,50]) that have never been well-characterized in genomics. Such a conventional scheme is hit-or-miss due to the risk of erroneous knock-out phenotypes in combination with off-targeting of other genes. DANGER analysis can provide transcriptome phenotype-aware on-/off-target profiles as well as sequence information of the expressed genes. This information can be used for the safer design of genome editing, which enables the optimized design of CRISPR editing by repeatedly looping back through genome-editing experiments without a reference genome. We believe that the DANGER analysis pipeline will expand the scope of genomic studies and industrial applications using genome editing.

DATA AVAILABILITY

The datasets were derived from the following public domain resources: https://www.ncbi.nlm.nih.gov/geo. The analyses were performed using DANGER analysis version 1.0. The Script for the DANGER analysis pipeline is available at https://github.com/KazukiNakamae/DANGER_analysis. In addition, the software provides a tutorial on reproducing the results presented in this article on the Readme page.

SUPPLEMENTARY DATA

Supplemental_Files - zip file

Supplementary Data are available at NAR online.

AUTHOR CONTRIBUTIONS

Kazuki Nakamae: Conceptualization, Software, Formal analysis, Methodology, Validation, Visualization, Writing of the original draft. Hidemasa Bono: Supervision, Writing – Review, and Editing.

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CONFLICT OF INTEREST

K.N. was employed by PtBio Inc. H.B. was a consultant for PtBio Inc. H.B. has a financial interest in PtBio Inc.

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TABLE AND FIGURES LEGENDS

Tables

Table 1. RNA-seq datasets evaluated for testing the DANGER analysis pipeline.

Table 2. D-index summary of GO terms related to previous GRIN2B research.

Figures

Figure 1. Scheme of CRISPR-Cas9 targeting, deleterious off-target editing, and DANGER analysis.

Figure 2. Overview of DANGER analysis and on-target region constructed by *de novo* transcriptome assembly. A. Bioinformatic workflow of DANGER analysis. Our analysis requires RNA-seq data derived from WT and Edited (each n≥3). DANGER analysis has two steps in the workflow: (1) *de novo* transcriptome assembly (light green background color) and (2) annotation analysis (light yellow background color). The *de novo* transcriptome assembly step is processed with Trinity and preprocessing tools such as cutadapt and bbduk.sh. Crisflash performs the search of on/off-target sequences. The RSEM quantifies gene expression in Edited RNA-seq samples in comparison to the WT *de novo* transcriptome (dot allow). The step of annotation analysis was involved processing with TransDedoder, ggsearch, org.XX.eg.db (e.g., org.Hs.eg.db in the transcriptome related to humans), and topGO. We implemented specific modules, colored in pink, for considering the phenotypic effect of deleterious off-targets. B. Comparison between the hg38 reference genome and transcript sequence constructed by *de novo* assembly of RNA-seq samples derived from WT iPSC-derived cortical neurons on the GRIN2B on-target region. The on-target region of the hg38 reference genome is illustrated with annotations of the *GRIN2B* CDS, the protospacer, and the NGG PAM sequence of SpCas9. The detected GRIN2B isoforms (1–5) are lined up in

green in the black box. The Cas9-sgRNA binding sites are highlighted in blue. C. Genome completeness of *de novo* transcriptome assembly RNA-seq data derived from WT iPSC-derived cortical neurons was assessed using conserved mammal BUSCO genes (mammalia_odb10). The result was shortly 90.9% of complete, 42.1% of single-copy, 48.8% of duplecated, 2.5% fragmented, and 6.6% missing BUSCO genes (n = 3640).

Figure 3. Benchmark for the methods of expression analysis in comparison with reference-based RNA-seq analysis using RNA-seq data derived from WT and GRIN2B edited iPSC-derived cortical neurons. A. Comparison of different expression analyses. A Venn diagram comparing the *de novo* transcripts (duplicate counts on a predicted ORF basis), which had potential off-target sites with up to 11 nt mismatches, was detected by the dTPM and dDE approaches. dTPM indicates that the expression is decreased based on the ratio of TPM counts between WT and Edited samples (left callout). dDE means the expression is reduced based on DEG analysis between WT and Edited samples (right callout). B. Comparison of *de novo* transcriptome assembly-based and reference-based analysis on the deleterious off-target detection. A Venn diagram comparing the off-target genes identified from *de novo* transcriptome analysis (dTPM and dDE approaches) and reference-based RNA-seq analysis (CRISPRroots, "RISK: CRITICAL"). C. Genomic sequence map of off-targets located outside of GALR2 mRNA. The sequence is a part dDE of the hg38 reference genome with annotations of GALR2 mRNA (XM_047436984.1) and the *de novo* transcript (TRINITY_DN86617_c0_g1_i1) and an off-target site with three mismatches compared to the on-target sequence. D. Summary of deleterious off-target sites detected by *de novo* transcriptome analysis (dTPM) and reference-based RNA-seq analysis (CRISPRroots, "RISK: CRITICAL"). D. The counts of off-target sites are annotated with genes and classified by mismatch number related to the on-target sequence. The blanket indicates the number of transcripts, including those with and without identified gene annotations. The number of genes and transcripts with ≤4 nt mismatches is colored red.

Figure 4. The result of risk assessment in DANGER analysis using RNA-seq data derived from WT and GRIN2B edited iPSC-derived cortical neurons. A. An example of the annotation table for DANGER analysis. The table includes GO ID, GO term, count of genes (Count), and the number of mismatches of genes belonging to a specific GO term. B. The formula for phenotypic off-target risk (D-index). An example of the calculation is shown on a yellow

background. C. Distribution of the D-index of each GO term associated with Biological Process (orange). The sum of all D-indexes is labeled on the top right.

Figure 5. DANGER analysis result using RNA-seq data derived from WT and park7 (dj1) Edited brains of *Danio rerio*. A. Comparison between the GRCz11 reference genome and transcript sequence constructed by *de novo* assembly of RNA-seq samples derived from WT brain on park7 on-target region. The on-target region of the GRCz11 reference genome is illustrated with annotations of the park7 CDS, the protospacer, and the NGG PAM sequence of SpCas9. The detected park7 isoforms (1-2) are lined up in green in the black box. The Cas9-sgRNA binding sites are highlighted in blue. B. Comparison of TPM values of park1. The TPM was measured from WT and Edited RNA-seq samples (Each n=3); data were expressed as the means±SEM. *** p-value < 0.001. C. The gene counts are classified by mismatch number related to the on-target sequence. The blanket indicates the number of transcripts, including those with and without identified gene annotations. The number of genes and transcripts with ≤4 nt mismatches is colored red. D. Distribution of the D-index of each GO term associated with Biological Process (orange). The sum of all D-indexes is labeled on the top right.

Figure 6. Our proposal for the usage of DANGER-analysis in organisms with and without a reference genome. The workflow is shown as black arrows. The dotted black arrows indicate the front of the arrow and refer to the arrow base information. The image of book is from TogoTV (© 2016 DBCLS TogoTV, CC-BY-4.0, https://creativecommons.org/licenses/by/4.0/).

Table1.

Dataset	Layout	Length of raw reads [nt]	Total raw reads (Spots)	The average length of raw reads [nt]	Run	WT/Edited	Ref
GRIN2B	paired-end	125	44,621,372	125	SRR7187518	WT	([23])
		125	39,290,710		SRR7187519	WT	
		125	42,356,322		SRR7187520	WT	
		125	37,725,951		SRR7187521	WT	
		125	40,423,942	125	SRR7187524	Edited	
		125	43,003,607		SRR7187525	Edited	
		125	35,134,095		SRR7187526	Edited	

		125	39,725,694		SRR7187527	Edited	
park7 (dj1)	paired-end	151	55,801,577	151	SRR9886606	WT	([20])
		151	58,680,708		SRR9886607	WT	
		151	52,584,965		SRR9886608	WT	
		151	57,060,988	151	SRR9886609	Edited	
		151	48,882,852		SRR9886610	Edited	
		151	53,633,192		SRR9886611	Edited	

Table2.

	GRIN2B			**park7**		
GO term	**D-index**	**Rank**	**%Rank**	**D-index**	**Rank**	**%Rank**
cell differentiation	35.0911614	52	0.490	0.81063635	65	2.551
cell population proliferation (cell proliferation)	16.3983262	128	1.206	0.0631758	596	23.391
cell cycle	13.7388691	153	1.441	0.29190039	133	5.220
central nervous system development	7.78966815	267	2.515	0.18076129	233	9.144
brain development	6.18288043	332	3.127	0.11031031	365	14.325
cell division	3.49649771	544	5.123	0.12545244	334	13.108
chromosome segregation	2.70573054	673	6.338	0.0144311	1300	51.020

Figure 1.

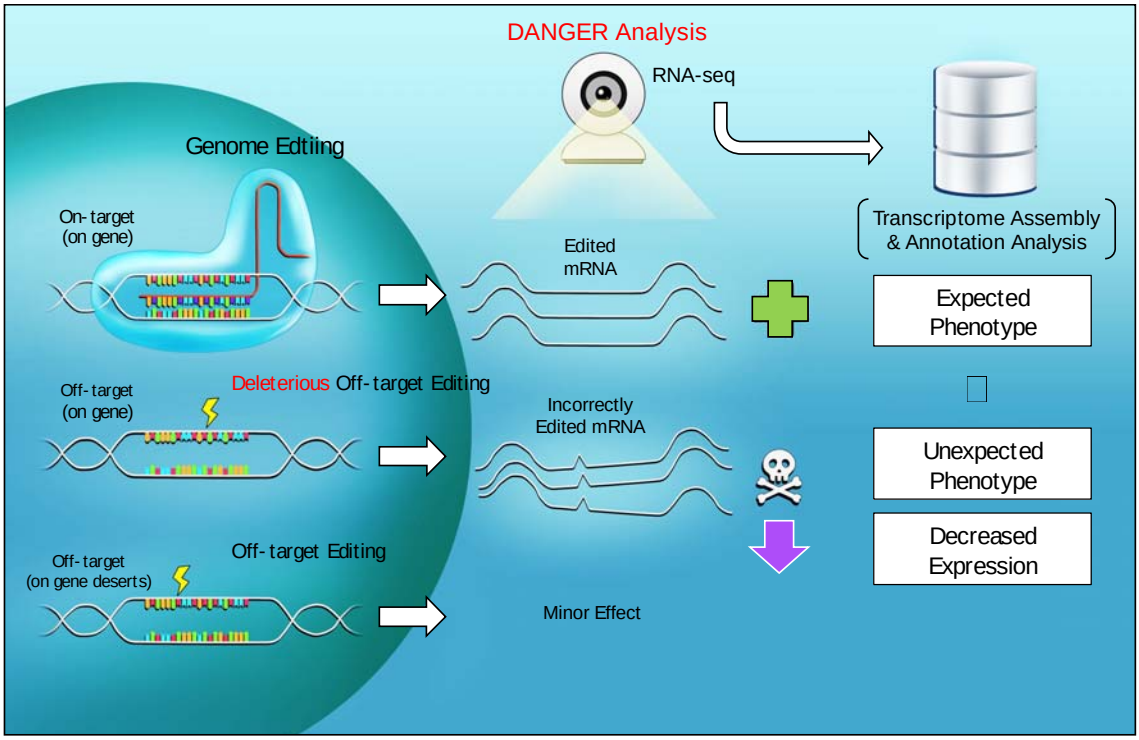
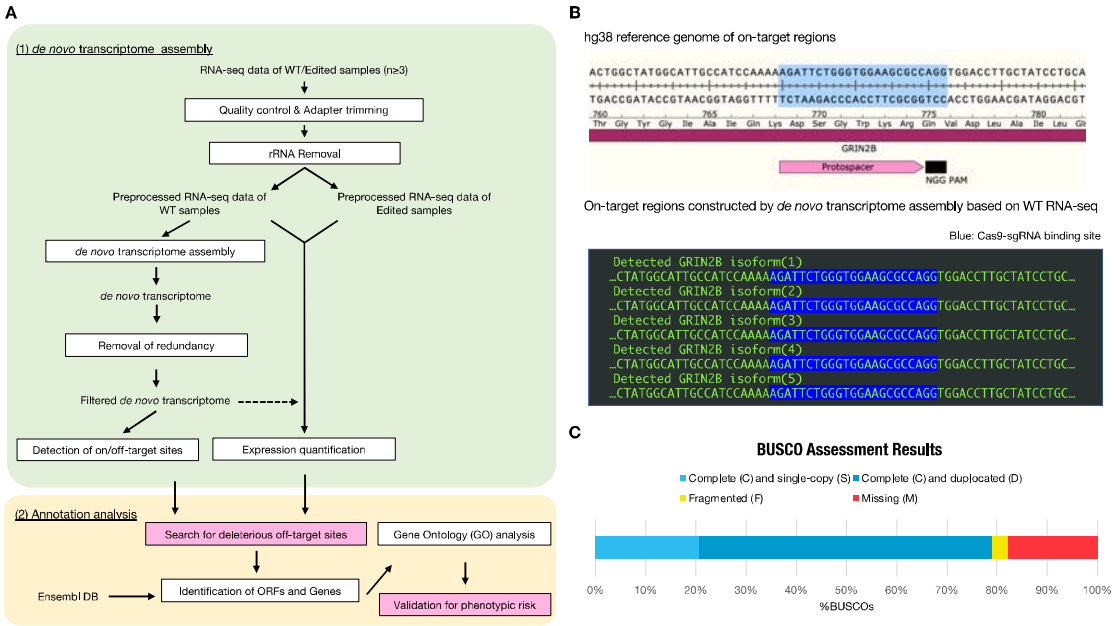


Figure 2.



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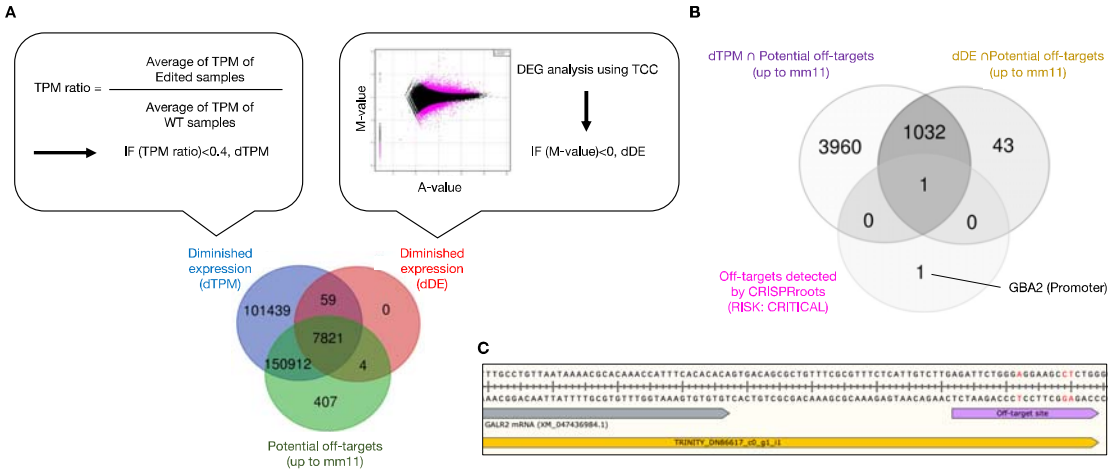


D

(): number of transcripts

Mismatch number	0	1	2	3	4	5	6	7	8	9	10	11
DANGER analysis (dTPM)	0	0	0	1 (1)	2 (14)	35 (193)	221 (1,253)	984 (7,341)	3415 (30,810)	6508 (83,203)	8288 (137,174)	8606 (157,417)
CRISPRroots (RISK: CRITICAL)	0	0	0	0	0	0	2	0	0	0	0	0

Figure 4.

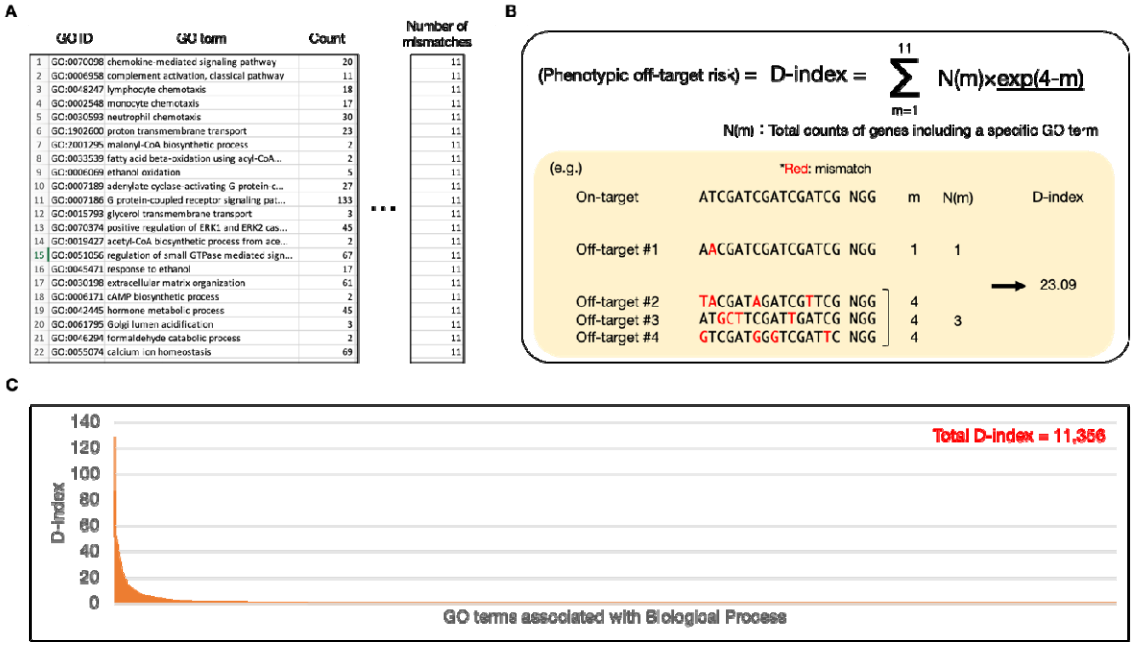
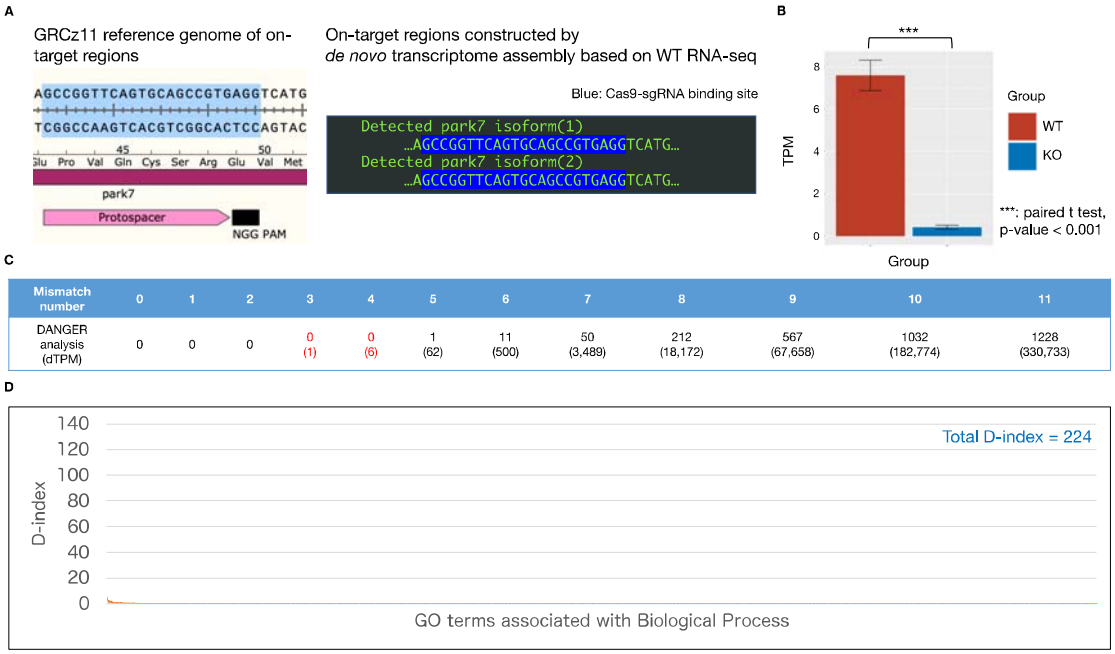


Figure 5.



Mismatch number	0	1	2	3	4	5	6	7	8	9	10	11
DANGER analysis (dTPM)	0	0	0	0 (1)	0 (6)	1 (62)	11 (500)	50 (3,489)	212 (18,172)	567 (67,658)	1032 (182,774)	1228 (330,733)

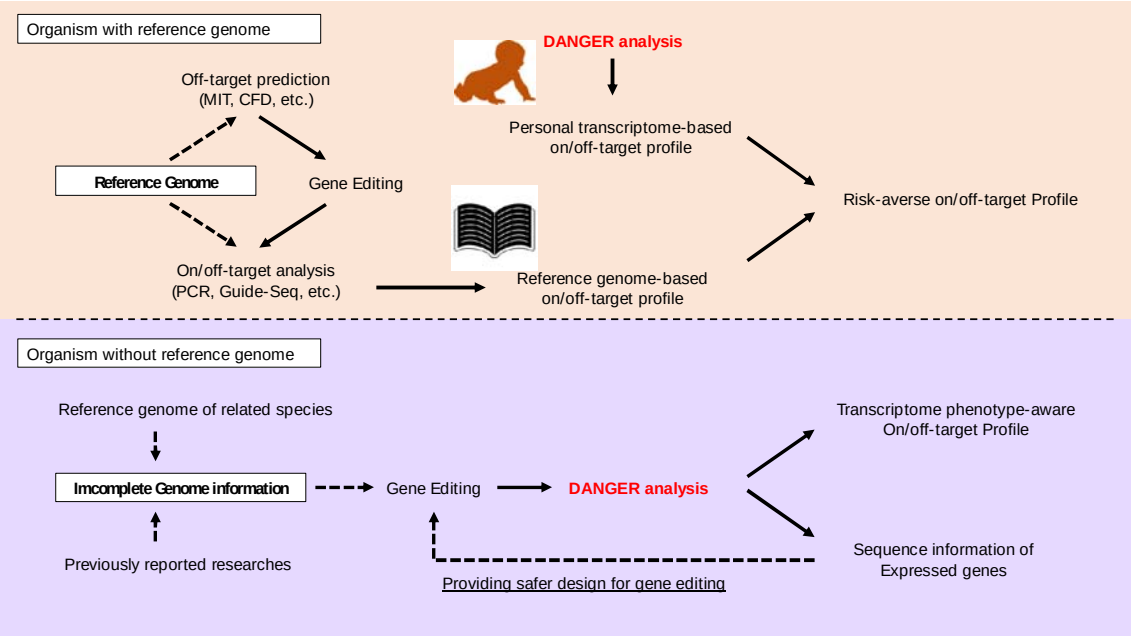



Figure 6.