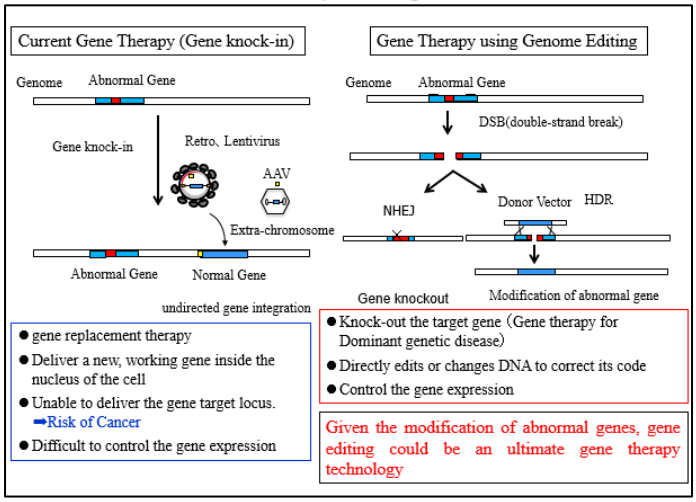
White-paper for quality and safety for gene therapy products using gene editing technology

1. Introduction

The development of genome editing technologies (1,2), which allow them to break, modify, and edit specific genes have been extensively proceeded. The implementation of genome editing technology is expected to use as a new gene therapy tool.

The reason to expect these new technologies is that the gene therapy up-to now has been defined as the addition of new genes to human cells/tissue. Genome editing can knock-out specific genes or modify specific genes that cause diseases. Gene editing could be an ultimate gene therapy technology.

Genome editing technologies rely on introduction of double-strand breaks (DSBs) into specific DNA sites and DSB repair mechanisms of cells. DSBs can be repaired through non-homologous end joining (NHEJ) and homology-directed repair (HDR) through homologous recombination (HR) (3) The NHEJ-based repair is a "first-aid (emergent)" response that occurs throughout the cell cycle. This repair mechanism, which often insert and delete several bases at a DSB site during end joining, can be used to cause the loss of target genes. HR occurs mainly in the S and G2 phases of the cell cycle. This pathway repairs DNA through recombination with a homologous sequence. Introduction of a DNA template carrying a desired sequence induces HDR at the break site, which corrects the abnormal gene related with the disease. Genome editing technologies are also to utilize to introduce and replace target genes at specific genome sites using HDR.



Zinc-finger nuclease (ZFN) (4) and transcription activator-like effector nuclease (TALEN) (1) are artificial restriction enzymes that cause DSBs specifically to DNA sequences in early phase of development. These engineered nucleases use proteins to recognize specific DNA sequences. Manufacturing these proteins require sophisticated technology, time, and a huge amount of money. On the other hand, a recently developed technique "clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR associated proteins (Cas)" (2) uses a single-guide RNA (sgRNA) to recognize the DNA sequence of a target gene. The design of CRISPR/Cas is easy to use and low cost, and then, this tool accelerated its development as a versatile genetic engineering technique (5). In fact, clinical trials of gene therapies for infections, cancers, and single-

gene diseases have been conducted overseas using this new genome editing technologies. The application for gene therapy products, etc. might be submitted for marketing authorization within several years. A similar clinical trial may be also about to start in Japan. Given this, a clear concept of the quality and safety of gene therapy products, using genome editing, should be clarified.

At first, this document addresses issues specific to genome editing currently under development, and then classifies them according to editing techniques and technologies (tools) that introduce them into a cell or body, and then purposes of genome editing, in order to clarify the characteristics of each technique/technology. The document also summarizes issues to be considered in conducting quality and safety evaluation and long-term follow-up in clinical application based on the characteristics of the genome editing techniques. Interpretations of risks associated with genome editing will depend on the type and seriousness of disease. Each genome editing technique should be individually evaluated for clinical application considering their risks and benefits. As genome editing is rapidly advancing, these issues need reviews as necessary.

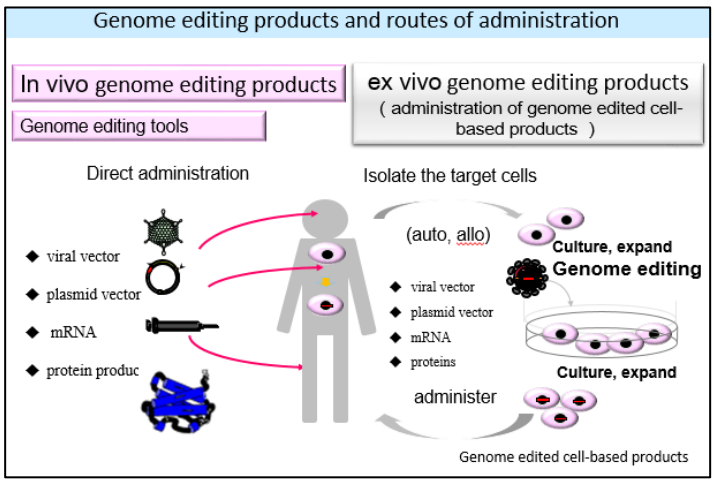
2. Definition

In this document, gene therapy products using genome editing techniques are defined as follows;

(1) *In vivo* genome editing product (products that directly administer a gene therapy product using a genome editing technique in the body)

1) Genome editing gene therapy products (gene therapy products consisting of a viral vector or plasmid vector that expresses a desired protein for genome editing)

2) Genome editing mRNA products (mRNA. that expresses a desired protein for genome editing)



3) Genome editing protein products (a desired protein as ingredient for gene editing which may include sgRNA)

(2) *Ex vivo* genome editing product (human cell-based products manufactured by *ex vivo* genome editing using a genome editing tool)

1) Genome edited cell-based products

3. Specific Issues on Genome Editing

(1) Cancer Risk of Gene-edited Cells

Genome editing technologies allow researchers to break, modify, and edit specific genes in a DNA sequence-specific manner. However, genome editing has the inherent risk of unintended editing of genes that have similar DNA sequences, which is called off-target effect. Of particular concern is that off-target effects may result in tumorigenicity (cancer) of cells. Off-target effects may directly activate oncogenes or inactivate tumor-suppressor genes. Genetic modifications by genome editing have the potential to cause permanent alterations in genome.

It has been reported that genome editing techniques that induce DSBs have the risk to induce genome instability which is associated with chromosomal break. With such techniques, large-scale chromosomal defects and insertion of off-target chromosomal sequences into DSB sites that cannot be detected by current analytical technologies have also been reported. The risk of tumorigenicity (cancer) attributable to chromosomal aberrations should also be clarified.

(2) Risk of Unintended Gene Modification in Germline Cells

In vivo genome editing that directly administers a genome editing gene therapy product in the patients may unintentionally result in genome editing of off-target cells or modification of off-target genes. It is difficult to identify and eliminate these off-target alterations when they occur.

In particular, *in vivo* genome editing for pediatric patients and patients of reproductive age may affect germline cells. Possible genetic effects in subsequent generations should be fully understood and justified. Recently, while to avoid the risk of chromosomal mutation attributable to genomic cleavage, new technologies that allow genetic engineering without genomic cleavage have been developed, for *in vivo* application of genome editing, the effect to next generation should be evaluated.

4. Classification of Genome Editing Technologies and Challenges Related to Their Quality Characteristics

(1) Classification by Genome Editing Tool and Point-to-consider

1) ZFN (4) and TALEN (1)

ZFNs are artificial nucleases engineered by fusing a domain that contains three to six zinc-finger protein motifs that recognize three specific base pairs and bind a targeted DNA sequence to a DNA-cleavage enzyme *Fok*I nuclease (*Fok*I). Designing this DNA-bind protein requires highly sophisticated technologies. TALENs were developed in order to simplify the complicated ZFN designing process. In the TALENs, each TAL module consisting of 34 amino acids of TAL, a plant-derived transcription factor, recognizes one nucleotide. By binding four different types of TAL module that each recognizes nucleotide base A, G, C, or T, targeted DNA sequences can be recognized to cut specific DNA

sequences by Fok1 nuclease which is fused to TAL modules. Typically, the TALEs are designed to recognize 15 to 20 nucleotides by binding 15 to 20 TAL modules.

Since the *Fok*I domains of ZFNs and TALENs cut only single chain of the DNA double strands, two artificial enzymes that recognize DNA sequences upstream and downstream of the target cleavage site is required. A recognition sequence of 18 to 40 bases is required to form one DSB, which is twice the length of the sequence recognized by one artificial enzyme. This makes the target DNA recognition specificity of both ZFN and TALENs high. The TALENs are thought to be less likely to cause off-target effects than CRISPR/Cas9 (1). Although the off-target effects of ZFNs and TALENs have not been reported as frequently as CRISPR/Cas9, they need to be evaluated carefully because currently there is no sufficient information available.

2) CRISPR/Cas

Unlike ZFNs and TALENs, a sgRNA complementary to the target DNA sequence is responsible for the recognition of specific DNA sequences in CRISPR/Cas. The sgRNA, therefore, could be designed to have a guide sequence that complementarily binds to 20 target nucleotides of the target DNA sequence and a Proto-spacer Adjacent Motif (PAM) that is adjacent to the target sequence.

The sgRNA and Cas9, an enzyme that cleaves the double strands of DNA, form a complex to cleave a gene having the sequence recognized by the sgRNA. The sgRNA is known to bind to the target DNA sequence even in the presence of up to five base pair mismatches (base pairs other than A:T and G:C) (6). The risk of this CRISPR/Cas system inducing off-target effects, such as insertions and deletions of off-target sequences, other than cleavage of the target gene will be considerably high (7-9). Many reports on the off-target effects by CRISPR/Cas have been published. It is however difficult to characterize the exact off-target effects that occur rarely, particularly those seen only in a small number of cells.

In order to mitigate the off-target effects of CRISPR/Cas, the effects of the length of the guide RNA or the second structure of the target DNA sequence have been investigated. However, enough resolution for this has been established yet. It is essential to design a sgRNA based on the latest knowledge and evaluate the frequency of off-target effects.

3) Genome Editing without Genomic Break (10,11)

To mitigate genome instability resulting from a DSB, genome editing without genomic break (e.g. single base editing with deaminase) and other editing technologies have been developed. For each newly developed genome editing technology, the mechanism to reduce off-target effects should be justified together with its evaluation methodology.

(2) Cautions Regarding Genome Editing Tools and Gene-modified Cells

1) Viral Vectors and Plasmid Vectors

Clinical trials of viral vectors, including adenovirus, adeno-associated virus (AAV) and so on, have been conducted to introduce ZFN or CRISPR/Cas into a cell for genome editing (12,13). The quality control for current gene therapy products can also be applied to viral or plasmid vectors coding a genome editing enzyme gene (14). The quality control and characterization for the manufactured vectors, and the establishment and characterization of cell bank systems should be evaluated in the same manner as current gene therapy products.

In many cases, viral promoters are utilized for efficient expression of the target protein. Insertion of a viral promoter sequences adjacent to a cancer-related gene possibly could cause tumorigenicity (cancer). In some case, genome editing also use viral promoters to express ZFNs, TALENs, or Cas9/sgRNA. Until now, oncogenesis by promoter insertion has not been reported. On the other hand, a transfected plasmid DNA has been integrated into the DSB site. Genome editing gene therapy products using a viral or plasmid vector require nonclinical safety evaluation similar to conventional gene therapy products. The cell or tissue directionality should also be assessed together with its biodistribution.

When a viral vector is used to introduce a genome editing tool into a cell, the extent and frequency of the gene modification of the target cell and off-target cells need to be analyzed from the viewpoints of infectivity and cell tropism. It should also be considered that the continued expression of a genome editing enzyme can increase in possibility of off-target effects. In particular, since viral vector-mediated genome editing can cause the long-term persistent expression of the genome editing enzyme. The persistent expression of genome editing enzymes should also be evaluated from a safety viewpoint.

2) mRNA

In order to express genome editing proteins, such as Cas, TALEN, and ZFN, in a cell, intracellular transfection of the mRNA that codes for these proteins has been utilized (15-17). In accordance with the Act on Securing Quality, Efficacy and Safety of Products Including Pharmaceuticals and Medical Devices (PMD Act), mRNA products are defined as “Gene expression products for treatment” as one kind of gene therapy products. While mRNA-based products have also been developed in fields other than genome editing, the guidelines for the Quality and Safety **assurance** of Gene Therapy Products do not cover the quality or safety of mRNA. Currently, however, no mRNA product has not been approved for marketing in Japan or overseas. The manufacturing method and quality control of mRNA-based products should be clarified in the future. In particular, gene editing using mRNA manufactured by chemical modification such as methylated Cap, etc., which does not naturally occur, to ensure the intracellular stability of mRNA also requires safety evaluation of the applied chemical

modification. The sponsors are encouraged to have full consultation with relevant regulatory authorities about quality control and safety evaluation of mRNA. To chemically synthesized mRNA products, the same product management methods as nucleic acid products can be applied. mRNA synthesized by *in vitro* transcription using a plasmid or PCR product as a template should be considered to have additional safety evaluation for process-derived impurities.

3) Proteins and Guide RNA

ZFN- or TALEN-based genome editing technologies could be utilized to modify the target gene by directly introducing an artificial nuclease protein into a cell (18,19). A CRISPR/Cas technique can be conducted to introduce a complex (ribonucleoprotein or RNP) of sgRNA complementary to the target DNA sequence and Cas9 into a cell (20,21). Gene therapy product using these genome editing protein is not categorized to the definition of current gene therapy product, which introduces a gene into a cell or body. However, protein-based genome editing also has a risk with either off-target effects or adverse events accompanied this, and they should be required to conduct similar safety measures to gene therapy products coding genes of interest. In this sense, genome editing protein products are classified as products that modify the target gene. They need to be evaluated as gene therapy products as with current products used for gene transfer technologies. The Guidance for Industry: Gene Therapy Clinical Trials (Ministerial Announcement No. 344 of Ministry of Health, Labour, and Welfare in 2015), which was revised as of February 28, 2019, defines protein-based genome editing as gene therapy.

The quality of artificial nuclease proteins, such as ZFN and TALEN, should be evaluated with reference to the ICH guidelines for evaluation and quality control of cell banks for biotechnological products. In order to evaluate the quality attributes of sgRNA complementary to the target DNA sequence, the Considerations for Quality Assurance and Evaluation of Nucleic Acid Products (Notification No. 0927-3 of the Pharmaceutical Evaluation Division, Pharmaceutical Safety and Environmental Health Bureau, Ministry of Health, Labour, and Welfare) may be useful. Genome editing proteins transfected in cells need to be characterized for the persistence of their activities and kinetics.

4) Human Cell-based Products modified by Genome Editing technology

The quality control for human cell-based products manufactured from current gene-transfected cells can be applied to human cell-based products manufactured from *ex vivo* genome-edited cells. The quality control and characterization for the manufacture of vectors, and the establishment and characterization of cell bank systems should be evaluated in the same manner as current ICH guidelines. To assure the safety of administering genome edited cell-based products, nonclinical safety

assessment similar to that for human cell-based products made from conventional gene-transfected cells is essential.

(3) Classification by Purpose of Genome Editing

1) Gene Knockout (22-27) and Homologous Recombination (7,28,29)

When gene editing is intended to knock out genes, the frequency of gene knockouts in the target cells and the non-uniformity of targeted gene modifications should be analyzed. For example, in CRISPR/Cas-based gene editing, an justification of sgRNA design should include conclusions on the efficiency and non-uniformity of this gene engineering technique. Gene editing by HDR is based on the DSB repair mechanism of cells. The activity of homologous recombination is high in ES cells which have a high ability to repair DSB. It should be noted that the DSB repair efficiency can be very low in some type of cell. Therefore, the frequency of homologous recombination should also be clarified. It might be necessary to select cells that have undergone homologous recombination for treatment. To select and purify gene-modified cells, the methods for cell selection/purification should be justified.

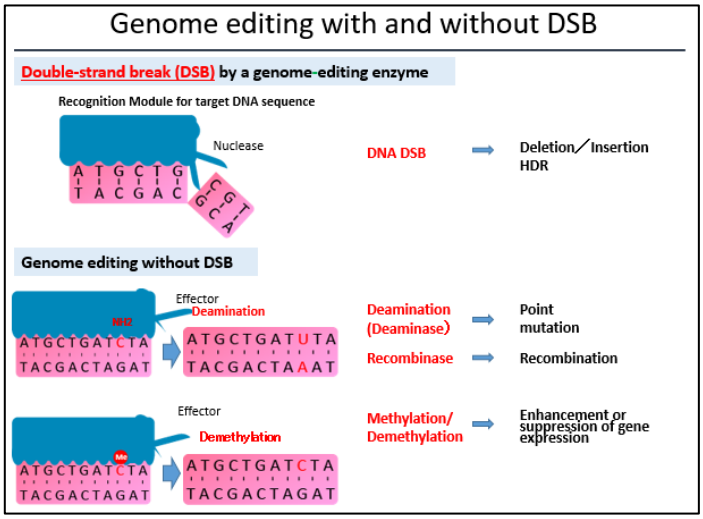
Homologous recombination involves introduction of donor DNA for gene recombination. To modify short DNA sequences such as single nucleotide polymorphism (SNP), single-strand DNA (ssDNA) that has a homologous sequence both upstream and downstream of the DSB site is to induce homologous recombination. In HDR to replace the entire gene coding for a protein, a plasmid is typically used as a DNA template. In this case, donor DNA having a homologous sequence of several hundred DNA base pairs from upstream to downstream of the DSB should be introduced to the target site. For this, it is crucial to evaluate the design of the donor DNA and the efficiency of homologous recombination (30). Even though some report suggests no correlation between a DNA length that can undergo homologous recombination and recombination efficiency, the homologous recombination efficiency should be determined together with the effects of the genome length (31).

For either simultaneous knockouts of more than one gene or more efficient homologous recombination, gene editing involving two DSBs has been explored. The existence of two or more DSBs is likely to be associated with significant chromosomal aberrations such as chromosomal translocations and deletions. Particularly in these cases, full evaluation to detect chromosomal aberration should be considered. (32).

2) Gene Modification without Genomic Cleavage (DNA modification without DSB such as Dead Cas9, deaminase, or, DNA methylation/demethylation)

To prevent chromosomal breaks, translocations, and large deletions associated with genome editing, genome editing technologies without DSB have been developed. These include a technique that breaks

only a single strand of the target DNA sequence, conversion of C to T or A to G with a deaminase, and introduction of epigenetic mutations, such as DNA methylation. However, these genome editing technologies without DSB may also cause adverse events attributable to the persistence of its activities and off-target effects. Their quality and safety as gene therapy products should be fully evaluated. As with DSB-based genome editing, the efficacy or specificity of non-DSB-based gene engineering without DSB may alter in each cell. Since selection and purification of gene-modified cells may be necessary, quality assessment of techniques without DSB should be conducted on these assumptions. In addition, the adequacy of each genome editing technology must be explained using the optimal analysis technique according to the nature of the technology.



5. Concept of Safety evaluation

(1) Issues Among Gene Therapy Products Using Genome Editing Techniques

1) Off-target Effects

To characterize the off-target effects of genome editing gene therapy products, it is necessary not only to predict the existence of sequences similar to the target gene sequence by *in silico* analysis and but also to explore experimentally candidate off-target sites throughout the entire human genome (33-36). Such off-target profiling methods include GUIDE-seq (37), which involves introduction of a tag of synthetic DNA in the cleavage site for genome-wide sequencing of the tag, and DIGENOME-seq (38), CIRCLE-seq (39), and SITE-seq (40), which explore potential off-target cleavage sites of genome editing enzymes using genome DNA extracted from cells. These analyses will focus on identifying mutations such as SNV/Indel and copy number variation (CNV) of cancer-related genes (41). Whether breaks or deletions have actually occurred at off-target sites predicted by *in silico* analysis or experimental methods should be clarified by the whole genome sequence (WGS) (34,36) of the genome-edited cell and amplicon sequence (42), which involves PCR amplification of candidate off-target sites followed by deep sequencing. While the detection sensitivity of these analyses depends on the read depth of DNA sequencing, it is very difficult to detect off-target effects that occur with a frequency of 0.1% or less.

To mitigate the off-target effects of CRISPR/Cas, the design of sgRNA may be most critical. It is

also important to select DNA sequences having few homologous sequences in other genomic regions by *in silico* analysis. However, it is noted that *in silico* analysis may not be able to predict all candidate off-target sites. Combination of *in silico* analysis and *in vitro* analysis is useful in identifying candidate off-target sites, and understanding the frequency of off-target effects and their influence are very important. Since in *in vitro* analysis, natural gene mutations may occur in cells during culture, such background mutations should be excluded in assessing gene mutations associated with genome editing procedures.

Investigation of off-target effects of genome editing in animals as non-clinical study is not appropriate because of species differences in the genome sequence between humans and animals. As characterization studies of genome editing, therefore, the frequency of off-target events and affected DNA sequences should be analyzed in detail in *in vitro* assays using human cells. If the results of characterization of an *ex vivo* genome editing-based product show the off-target effects of the gene editing, the risk of the off-target effects causing cancer and other possible adverse effects on the safety of the gene therapy itself should be evaluated. Clonality analysis of gene-modified cells may be required if necessary. For *in vivo* genome editing products, *in vitro* analysis using an established cell line harboring many DNA mutations may not provide useful data. In order to assess the off-target effects of *in vivo* genome editing, it is encouraged to consider analysis using primary cells. iPS or ES cell-derived functional cells are also useful. iPS and ES cell-derived cells are very promising tools for assessment of off-target effects on human primary cultured cells that are not easily available.

2) Genome Deletions/Insertions of unintended DNA and Chromosomal Translocations/Inversions

It is reported that large deletions (of several kb), and insertions and inversions of gene fragments during the DSB repair process may occur in process of genome editing. The insertion of the genome DNA of a viral vector used for genome editing in the target site are also reported. This may be because gene modifications by genome editing depend on the DSB-induced genome repair mechanism of cells. The directionality of genome editing how to modify or repair the target genome is not involved in genome editing tool (43). Therefore, the genome sequence adjacent to the target gene in cells/tissues as similar as possible to the actual target cells should be analyzed in detail. As aforementioned, the risk of chromosomal translocations and deletions following a DSB has been reported. In particular, formation of two DSBs in the genome is reportedly associated with an significant increase in the risk of chromosomal translocations. Such chromosomal aberrations should be analyzed using G-band analysis, Q-band analysis, multicolor fluorescent *in situ* hybridization (mFISH) using false colors, comparative genomic hybridization (CGH), etc. However, it should be understood that these analyses also have certain limitations. For example, G-band analysis and mFISH only analyze cells in the

metaphase stage. For G-band analysis, it is difficult to deal with many cells at once and detect a very small group of cells having chromosomal aberrations. On the other hand, mFISH is suitable for detection of translocations between different chromosomes and large chromosomal deletions. However, this analysis does not detect inversions within the same chromosome. CGH can detect abnormal gene amplifications and deletions when they occur in many cells. However, the sensitivity of CGH is too low to detect DNA aberrations when they are not uniform across cells or occur only in some cells. In the risk assessment of genome editing-associated chromosomal aberrations, the characteristics of these analysis methods should be fully taken into consideration.

3) Risk of DNA-Repair Gene Mutations Such as p53 in Genome-edited Cells

Mutations of p53 tumor-suppressor gene in cells of which a gene modification was caused by HDR-based genome editing and an increase in HDR efficiency in cells knocked out for p53 gene have been reported (47,48). These phenomena can be mainly explained by the fact that p53 mutations increase resistance to cell death. The occurrence of gene mutations related to genome repair factors, such as p53 mutations, should be investigated in HDR-derived gene introduction.

4) Difference in Risk of Cancerization Among Target Cells

Both the genome editing technologies and the current gene therapies using chromosomally-integrated vectors such as retroviral vector and lentiviral vector are intended to modify genes themselves. From this perspective, the risk of their off-target effects in both products will be similar. From the early phase of the development of gene therapy, the most concerned risk associated with chromosomally-integrated vectors was to induce the cancer in the cells due to insertional mutagenesis. In fact, gene therapy using hematopoietic stem cells for the treatment of X-linked severe combined immunodeficiency (X-SCID) or Wiskott-Aldrich syndrome (WAS) were reported to cause leukemia (8). Currently, therefore, these gene therapies require long-term follow-up. On the other hand, there is no report of cancer caused by gene therapies where a chromosomally-integrated vector is introduced into cells other than hematopoietic cells.

Hematopoietic cell-based gene therapies involve insertion of a vector having a viral promotor/enhancer adjacent to a cancer-related chromosomal gene (49), which is thought to be the mechanism to cause cancer associated with hematopoietic cell-based gene therapies. Genome editing using neither promoter nor enhancer is unlikely to cause inserted mutations to promote cell proliferation. In particular, the direct transfection of genome editing tool as a protein or mRNA into cells is very unlikely to cause such cancer. However, other concerns exist in such a genome editing tool, because genome editing may cause chromosomal translocations, deletions, and other aberrations as aforementioned. Chromosomal translocations may cause cancer chimera proteins such as *Bcr-abl*

or destroy tumor-suppressor genes (50). Genome editing with homologous recombination may cause the increase in number of cells harboring mutations in tumor-suppressor genes such as p53 as described earlier. The risk of genome editing causing cancer due to chromosomal aberrations or destruction of tumor-suppressor genes has not been fully investigated yet. From the experience with current gene therapies where genes are integrated into cells, the risk of carcinogenesis should depend on the type of cell. Differentiated cells are likely to be more robust to the risk of carcinogenesis than those undifferentiated. On the other hand, iPS/ES cells and hematopoietic cells have a higher risk of carcinogenesis than the other somatic cells.

5) Immunogenicity of Genome Editing Enzymes

DNA breaking enzymes used for genome editing, such as Cas protein, are derived from bacteria. Even in *ex vivo* gene therapy, therefore, when genome-edited cells administered *in vivo* express the enzymes, the enzymes are recognized as heterologous antigens. The immunogenicity in human could not be predicted from animal studies. Clinical trials should be designed taking into consideration the potential immunotoxicity of genome editing enzymes, i.e., immune response to these enzymes, including the attenuation of clinical effects and anaphylaxis.

(2) *In Vivo* Genome Editing

1) Safety Evaluation of Modified Target Genes

When some safety concerns about the expression of modified target genes exist, a POC study in animals having the modified homologous gene may provide information about kinetics and safety related to the modified target gene, together with data supporting the efficacy or performance of the gene therapy.（Table 1）

Table 1. Immunogenicity of DNA breaking enzymes used for genome editing

● AAV-CRISPR-Cas9 activates the immune system, resulting the elevation of not only humoral but also cellular immune response. (Chew et al., Nature Methods, 2016)
● Seventy-nine % of healthy donors staining against SaCas9, 65% of donors staining against SpCas9 and 46 % staining against SaCas9 were observed, respectively. (Charlesworth et al., BioRxiv, 2018)
● From screening for pre-existing antibodies in 200 human serum, the prevalence of anti-SaCas9 and anti-SpCas9 antibodies to be 10% and 2.5% were detected, respectively. (Simhadri et al., Mol Ther Methods Clin Dev, 2018)
Immunogenicity; Serum type of vector, Route of administration, Dose, Promotor, Host etc.

2) Targeting and Modification Efficiency of Genome Editing Enzymes

In *in vivo* genome editing, targeting in tissues/cells to be modified is important (51). It is necessary to characterize the distribution of a genome editing enzyme *in vivo* in order to understand its distribution to not only targeted cells/tissues but also non-targeted cells/tissues. The persistence of the genome editing enzyme in tissues/cells also should be understood. In particular, when an *in vivo* distribution study shows the distribution signal of a genome editing enzyme in germline cells, the risk of germline gene modification should be clarified in nonclinical studies with reference to the ICH opinion "General Principles to Address the Risk of Inadvertent Germline Integration of Gene Therapy Vectors."

In vivo genome editing may not provide sufficient effects because of its low genome editing efficiency in target cells/tissues. A variety of technologies to improve the efficiency have been under development. For example, homology-independent targeted integration (HITI) is the method where the same sequence as that at the target DBS site is inversely inserted into the donor vector to cut the genome and the donor vector simultaneously (52). This technique allows efficient *in vivo* genome editing. Introduction of CRISPR/Cas using AAV to express CRISPR/Cas for a prolonged period of time is reported to enable efficient genome editing of non-dividing cells (53). However, the long-term expression of CRISPR/Cas cause increase in risks of off-target effects and other undesirable gene modifications at the target sequence. It should be noted that off-target effects of *in vivo* genome editing, if they occur, are difficult to remove, unlike *ex vivo* genome editing.

3) Others

Nonclinical studies using model animals are unlikely to provide useful information about the off-target effects of *in vivo* genome editing-based products. Limited but somewhat meaningful information about off-target effects may be obtained from *in silico* analysis and *in vitro* analysis using human cells. Clinical development studies for *in vivo* genome editing technologies should be designed on the basis the assessment of their potential risks using these analyses. Clinical design should be considered about both identified potential risks and potential usefulness for each indication.

6. Important Issues in Clinical Trials (including Long-term Follow-up)

Since genome editing technologies are intended to modify target genes, genome editing requires long-term follow-up of patients for having similar risk to current gene therapy products using a chromosomally integrated vector. Genome editing, which is utilized to deletes or inserts genes at specific sites, could be safer than current gene therapies involving random gene insertions unless no safety concern associated with off-target effect exists. However, genome editing using homologous

recombination-possibly increases in mutation risk of DNA-repair genes such as p53 and is associated with the risk of chromosomal translocation. To identify adverse events related to these risks, the period of follow-up should be set in according to each risk (54).

The length of follow-up should be set in dependent to the genome editing technology (e.g., gene modifications through direct introduction of a protein, etc. or introduction/modification using a viral vector), type of target cells, and targeted gene. Considering experiences with current gene therapy products, especially the application of genome editing to hematopoietic stem cells is associated with a high risk of adverse events. It is strongly encouraged to make long-term follow-up plans including periodic examinations.

The risk related to *in vivo* genome editing, should be considered to cause the gene modification in off-target tissues/cells, especially germline cells. In particular, when there is the risk of gene modification in germline, measures to prevent the modification from affecting subsequent generations, such as setting an adequate contraception period, should be taken. The risk control measures for genotoxic antineoplastic drugs can help to establish such measures (55). Since it is difficult to identify gene mutations in germ cells and fertilized eggs, careful long-term follow-up is required to investigate the off-target effects of *in vivo* genome editing.

7. Overview Summary

This document summarizes recent discussions about development of the gene therapy products using genome editing technology by experts in gene therapy and genome editing study in Japan. We hope that this documents will help companies and researchers to develop new genome editing-based gene therapy approaches, as well as reviewers to conduct regulatory reviews of genome editing products. However, genome editing technologies are rapidly advancing with a greater variety of technologies. This document needs to be revised as necessary according to technological advances.

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