参考資料７

過去の報告書・英訳・論文掲載の事例

第４期：ゲノム編集専門部会

- ゲノム編集技術を用いた遺伝子治療用製品等の品質・安全性等の考慮事項に関する報告書・・・P2

- 事務連絡（厚生労働省から自治体あて）
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令 和 ２ 年 ２ 月 ７ 日

独立行政法人医薬品医療機器総合機構
理 事 長　　藤 原 康 弘　殿

科 学 委 員 会
委員長 井上 純一郎

科学委員会では、今般、下記について科学的見地からの議論をまとめました。
独立行政法人医薬品医療機器総合機構における通常業務にご活用ください。

記

ゲノム編集技術を用いた遺伝子治療用製品等の
品質・安全性等の考慮事項に関する報告書

以上

ゲノム編集技術を用いた遺伝子治療用製品等の品質・安全性等の考慮事項に関する報告書

令和２年２月７日
科　学　委　員　会

【目次】

1. 序論

特定の遺伝子を特異的に切断、改変、編集できる画期的な技術としてゲノム編集技術[1, 2]の開発が精力的に進められ、新たな遺伝子治療法として、その実用化が期待されている。

この技術が特に注目される理由は、これまでの遺伝子治療は新たな遺伝子を付加することで疾患を治療するのに対し、ゲノム編集は、特定の遺伝子の機能を失わせたり、疾患の原因となっている遺伝子異常を修正したりすることが可能なため、究極の遺伝子治療技術となる可能性があることによる。

ゲノム編集技術の基本は、DNA の特定の部位への二重鎖切断（double strand break：DSB）の導入と細胞のもつ修復機構の利用である。DSB の修復機構としては非相同末端結合（non-homologous end joining：NHEJ）と、相同組換え（homologous recombination:HR)を利用した修復(homology-directed repair: HDR）がある。NHEJ による修復は細胞周期を通して起こる応急処置的な反応で、結合時に末端で数塩基の挿入や欠失を伴う場合があるため遺伝子破壊に利用できる(図 1)。一方、主として細胞周期の S/G2 期に起きる HR[3]では相同配列との組換えによる修復が起こり、正常遺伝子配列を持つテンプレート DNA を導入して HDR を起こすことにより疾患の原因となっている遺伝子異常の正常化も可能となる。また、HDR を利用して特定のゲノム部位に目的とする遺伝子を導入・置換するようなゲノム編集も試みられている。

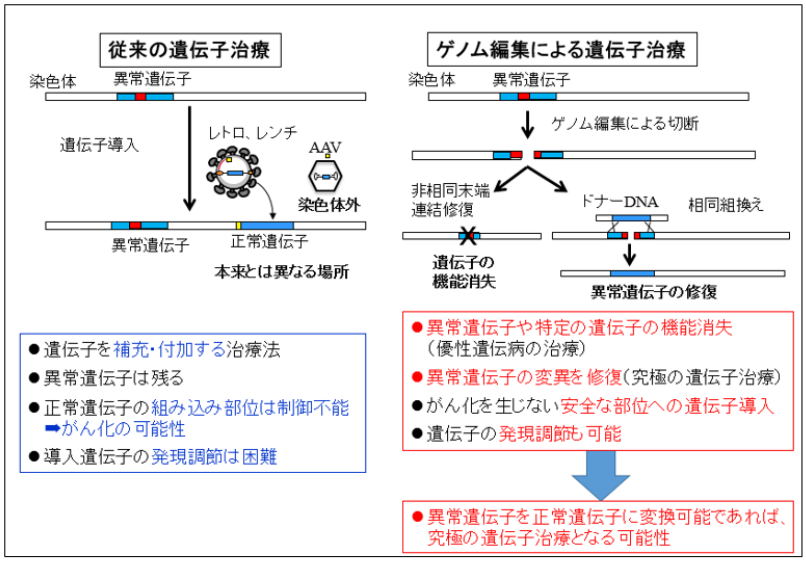

図１　遺伝子治療技術とゲノム編集技術の違い

特定の塩基配列特異的に DSB を導入できる人工ヌクレアーゼとして初期に開発されたのが zinc-finger nuclease （ZFN）[4]と transcription activator-like effector nuclease （TALEN）[1]である。しかしながら、これら人工ヌクレアーゼは特定の塩基配列の認識をタンパク質によって行うため、その作製に高度の技術と時間を要し、莫大な費用もかかる。一方、近年開発された clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR associated proteins (Cas) [2]では、目的遺伝子の塩基配列認識を一本鎖ガイド RNA（single-guide RNA; sgRNA）により行うため、その設計は容易で、費用も安価であることから、汎用性の高い遺伝子改変技術として急速に発展した[5]。事実、海外では感染症やがん、単一遺伝子疾患等を対象にゲノム編集を用いた遺

伝子治療臨床試験が実施されており、数年以内にこれら遺伝子治療用製品等の製造販売承認申請が行われる可能性がある。そして、これを受け、国内においても治験が始まる可能性が高く、ゲノム編集技術を用いた遺伝子治療用製品等の品質及び安全性に関する考え方を整理しておく必要があると思われる。

本文書は、始めに、現在開発中のゲノム編集技術に特有の課題を提示し、次にその手法とそれを細胞内や体内に導入するための技術（ツール）並びにゲノム編集の目的によって分類し、それぞれの特性を整理すると共に、ゲノム編集技術の特性を踏まえた品質や安全性評価及び臨床における長期フォローアップに関する考慮事項をまとめたものである。なお、ゲノム編集に伴うリスクに対する考え方は対象疾患の種類や重篤度によって異なると考えられ、その臨床応用に関してはリスクベネフィットを考慮した個別の評価が必要になる。さらに、ゲノム編集技術は現在急速に進歩しているため、本考慮事項に関しても、適宜見直しを行うことが必要である。

2. 定義

本文書では、ゲノム編集技術を用いた遺伝子治療用製品等を以下のとおり定義する（図 2）。

(1) *in vivo* ゲノム編集製品（直接、体内に投与して体内でゲノム編集を行うための製品）

①ゲノム編集遺伝子治療用製品（ゲノム編集に用いる酵素タンパク質（以下、「ゲノム編集酵素」という。）を発現させるウイルスベクター又はプラスミドベクターを主成分とする製品）

②ゲノム編集 mRNA 製品（ゲノム編集酵素を発現させる mRNA を主成分とする製品）

③ゲノム編集タンパク質製品（ゲノム編集酵素を主成分とする製品（sgRNA を含む場合もある））

(2) *ex vivo* ゲノム編集製品（ゲノム編集ツールにより体外で遺伝子改変した細胞であり、体内に投与するための製品）

①ゲノム編集細胞加工製品（ゲノム編集ツールにより体外で遺伝子改変したヒト細胞加工製品）

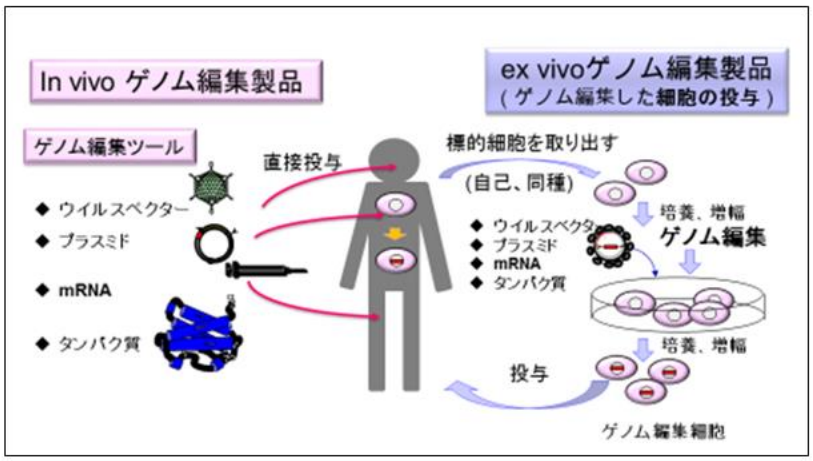

図２　ゲノム編集製品と投与方法

3. ゲノム編集技術特有の課題

(1) 遺伝子改変細胞のがん化等のリスク

ゲノム編集技術は細胞の特定の遺伝子を塩基配列特異的に切断、改変、編集できる技術であるが、同時に類似の塩基配列をもつ目的外の遺伝子の編集リスク、すなわちオフターゲット作用のリスクが存在する。このオフターゲット作用による結果として特に懸念されるのが、細胞のがん化である。オフターゲット作用により、直接がん遺伝子の活性化やがん抑制遺伝子の不活化が起こる可能性があり、またゲノム編集の遺伝子改変は永続的な効果をもたらすため、その危険性は増大する。

また、DSB を誘導するゲノム編集技術では染色体切断に伴いゲノムが不安定化したり、従来の評価法では検出できない染色体の大規模欠損や切断部位への目的外配列の挿入が起きたりするリスクも報告されていることから、染色体異常によるがん化のリスクについても検討する必要がある。

(2) 生殖細胞における意図しない遺伝子改変リスク

in vivo ゲノム編集では、標的細胞以外でのゲノム編集や、目的遺伝子以外の遺伝子改変が生じても、それらを確認したり排除したりすることは困難である。

特に、小児や生殖可能年齢の患者を対象とする *in vivo* ゲノム編集では生殖細胞への影響が懸念され、最近ではゲノム切断に伴う染色体異常等のリスクを避けるために、ゲノム切断を介することなく遺伝子改変を行う新たな技術も開発されているが、次世代への遺伝的な影響を十分に検討する必要がある。

4. ゲノム編集技術の分類とその品質特性に関する課題

(1) ゲノム編集ツールによる分類とその留意事項

1) ZFN[4]、TALEN[1]

ZFN は、特定の 3 塩基配列を認識する zinc-finger タンパク質モチーフを 3～6 個有し、目的とする塩基配列に結合するドメインと、DNA 切断酵素である *Fok* I nuclease を連結させた人工ヌクレアーゼであり、この DNA 結合タンパク質の設計には高度な技術が要求される。一方、ZFN の複雑さを改良した TALEN は、植物由来の転写因子である TAL の 34 アミノ酸からなるモジュールが 1 塩基を認識することを利用しており、A、G、C、T の各塩基をそれぞれ認識する 4 種類の TAL モジュールを連結することで目的塩基配列を認識し、特定の塩基配列を切断する。TALEN では TAL モジュールを 15～20 結合させることで 15～20 個の塩基を認識するように設計される場合が多い。

ZFN も TALEN も *Fok* I が二本鎖 DNA のうち一方の DNA 鎖しか切断しないため、目的とする切断部位を挟んで上流と下流の塩基配列を認識する２つの人工酵素を設計する必要があり、1 か所の DSB に必要な認識塩基配列は一つの人工酵素が認識する配列の 2 倍となり、その数は 18～40 塩基程度となる。このため塩基配列の認識特異性は高く、オフターゲット作用が起こる頻度は CRISPR/Cas9 より低いとされている[1]。ZFN や TALEN におけるオフターゲット作用は CRISPR/Cas9 ほど報告されていないが、現時点で十分な情報が得られていないことから、慎重

に評価する必要がある。

2) CRISPR/Cas[2]

CRISPR/Cas における特定の塩基配列の認識は、ZFN や TALEN と異なり、標的となる DNA 配列と相補的な sgRNA が担っている。すなわち、sgRNA は染色体 DNA 上の標的となる 20 塩基と相補的に結合するガイド配列と、その標的配列に隣接する PAM（proto-spacer adjacent motif：プロトスペーサー隣接モチーフ）と呼ばれる配列を持つ必要がある。この sgRNA と DNA 二重鎖切断酵素である Cas9 が複合体を形成し、sgRNA が認識する塩基配列を有する遺伝子を切断する。一方、この CRISPR/Cas システムでは、sgRNA が最大 5 個のミスマッチ（A:T または、G:C 以外）があっても結合することが知られ、目的外遺伝子の切断とそれに伴う目的外配列の挿入や欠損が起きるオフターゲット作用の可能性は高くなる[6]。一方、これまでにオフターゲット作用が起こる頻度に関する論文が数多くあるが[7-9]、その評価、特に少数の細胞で起こる低頻度のオフターゲット作用を正確に評価することは難しいとされている。

CRISPR/Cas のオフターゲット作用を低減化するための取組みとして、ガイド RNA の長さや目的とする配列の立体構造等の影響が検討されているが、現時点では十分な技術が確立している状況ではない。したがって、随時刷新される新たな知見をもとに、sgRNA を設計し、オフターゲット作用の頻度を評価する必要がある。

3) ゲノム切断を行わないゲノム編集[10, 11]

DSB に伴うゲノムの不安定化を低減させるため、ゲノム切断を行わないゲノム編集（デアミナーゼによる 1 塩基編集）等の様々なゲノム編集技術が開発されている。このような新規ゲノム編集技術を適用する場合でも、どのような機序においてオフターゲット作用が低減できるのかを、評価法を含め、説明する必要がある。

(2) ゲノム編集ツール及び遺伝子改変した細胞における留意事項（図 2）

1) ウイルスベクター、プラスミドベクター

ゲノム編集に用いる ZFN や CRISPR/Cas を細胞内に導入するためにアデノウイルスやアデノ随伴ウイルス（AAV）等のウイルスベクターを用いた臨床試験が実施されている[12, 13]。このように、ゲノム編集酵素遺伝子を搭載したウイルスベクターやプラスミドベクター[14] を用いる場合、その品質管理に関しては従来の遺伝子治療用製品と同様の考え方が適用可能であり、ベクター製造に関する品質管理と特性解析並びにセルバンクシステムの構築とその特性解析等は従来の遺伝子治療用製品と同様の評価を適用するべきである。

一方、従来の遺伝子治療用製品では、目的タンパク質の効率的な発現のためにウイルスプロモーターが搭載される場合が多く、このプロモーターががん関連遺伝子近傍に挿入されることでがん化も起こりうることが報告されている[8]。ゲノム編集において ZFN・TALEN・Cas9/sgRNA を発現させるため同様のプロモー

ターが用いられる場合もあるが、現時点ではプロモーター等の挿入発がんの報告はない。一方で、導入したプラスミドが DSB 部位に組み込まれた例も報告されていることから、ゲノム編集遺伝子治療用製品においても従来の遺伝子治療用製品と同様の非臨床安全性評価が必要と考えられる。また、細胞や組織指向性については、生体内分布を含めた評価が重要である。

ゲノム編集ツールの細胞内への導入法としてウイルスベクターを用いる場合、感染性や細胞指向性の観点から「目的とする細胞での遺伝子改変の程度及び頻度」と「目的としない細胞での遺伝子改変の程度及び頻度」について解析する必要がある。さらに、ゲノム編集酵素の発現が持続するとオフターゲット作用の危険性が増加することも留意すべきである。特に、ウイルスベクターを用いた場合には長期にわたって持続的にゲノム編集酵素が発現する可能性があり、安全性の観点からもゲノム編集酵素の発現持続性を解析しておく必要がある。

2) mRNA

細胞内で Cas、TALEN、ZFN 等のタンパク質を発現するために、これらタンパク質をコードする mRNA の細胞内導入にて行う方法が報告されている[13, 15, 16] 。「医薬品、医療機器等の品質、有効性及び安全性の確保等に関する法律（薬機法)」の上では、mRNA は遺伝子治療用製品の中の「遺伝子発現治療製品」に含まれているが、「遺伝子治療用製品等の品質及び安全性の確保に関する指針」には mRNA の品質や安全性に関する記載はない。現状としては、ゲノム編集の分野以外でも mRNA を用いた製品が開発されているが、現時点において、国内外でいまだ製造販売承認されたものはなく、今後、mRNA 製品の製法や品質管理についての評価法を明確にしていく必要がある。特に、細胞内での安定性を確保するためにメチル化 Cap 等の天然型にない化学修飾を加えた mRNA を利用する場合には当該修飾に関する安全性評価も必要と考えられることから、mRNA の品質管理や安全性評価について開発初期から規制当局と十分な相談を行うことが求められる。mRNA 製品を化学合成によって製造する場合は核酸医薬品に準じた製品管理の手法が適用できるが、プラスミドや PCR 産物を鋳型として *in vitro* 転写により合成する場合には、追加の製造工程由来の不純物について安全性評価が必要となる。

3) タンパク質、ガイド RNA

ZFN や TALEN によるゲノム編集技術では、人工ヌクレアーゼタンパク質を直接細胞内に導入することで目的の遺伝子改変が可能である[17, 18]。また、CRISPR/Cas では、標的 DNA 配列に相補的な sgRNA と Cas9 タンパク質との複合体（ribonucleoprotein; RNP）をあらかじめ形成し、細胞に導入する方法も報告されている[19, 20]。この様なゲノム編集タンパク質製品による遺伝子治療は、「細胞内や生体に遺伝子導入する」という従来の遺伝子治療の定義には当てはまらない。しかしながら、遺伝子を導入する遺伝子治療と同様、目的外の遺伝子を改変する危険性やそれに伴う有害事象の発現が懸念されるため、遺伝子を使用する遺伝子治療用製品と同様の安全対策が必要である。したがって、ゲノム編

集タンパク質製品も標的遺伝子の改変を目的として用いられることから、従来の遺伝子治療用技術の視点を踏まえて、同様の品質・安全性評価を行う必要がある。なお、平成 31 年 2 月 28 日付けで改正された「遺伝子治療等臨床研究に関する指針（平成 27 年厚生労働省告示第 344 号)」ではこのようなタンパク質を用いたゲノム編集技術も遺伝子治療等として定義している。

ZFN や TALEN などの人工ヌクレアーゼタンパク質の品質評価については、バイオ医薬品の細胞バンク評価や品質管理に関する ICH ガイドライン（Q5A,Q5B,Q5D,Q6B）が参考となる。また、標的 DNA 配列に相補的な sgRNA の品質評価については、「核酸医薬品の品質の担保と評価において考慮すべき事項について」（平成 30 年 9 月 27 日付薬生薬審発 0927 第３号）が参考となる。さらに細胞内に導入されたゲノム編集酵素について、細胞内での活性の持続性や動態等の評価も必要となる。

4) ゲノム編集ツールを用いて加工したヒト細胞加工製品

ex vivo でゲノム編集した細胞からなるヒト細胞加工製品の場合、その品質に関しては従来の遺伝子導入細胞からなるヒト細胞加工製品と同様の考え方が適用できる。ベクターを用いる場合には、その製造に関する品質管理と特性解析並びにセルバンクシステムの構築とその特性解析等は従来の製品と同様の評価を適用するべきである。ゲノム編集細胞加工製品の投与に際しても従来型の遺伝子導入細胞からなるヒト細胞加工製品と同様の非臨床安全性評価が必要と考えられる。

(3) ゲノム編集の目的による分類

1) 遺伝子破壊[21-26]及び相同組換え[7, 27, 28]

遺伝子破壊を目的とする場合は、目的とする細胞での遺伝子破壊の頻度や目的遺伝子改変の不均一性について評価する必要がある。例えば、CRISPR/Cas を用いる場合は、sgRNA の設計の適切性を説明する際に、このような遺伝子改変の効率や不均一性についてどのような検討がなされたかを含める必要がある。相同組換えを目的とする場合も、細胞の DSB 修復機構を利用する反応であるため、その活性が高い ES 細胞等では高効率で組換えが起こる[29]が、細胞によってはその効率が極めて低い場合があることに留意すべきである。また、相同組換えの頻度を評価する必要があり、場合によっては相同組換えが起きた細胞のみを選択し、治療に用いることも想定される。このように遺伝子改変した細胞の選別・純化を行う場合にはその手法の適切性を示す必要がある。

HDR の場合には遺伝子組換え用ドナーDNA を導入する必要があるが、一塩基多型（single nucleotide polymorphism：SNP）のような短い DNA の改変[30] では、切断した配列の上流及び下流の両方に相同な配列を持つ一本鎖 DNA(single-strand DNA; ssDNA) を導入することで相同組換えが可能である。一方、タンパク質をコードする遺伝子全体を HDR で置換する場合は、供与するテンプレート DNA としてプラスミドを用いる場合が多い。この場合は切断部位の上流及び下流にわたって数百塩基の相同配列を持つドナーDNA を導入する必要があるが、その

際にはドナーDNA の設計と相同組換えの効率の評価が重要になる。また、これまでに相同組換え可能な DNA 長について、DNA 長と組換え効率には相関がないとの報告もあるが、相同組換えの効率についてはゲノム長の影響も含めて評価しておく必要がある。

なお、複数の遺伝子を同時に破壊したり、あるいは相同組換えをより効率的に引き起こすために DSB を 2 箇所に入れたりすることも試みられているが、DSB を 2 箇所以上入れた場合には染色体の転座や欠失等の大きな染色体異常が発現しやすいとされており、特に染色体異常について検討を考慮するべきである[31]。

2) ゲノム切断を伴わない遺伝子改変（Dead Cas9 やデアミナーゼ等による非切断改変、DNA メチル化・脱メチル化）

ゲノム編集による染色体切断や転座・大規模欠失を防ぐ手法として、DSB を起こさないゲノム編集技術の開発が行われており、標的 DNA 配列の一方の鎖のみを切断する反応や、デアミナーゼによる C→T 変換や A→G 変換、さらには DNA のメチル化等のエピジェネティック変異の導入等が試みられている（図 3）。しかしながら、これらの DSB を起こさないゲノム編集技術についても、その持続性やオフターゲット作用による有害事象の可能性があり、遺伝子治療用製品としての品質や安全性評価が必要と考えられる。この場合、DSB を引き起こすゲノム編集の場合と同様に、細胞ごとにゲノムの改変の効率や特異性が変わり得ること、場合により改変された細胞の選別・純化等も必要になることを前提に品質評価を行う必要があり、さらに各遺伝子改変技術の内容に応じた最適な解析手法を用いて、使用する技術の妥当性を説明しなければならない。

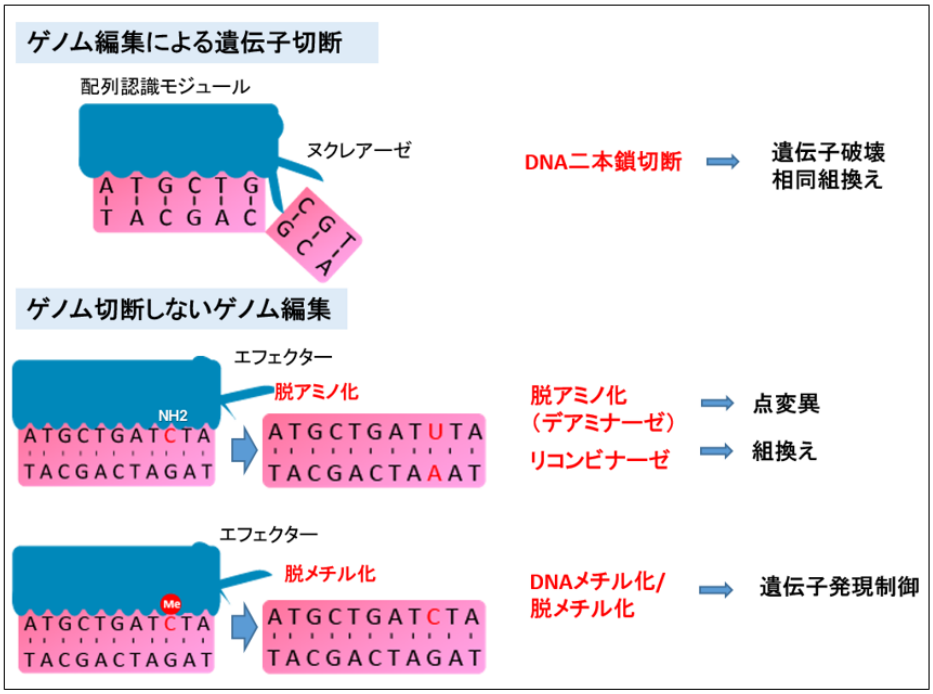

図３　ゲノム編集による遺伝子切断とゲノム切断しないゲノム編集

5. 安全性評価の考え方

(1) ゲノム編集技術を用いた遺伝子治療用製品等の共通事項

1) オフターゲット作用

ゲノム編集技術を用いた製品によるオフターゲット作用の特性を把握するために、標的とする遺伝子配列に類似した配列の存在を *in silico* 解析により予測するだけでなく、実験的手法を用いてヒトゲノム全体にわたりオフターゲット候補サイトを解析することが必要である[32-35]。実験的手法でオフターゲット候補サイトを検索する方法としては、ゲノム編集の際、切断部位に合成 DNA のタグを導入し、タグの取込みをゲノム全体にわたって塩基配列解析する方法（GUIDE-seq）[36]や、細胞から抽出したゲノムを用いてゲノム編集酵素による切断可能部位を探索する DIGENOME-seq[37, 38]、CIRCLE-seq[39]、SITE-seq[40]等の方法がある。これらの解析では、例えば、がん関連遺伝子[41]の SNV（single nucleotide variant：一塩基亜型）/Indel（insertion and deletion：挿入や欠失）やコピー数異常（copy number variant：CNV）等の変異を確認すること等が考えられる。*In silico* 解析及び実験的手法により検出されたオフターゲット候補サイトにおいて、実際に切断や欠失が起こっているか否かを確認する手法としては、ゲノム編集を実施した細胞の全ゲノムシークエンス（whole genome sequence：WGS）の確認[33, 35]や、候補サイトを PCR 増幅しディープシーケンスする amplicon sequence 等[42] が想定される。これらの解析では、塩基配列をどの程度深く読むかによって検出感度が左右されるが、次世代シークエンシング技術（next-generation sequencing：NGS）のエラー頻度のために、0.1%以下の頻度で起こるオフターゲット作用を検出することは極めて困難である（表 1）。

表１　オフターゲット作用の検出方法

カテゴリー	方法	例	長所	短所
全ゲノムシークエンス	Hi-seq など		正確性	高価 低感度
in silico	DNA 配列の相同性など		容易	不正確
細胞内（*in vitro*）	DNA 二本鎖切断を同定	BLESS, BLISS, GUIDE-seq	実際の細胞内での二本鎖切断	一部の細胞腫でのみ可能
試験管内	酵素反応	Digenome-seq, CIRCLE-seq	高感度 SNP も区別	細胞内反応ではない

CRISPR/Cas のオフターゲット作用を可能な限り低減化するためには、sgRNA の設計が最も重要であり、*in silico* 解析により他のゲノム領域に相同配列の少

ない塩基配列を選ぶことが重要である。しかし、*in silico*解析では全てのオフターゲット候補部位を予測できない可能性もあるため、これに加えて *in vitro* 解析を組み合わせたオフターゲット候補部位の検索やオフターゲット作用の生じる頻度とその影響をできるだけ評価しておくことが有用である。ただし *in vitro*解析では、培養中に細胞に自然発生的な遺伝子の変異が起こる可能性があるため、このようなバックグラウンド変異も考えられ、場合によってはバックグランド変異を差し引いてゲノム編集操作によって起こる遺伝子の変異を評価する必要もある。

ゲノムの塩基配列にはヒトと動物で種差が存在することから、ゲノム編集におけるオフターゲット作用について動物で評価することは困難と考えられる。このため、特性解析の一環として、ヒト細胞を用いた *in vitro* 試験の中でオフターゲット編集の発生頻度やオフターゲット編集が起きた塩基配列を詳細に解析する必要がある。*ex vivo* ゲノム編集の場合、ゲノム編集した製品の特性解析の結果からオフターゲット作用が確認された際には、オフターゲット作用によるがん化のリスク等、当該遺伝子治療自体の安全性にどこまで影響を与えるかを評価し、必要に応じてその遺伝子改変細胞のクローナリティー解析が必要な場合もある。一方、*in vivo* ゲノム編集の場合、多くの変異を持つ株化細胞を用いた *in vitro* 解析では有用なデータが得られない可能性もあり、初代細胞を用いた解析を考慮することが望ましい。このためヒトでの *in vivo* ゲノム編集のオフターゲット作用を評価するために iPS 細胞や ES 細胞由来細胞を用いることも有用と考えられる。iPS 細胞や ES 細胞由来細胞は、ヒト初代培養細胞の入手が困難な細胞に対する作用を評価する場合に有用なツールになる可能性が高い。

2) ゲノム欠失・目的外配列の挿入、染色体の転座、逆位

ゲノム編集では DSB の修復過程で数 kb にわたる大きな欠失や遺伝子断片の挿入、逆位が生じることが報告されている[43]。また、ゲノム編集に使用したウイルスベクターのゲノム DNA がその部位に挿入されている例も報告されている[44, 45]。これはゲノム編集による遺伝子改変が、DSB により誘導される細胞のゲノム修復機構に依存しているためで、どのようにゲノムを編集するのかの指向性（修復指向性）が定まっていないことによる[43]。したがって、ゲノム編集により編集された標的遺伝子近傍のゲノム配列の状態を、できるだけ実際の標的に近い細胞・組織を用いて詳細に解析しておく必要がある。先に述べたように、DSB に続いて染色体転座や欠失等を引き起こすリスクが指摘されており、特に、ゲノム上 2 カ所に DSB を導入する場合は有意に染色体転座の危険性が増大することが報告されている[46-48]。そのため、G バンド解析や Q バンド解析、疑似カラーを用いた multicolor fluorescent *in situ* hybridization (mFISH)、さらには比較ゲノムハイブリダイゼーション（comparative genomic hybridization：CGH）等を利用して、染色体異常を解析する必要がある。ただし、これらの解析には一定の限界があることも理解しておく必要がある。例えば、G バンド解析や mFISH ではメタフェーズ（分裂中期）にある細胞しか解析できない。また、G バンド解析は多数の細胞を一度に解析することは困難で、染色体異常を有するご

く少数の細胞集団を検出することは難しい。一方、mFISH は異なる染色体間での転座の検出や染色体の大きな欠失を検出するのには適しているが、同じ染色体内での逆位等は検出できない。さらに CGH では異常な遺伝子の増幅や欠失が多くの細胞に起きている場合には検出可能であるが、細胞ごとに不均一性がある場合や一部の細胞でのみ生じた異常を検出できる感度は有していない。これら解析法の特性を十分考慮した上で、ゲノム編集による染色体異常のリスクを評価する必要がある。

3) ゲノム編集細胞における p53 等のゲノム修復遺伝子の変異リスク

相同組換え修復を利用したゲノム編集により遺伝子改変された細胞でがん抑制遺伝子 p53 の変異が検出され、さらに p53 遺伝子をノックアウトした細胞では HDR の効率が上昇するとの報告がある[49, 50]。これは、p53 変異を持つ方が細胞死の誘導がされにくいことなどがその大きな要因と考えられている。従って、相同組換えによる遺伝子導入では p53 をはじめとするゲノム修復に関与する因子に関する遺伝子変異の有無を確認する必要がある。

4) 標的細胞によるがん化リスクの違い

ゲノム編集におけるオフターゲット作用の影響は、遺伝子そのものを改変するという点において、従来のレトロウイルスベクターやレンチウイルスベクター等の染色体組込み型ベクターによるリスクと同様とも想定される。遺伝子治療開発が始まった当時から、染色体組込み型ベクターのリスクとして、挿入変異による細胞のがん化は最大の懸念として挙げられ、実際、X-SCID（X 連鎖重症複合免疫不全症）や WAS（Wiskott-Aldrich 症候群）等の造血幹細胞を用いた遺伝子治療においてレトロウイルスベクターを用いた遺伝子導入では白血病が発症[8]したため、これらの遺伝子治療では長期にわたるフォローアップが必須とされている。一方、同じ染色体組込み型ベクターを用いた遺伝子治療であっても、造血幹細胞以外の細胞への遺伝子導入ではがん化は報告されていない。

造血幹細胞遺伝子治療における腫瘍化のメカニズムは、ウイルス由来プロモーター/エンハンサーを持つベクターが染色体上のがん関連遺伝子近傍へ挿入[51] されたためと考えられており、プロモーターやエンハンサーを用いないゲノム編集では、このような挿入変異による細胞増殖促進が起こることは考えられない。一方、前述のようにゲノム編集では、染色体の転座や欠失等は起こり得るため、染色体転座による Bcr-abl のようながんキメラタンパク質が生じる懸念や、がん抑制遺伝子が破壊されること等がリスクとして考えられる[52]。また相同組換えを目指すゲノム編集では、前述のように p53 のようながん抑制遺伝子に変異が生じた細胞が増加する懸念がある。ゲノム編集における染色体異常やがん抑制遺伝子の破壊等によるがん化リスクについては、現時点で十分に評価されているとは言えないが、遺伝子付加型の従来の遺伝子治療での経験を踏まえると、各細胞種におけるがん化リスクは必ずしも同等と考えるべきではなく、分化した細胞でのリスクは、増殖能を持つ未分化な細胞に比べてより低いと考えられる。逆に、iPS／ES 細胞や造血幹細胞では、造血幹細胞以外の体細胞等

に比べてリスクが高いと想定される。

5) ゲノム編集酵素の免疫原性

Cas タンパク質等のゲノム編集に用いられる DNA 切断酵素は細菌由来タンパク質であり、*ex vivo* 遺伝子治療であっても、ゲノム編集された細胞がゲノム編集酵素を発現する場合には生体内で異種抗原として認識される可能性がある（表 2)。動物試験ではヒトでの免疫原性を予測することは困難であるため、ゲノム編集酵素に対する免疫反応により臨床効果の減弱やアナフィラキシー等の免疫毒性が生じる可能性を考慮して、臨床試験を計画する必要がある。

(2) *in vivo* ゲノム編集

1) 標的遺伝子の改変に関する安全性評価

改変された標的遺伝子の作用について何らかの安全性上の懸念がある場合には、同じ標的遺伝子を改変した動物を用いた POC 試験において、効力又は性能を裏付ける成績等と共に標的遺伝子の改変に関連した体内動態や安全性に関する情報が得られる可能性がある（表 2)。

表 2　ゲノム編集に用いられる DNA 切断酵素の免疫原性

- AAV-CRISPR のマウス骨格筋投与後、Cas9 に対する液性及び細胞性免疫応答を解析 (Chew et al., Nature Methods, 2016)[53]

- ヒト血清を用いて 2 つの Cas９に対する抗体保有率を解析；65%に抗 spCas9 抗体、79%に抗 SaCas9 抗体、46%に抗 SaCas9 応答性 T 細胞を確認 (Charlesworth et al., BioRxiv, 2018)[54]

- ヒトが細菌由来の Cas9 に対する抗体をどの程度保有しているのかを血清を対象として ELISA で測定；2.5%に抗 spCas9 抗体、10%に抗 SaCas9 抗体 (Simhadri et al., Mol Ther Methods Clin Dev, 2018)[55]

免疫原性の決定因子：ベクターの血清型、投与経路、投与量、プロモーター特異性、宿主等

2) ゲノム編集酵素のターゲティングと改変効率

In vivo ゲノム編集では改変する組織・細胞へのターゲティングが重要[56]であり、どのようなゲノム編集ツールを用いるにせよ、ゲノム編集酵素の生体内分布の評価を行い、目的とする細胞や組織への分布だけでなく、目的としない部位への分布を評価しておく必要がある。また、ゲノム編集酵素の組織・細胞での持続性についても評価しておく必要がある。特に、生体内分布試験で生殖細胞への

分布が認められた場合には、生殖細胞の遺伝子改変のリスクについて、ICH 見解「生殖細胞への遺伝子治療用ベクターの意図しない組み込みリスクに対応するための基本的な考え方」[57]を参考に非臨床試験での評価が求められる。

また、*in vivo* ゲノム編集では、目的とする細胞や組織でのゲノム編集効率が低いことから十分な効果を発揮しない可能性があり、ゲノム編集効率を高めるための様々な技術開発が行われている。例えば、homology-independent targeted integration (HITI)法は、ゲノムの切断部位と同じ配列をドナーベクターに逆向きに入れることにより、ゲノムとドナーベクターが同時に切断され、*in vivo* でも高い効率でのゲノム編集が可能とされている[58] 。これに対応して CRISPR/Cas を長期にわたって発現させる目的で AAV を用いてこれらの遺伝子を導入すると、非分裂細胞でも高い効率でゲノム編集が可能であると報告されている[59] 。一方、長期にわたって CRISPR/Cas が発現し続けるということは標的以外へのオフターゲット作用や他の望ましくない標的部位での遺伝子改変リスクも高くなるという懸念がある。また *in vivo* ゲノム編集では、*ex vivo* ゲノム編集と異なり、目的外のゲノム編集が起きてもそれを排除することが困難であることに留意すべきである。

3) その他

In vivo ゲノム編集については、動物を用いた試験を実施してもオフターゲット作用に関する有用な情報が得られる可能性は低いが、*in silico* 解析やヒト細胞を用いた *in vitro* 解析での検討により、限定的ではあるものの、一定の意義ある情報が得られる可能性がある。したがって、*in vivo* ゲノム編集の開発ではこれらの方法を用いて潜在的なリスクを評価した上で、適用疾患での期待される有用性も踏まえて慎重に臨床開発を進める必要がある。

6. 治験において留意すべき事項（長期フォローアップ等）

ゲノム編集技術は目的とする遺伝子を改変する技術であり、その観点から染色体組込み型ベクターを用いた従来の遺伝子治療用製品と同様のリスク評価を想定した患者の長期フォローアップが必要である。一方、ゲノム編集は遺伝子の特定部位の欠失や遺伝子挿入を目指した技術であるため、オフターゲット作用による安全性上の懸念がなければ、遺伝子組込み部位がランダムな従来の遺伝子治療よりも安全な技術と考えられる。その一方で、ゲノム編集においては相同組換えにより p53 等のゲノム修復遺伝子の変異リスクが高まることや、DSB による染色体転座のリスクが指摘されていることから、これらに起因する有害事象を確認するフォローアップ期間を設定する必要がある[60](FDA LTF guideline)。

なお、どの程度の期間フォローアップが必要とされるかは、使用するゲノム編集技術（例えば、タンパク質等を直接導入することによる改変やウイルスベクターを用いた導入・改変等の違い）、ターゲットとなる細胞種、標的とする遺伝子等によって異なると考えられる。従来の遺伝子治療用製品での経験も踏まえると、特に造血幹細胞を対象としたゲノム編集では有害事象の発現リスクが高い[30]と想定され、定期検査を含めた長期フォローアップ計画を設定することが

望ましい。

また、*in vivo* ゲノム編集では目的外の組織・細胞、特に生殖細胞に導入されるリスクを十分に考慮する必要があり、特に、生殖細胞において遺伝子改変の可能性がある場合には、次世代への影響を回避するために、適切な避妊期間を設定する等の対応をとる必要がある。その際には、遺伝毒性を持つ抗悪性腫瘍薬でのリスク管理の手法も参考にできるであろう[61]（FDA Guidance for Industry）。また生殖細胞や受精卵の遺伝子に変異がないことを調べることは困難であることから、その影響については慎重な長期フォローアップが必要である。

7. おわりに

本文書は現時点で日本の遺伝子治療研究やゲノム編集の専門家の議論を結集し、ゲノム編集技術を用いた遺伝子治療用製品等の開発において道標となるべく作成された成果物である。それらを開発している企業や研究者、また、それらの審査を行う審査官にも参考にしていただくことを期待する。しかしながら、ゲノム編集技術の開発は、日々、急速に進歩しており、同時に、その適用範囲も拡大し、様々な評価技術の開発も進んでいる。最近では RNA ゲノム編集技術も展開しており、このような新たな製品に対しても本文書に示した考え方は適用可能な部分もあると思われるが、その開発動向に合わせ考え方を適宜見直していくことが必要であると考える。

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（五十音順）

写

事　務　連　絡
令和２年２月７日

各都道府県衛生主管部（局）薬務主管課　御中

厚生労働省医薬・生活衛生局医薬品審査管理課
厚生労働省医薬・生活衛生局医療機器審査管理課

「ゲノム編集技術を用いた遺伝子治療用製品等の品質・安全性等の考慮事項に関する報告書」の送付について

今般、独立行政法人医薬品医療機器総合機構に設置されている科学委員会において、別添「ゲノム編集技術を用いた遺伝子治療用製品等の品質・安全性等の考慮事項に関する報告書」が取りまとめられましたので、ゲノム編集技術を用いた遺伝子治療用製品等の開発に際し、参考とするよう、貴管下関係事業者に対し、周知願います。

なお、本事務連絡の写しを日本製薬団体連合会等の関係団体宛てに送付しますこと、念のため申し添えます。

（別添　略）

REVIEW

HGT

Aspects of Gene Therapy Products Using Current Genome-Editing Technology in Japan

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The development of genome-editing technology could lead to breakthrough gene therapy. Genome editing has made it possible to easily knock out or modify a target gene, while current gene therapy using a virus vector or plasmid hampering modification with respect to gene replacement therapies. Clinical development using these genome-editing tools is progressing rapidly. However, it is also becoming clear that there is a possibility of unintended gene sequence modification or deletion, or the insertion of undesired genes, or the selection of cells with abnormalities in the cancer suppressor gene *p53*; these unwanted actions are not possible with current gene therapy. The Science Board of the Pharmaceuticals and Medical Devices Agency of Japan has compiled a report on the expected aspects of such genome-editing technology and the risks associated with it. This article summarizes the history of that discussion and compares the key concepts with information provided by other regulatory authorities.

Keywords: gene therapy, genome editing, off-target effect, on-target mutagenesis, safety, double-strand break

INTRODUCTION

IN THE PAST decade, advances in genome-editing technologies[1,2] have provided the possibility of a dramatic improvement in gene therapy not only *in vitro* but also *in vivo*. Genome editing is a technology that can bring about either the knockout (KO) of a target gene or the homologous recombination of genes of interest (homology-directed repair [HDR]) through double-strand breaking (DSB) of a target DNA sequence. The targeting ability of genome-editing enzymes is dependent on the recognition by specific protein to be able to bind target DNA sequence (using a zinc-finger nuclease [ZFN],[3] or a transcription activator-like effector nuclease [TALEN][1] or by binding single-guide RNAs [sgRNAs], which form clustered regularly interspaced short palindromic repeats [CRISPR]/CRISPR-associated proteins [Cas] protein complexes[4]).

These genome-editing nucleases are able to cause a DSB in the target DNA sequence, and the targeted gene knockout could then be induced by a frame shift-induced mutation in generating DSBs during the repair process in a cell.[3] Genome-editing tools may also be utilized to induce homologous recombination with donor DNA fragments, resulting in modification of the hereditary disease gene with normal gene. Therefore, to effectively apply genome editing, it is critical to have a design that will introduce the DSB into the appropriate locus in the target gene sequence. Current gene therapy products using lentiviral or retroviral vectors that deliver a specific gene to introduce patient cells can integrate the gene of interest into patient cell chromosomes, but cannot yet target the gene into any specific chromosomal locus. Other viral vectors such as adenovirus or adeno-associated virus (AAV) have no genome integration machinery but may be integrated into nonspecific sites, although with extremely low efficiency. Thus, the targeting ability of genome-editing technologies provides new potentials for elimination-(knockout) or insertion of specific genes or the repair of damaged genes and the promise of ''correcting'' genetic disease or as novel treatments for infectious diseases.

Although no genome-editing gene therapy product has yet been approved worldwide, many clinical study protocols using genome-editing tools have been approved and

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are ongoing. The advantageous aspects of genome editing are utilized to exploit either knockout technology for a target gene by nonhomologous end joining or the modification of hereditarily abnormal genes with normal ones by HDR. In contrast, genome-editing technologies have been known to cause DSB on nontarget genes (off-target effect), or to yield insertions and deletions (indels), or templated repair from a separate donor DNA molecule (on-target mutation). To avoid off-target effects, genome-editing technologies that do not cause DSB, such as base editing and death editing, have been explored. New analytical methods and new technologies continue to be developed to combat the various undesirable effects of genome editing.

The Science Board of the Japan Pharmaceuticals and Medical Devices Agency has established an Expert Committee for Genome Editing to discuss the quality and safety issues of these technologies. In early 2020, the committee published its white paper on the evaluation of genome editing to educate both industry and regulatory scientists. In this article, we discuss the key points of the white paper and compare them with information provided by other regulatory agencies as follows:

1. All information with respect to classifications; CLASSIFICATION OF GENOME-EDITING PRODUCTS FOR GENE THERAPY & ISSUES IN SPECIFIC GENOME EDITING COMPARED WITH THOSE OF CURRENT GENE THERAPY PRODUCTS
2. All quality aspects with respect to product development; Cautions regarding genome-editing tools and gene-modified cells
3. All kinetics and safety aspects with respect to product development; Classification by purpose of genome editing & SAFETY EVALUATION
4. All clinical aspects with respect to product development; IMPORTANT ISSUES IN CLINICAL TRIALS (INCLUDING LONG-TERM FOLLOW-UP)

CLASSIFICATION OF GENOME-EDITING PRODUCTS FOR GENE THERAPY

The white paper first classifies genome-editing products according to the editing techniques and technologies (tools) that introduce them into a cell or a specific tissue in the body, and then discusses the purposes of genome editing to clarify the characteristics of each technique/technology. Genome-editing products are categorized into three types depending on the method used to express or introduce genome-editing enzymes into cells, as shown in Table 1.

Table 1. *Definition of genome-editing products*

- *In vivo* genome-editing products (gene therapy products that use at least one genome-editing technology and that are administered directly in the body)
  - ➢ Gene therapy vector products for genome editing (gene therapy products consisting of a viral or plasmid vector that expresses desired proteins, that is, nucleases used for genome editing) and gRNAs for genome editing
  - ➢ mRNA products for genome editing (mRNA that expresses desired proteins for genome editing)
  - ➢ Protein products for genome editing (desired proteins or protein/gRNA complexes used for genome editing)
- *Ex vivo* genome-editing products (human cell-based products genetically modified by a genome-editing tool)

gRNA, guide RNA; mRNA, messenger RNA.

Early clinical studies made use of adenovirus,[5] adeno-associated viral vectors,[6] or plasmids[7] to express genome-editing enzymes, and mRNAs[8] coding ZFNs or TALENs were also used. The direct introduction of genome-editing enzymes to modify a target gene have also been explored in both *ex vivo* and *in vivo* treatments.

ISSUES IN SPECIFIC GENOME EDITING COMPARED WITH THOSE OF CURRENT GENE THERAPY PRODUCTS

Risk from undesired genome modification and cancer risk

Genome-editing technologies provide novel tools to replace, eliminate, or modify target genes in a DNA sequence-specific manner. These technologies are expected to provide novel and valuable products that will make it possible to insert, delete, or replace desired DNA in human genomes using engineered site-specific nucleases. However, genome editing has the inherent risk of unintended editing of genes that have similar DNA sequences; this is known as the off-target effect. In addition to the risk of unintended gene modification, the long-term effects of off-target genome editing remain unknown. Of particular concern is that off-target effects may result in tumorigenicity (cancer). Off-target effects may directly activate oncogenes or inactivate tumor-suppressor genes. Importantly genetic modifications by genome editing have the potential to cause permanent alterations in genomes.

It has been also reported that genome-editing techniques that induce DSBs may induce genome instability, which is associated with chromosomal breaks. With some technical limitations, undetectable large-scale defects and the insertion of DNA sequences into DSB sites in chromosomal sequences that cannot be detected by current analytical technologies have also been reported.[9] These undesired modifications of chromosomes may be caused not only in on-target sites but also in off-target sites. The potential risks that stem from these chromosomal aberrations due to undesired modification of chromosomes, including tumorigenicity, should be assessed preclinically.

Risk of unintended gene modification in germline cells

In vivo genome editing where a genome-editing gene therapy product is administered directly to the patient may unintentionally result in genome editing of unintended cells or the modification of off-target genes. The fact that it is difficult to identify and eliminate these unintended al-

terations of cells and genes when they occur is a key safety concerning *in vivo* genome editing.

Of particular concern is the fact that *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 elucidated.

To avoid the risk of chromosomal mutations attributable to genomic cleavage, new technologies that allow genetic engineering without genomic cleavage have recently been developed. For the *in vivo* application of these new types of genome editing, the effects on the next generation should be evaluated through nonclinical and clinical studies.

CLASSIFICATION OF GENOME-EDITING TECHNOLOGIES AND CHALLENGES RELATED TO THEIR CHARACTERISTICS

Many genome-editing methods and related technologies have been and continue to be developed, and suitable technologies from the genome-editing toolbox can now be selected. Gene therapies to specifically modify or knock out a target gene were much desired in the early phases of the development of gene therapy. Until genome-editing technologies were developed, it was very difficult to make such modifications. Let us now consider the characteristics of genome-editing technologies.

Classification by genome-editing tool and points to consider

ZFN and TALEN. 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 *FokI* nuclease (*Fok*I).[3] Since designing this DNA-binding protein requires highly sophisticated technologies, ZFNs have not been utilized as a genome-editing tool by many researchers. In contrast, TALENs were developed to simplify the complicated ZFN design process.[1] In TALENs, each TAL module consists of 34 amino acids of TAL, a plant-derived transcription factor, and each module recognizes one nucleotide. By binding four different types of TAL module, each of which recognizes nucleotide base A, G, C, or T, targeted DNA sequences can be recognized so that specific DNA sequences can be cut by *Fok1* nuclease, which is fused to TAL modules. Typically, the TALs are designed to recognize 15–20 nucleotides by binding 15–20 TAL modules.

Since the *Fok*I domains of ZFNs and TALENs cut only a single chain of DNA double strands, two artificial enzymes that recognize DNA sequences upstream and downstream of the target cleavage site are required to make a DSB on a target sequence. A recognition sequence of 18–40 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 ZFNs and TALENs high. TALENs are thought to be less likely to cause off-target effects than CRISPR/CRISPR-associated protein 9 nuclease (Cas9).[1,2]

CRISPR/Cas. A novel genome-editing tool based on bacterial Cas9 from *Streptococcus pyogenes* has been reported.[10] 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 easily be designed to have a guide sequence that complementarily binds to 20 target nucleotides of the target DNA sequence and a protospacer adjacent motif adjacent to the target sequence.

The sgRNA and Cas9, an enzyme that cleaves the double strands of DNA, together form a complex to cleave a gene having the sequence recognized by the sgRNA. It has been noted that the sgRNA binds 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). The risk of this CRISPR/Cas system inducing off-target effects, such as undesired insertions and deletions of DNA in off-target sequences, will thus be quite high.[6] Although many reports on off-target effects caused by CRISPR/Cas have been published,[11–13] there is as yet no standard method. It is, however, difficult to characterize or detect the exact off-target effects that occur rarely, particularly those seen in only a small number of cells.

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, not enough resolution for this has yet been established. Therefore, given the results of the off-target analysis described as follows, it is essential to design an sgRNA based on the latest knowledge and evaluate the frequency of off-target effects.

Genome editing without genomic breaking. To avoid undesirable effects due to the genome instability resulting from a DSB, genome editing without genomic breaking (*e.g.*, single-base editing with deaminase or dead Cas) and other editing technologies have been developed.[14,15] Although there are many new genome-editing technologies that do not cause DSB, they may potentially modify the genome sequence at sites other than the target loci. For each newly developed genome-editing technology, mechanisms to reduce off-target effects should be justified together with the evaluation methodology.

Cautions regarding genome-editing tools and gene-modified cells

As described earlier, there are several methods for introducing genome-editing tools into the target cells/tissues both *in vivo* and *ex vivo*. Based on the characteristics of different transfection methods, it is important to select the most suitable method to achieve the desired clinical effect.

Viral vectors and plasmid vectors. According to the information on the National Institutes of Health (NIH)

ClinicalTrial website, many clinical trials of viral vectors, including adenovirus, AAV and others, have been conducted to introduce ZFN or CRISPR/Cas into a cell for genome-editing gene therapy.[6,16] Quality control measures for current gene therapy products can also be applied to gene therapy using viral or plasmid vectors[17] coding a genome-editing enzyme gene. For the past three decades, ~4,000 clinical protocols for gene therapy have been conducted or are ongoing worldwide (NIH ClinicalTrials.gov). There has been sufficient clinical experience of gene therapy using viral or plasmid vectors for several gene therapy products using AAV, lentivirus, or retrovirus vectors to have been approved for market authorization, and many guidelines for quality, safety, and efficacy have been published. 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 to achieve efficient expression of the target protein. However, the insertion of viral promoter sequences adjacent to a cancer-related gene could possibly cause tumorigenicity.[12] In some cases, genome editing also makes use of viral promoters to express ZFNs, TALENs, or Cas9/sgRNA. To the best of our knowledge, oncogenesis by promoter insertion has not yet been reported, although 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 those used to evaluate conventional gene therapy products. The cells or tissue tropisms should also be assessed together with their 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 should be analyzed from the viewpoints of infectivity and cell tropism. It should also be taken into account that the persistent expression of a genome-editing enzyme can increase the possibility of off-target effects, especially 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 be evaluated from the viewpoint of safety.

mRNA.

To express genome-editing proteins such as Cas, TALEN, and ZFN, intracellular transfection of the mRNA that codes for these proteins has been utilized.[16,18,19] 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 product. Although 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 yet been approved for marketing in Japan or overseas. The manufacturing method and quality control of mRNA-based products remain to be clarified in the future. In particular, gene editing using mRNA manufactured by chemical modification such as methylated Cap, which does not occur naturally, to ensure the intracellular stability of mRNA also requires safety evaluation of the applied chemical modification. Sponsors are encouraged to engage in full consultation with the relevant regulatory authorities about the quality control and safety evaluation of mRNA. The same product management methods that are applied to nucleic acid products can also be applied to chemically synthesized mRNA products. mRNA synthesized by *in vitro* transcription using a plasmid or PCR product as a template should be required to undergo additional safety evaluation for process-derived impurities such as RNA polymerase or template DNA.

Proteins and guide RNA.

Both ZFN and TALEN proteins consist of two components: a DNA binding domain and a DNA cleavage domain (*Fox1* nuclease).[20,21] The design of the DNA binding domain is the key element of genome-editing enzymes; for example, either the specificity of the target DNA or the efficiency of these artificial nucleases. A CRISPR/Cas technique can be conducted to introduce a complex (ribonucleoprotein) of sgRNA complementary to the target DNA sequence and Cas9 into a cell.[22,23] The specificity of CRISPR/Cas depends primarily on the design of the sgRNA sequence. As a genome-editing technology, the direct transfection of these genome-editing enzymes could cause DSB on the target sequence in the cells, resulting in KO or HDR in the target gene. Therefore, protein-based genome editing also carries a risk of either off-target effects or adverse events and there may also be potential on-target mutations in the target sequences accompanied by DSB (undesired large deletions or insertions of DNA).[24] In this sense, genome-editing products need to be evaluated as gene therapy products as with the current products used for gene transfer technologies.

In contrast, the quality of artificial nuclease protein products such as ZFN, TALEN, and CRIPR/Cas should be evaluated according to the International Conference on Harmonization (ICH) biotechnology guidelines for evaluation and quality control of cell banks for biotechnological products (ICH Q6B).

Human cell-based products modified by genome-editing technology.

The quality control strategy 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, that is, as per current ICH guidelines (ICH Q5A, Q5B, and Q5D). 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.

Classification by purpose of genome editing

Gene knockout[25–30] and homologous recombination[11,31,32]. When gene editing is intended to knock out genes, the frequency of gene knockouts in the target cells and the heterogeneity of targeted gene modifications should be analyzed. For example, in CRISPR/Cas-based gene editing, a justification of the sgRNA design should include conclusions on efficiency and heterogeneity in target cells. Although gene editing by HDR is based on the DSB repair mechanism of cells, it has been reported that the activity of homologous recombination was found to be high in embryonic stem (ES) cells, which have a high ability to repair DSB.[33] It should be noted that the DSB repair efficiency can be very low in some types of cells. Therefore, homologous recombination for the development of gene-editing products intended to cause HDR on target genes should also be clarified and justified for clinical efficiency. It might be supposed that the efficiency of HDR in target cells will be very low.[31,34] In this case, it might be necessary to select and purify gene-modified cells that have undergone homologous recombination for treatment, and the methods for cell selection/purification should then be justified.

Homologous recombination is required to introduce the donor DNA for the modification of the target gene. To modify short DNA sequences such as single-nucleotide polymorphisms,[33] single-strand DNA that has a homologous sequence both upstream and downstream of the DSB site is used to induce homologous recombination. In HDR to replace the entire gene coding for a protein, a plasmid is typically used as a donor DNA template. In this case, DNA with a homologous sequence of several hundred DNA sequences from upstream to downstream of the DSB should be introduced to the target site. Therefore, it is crucial to evaluate the design of the donor DNA and the efficiency of homologous recombination. Even though some reports suggest that there is no correlation between a DNA length that can undergo homologous recombination and recombination efficiency, homologous recombination efficiency should be evaluated together with the effects of the genome length.[35,36]

For either simultaneous knockouts of more than one gene or more efficient homologous recombination, gene editing involving two DSBs has been explored. The introduction of two or more DSBs is likely to be associated with significant chromosomal aberrations such as chromosomal translocations and/or deletions. In these cases, chromosomal aberrations should be fully evaluated.[37]

A new technology such as 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[38–40] as a new approach to genome editing that enables the direct irreversible conversion of one target DNA base into another in a programmable manner, such as the conversion of C to T or A to G with a deaminase.[41,42] In addition, genome editing to introduce epigenetic mutations in target sequence, such as DNA methylation, is actively being developed. However, these genome-editing technologies without DSB may also cause adverse events attributable to off-target effects on untargeted genes. Genome-editing products for human diseases should be regulated as gene therapy products, and many types of products have been developing, for example, products that cause KO or HDR of target genes that cause diseases, deadCas, and base-Editing. Recently, genome editing has been developed that does not alter DNA, but enhances the target gene through the modification of histone or merely binding to the target gene (Fig. 1). Since the efficacy or specificity of based gene engineering without the induction of DSB may be different in each cell, selection and/or purification of gene-modified cells may be necessary. Therefore, quality assessment of techniques without DSB should be conducted based on these assumptions. In addition, the adequacy of each genome-editing technology must be determined using the optimal analysis technique according to the nature of the technology.

SAFETY EVALUATION

Issues in the application of gene therapy products using genome-editing techniques

For the safety of genome-editing technologies, many issues associated with genome-editing nucleases should be considered, such as off-target mutations and unwanted chromosomal translocations associated with off-target and on-target DNA cleavages. Regarding the application of genome editing to induce HDR, the mutation of p53 protein should be analyzed. Furthermore, since genome-editing enzymes are derived from nonhuman proteins, the risk of an immune reaction to the genome-editing enzyme must also be assessed (Table 2).

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, but also to experimentally explore candidate off-target sites throughout the entire human genome (Fig. 2).[43–46] Such off-target profiling methods include GUIDE-seq,[47]

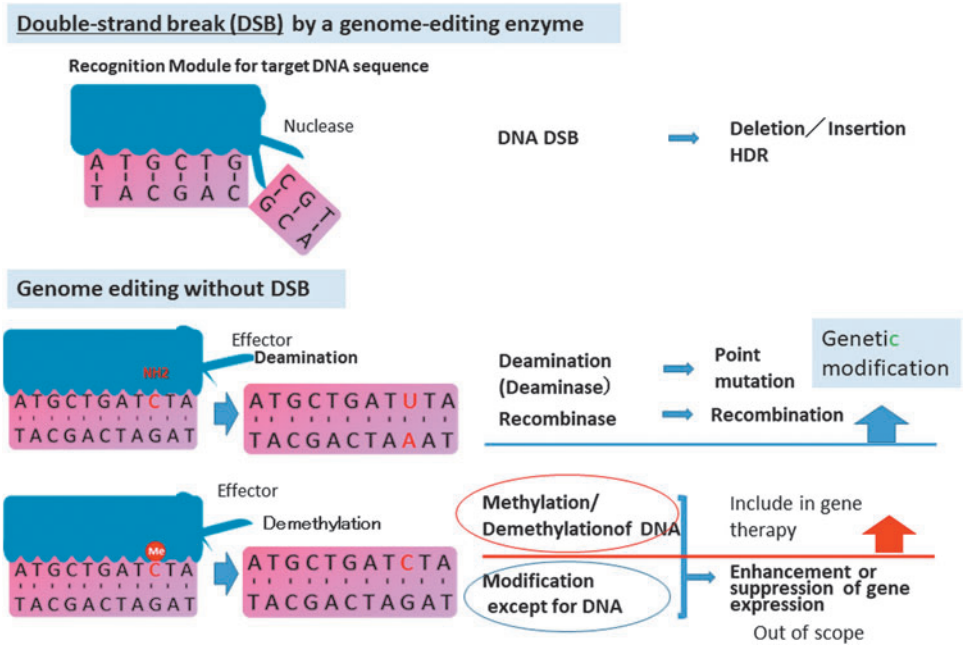

Figure 1. Definition and categories of genome editing.

which involves the introduction of a tag of synthetic DNA in the cleavage site for genome-wide sequencing of the tag, and DIGENOME-seq,[48,49] CIRCLE-seq,[50] and SITE-seq,[51] which explore potential off-target cleavage sites of genome-editing enzymes using genome DNA extracted from cells. These analyses will provide information focused on mutations such as SNV/Indel and the copy number variation of cancer-related genes.[52] It should be determined whether breaks or deletions have actually occurred at the off-target sites predicted by *in silico* analysis, and experimental methods should be evaluated based on the whole genome sequence[44,46] of the genome-edited cell and amplicon sequence, which involves PCR amplification of candidate off-target sites followed by deep sequencing.[53] It should be noted that although 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. If the results of characterization of an *ex vivo* genome-editing-based product show off-target effects of the gene editing, the risk of those off-target effects causing cancer or other adverse events should be evaluated. Clonality analysis such as linear-amplification-mediated PCR of gene-modified cells may be required.

Table 2. *Safety issues in gene therapy products using genome-editing techniques*

- Off-target effects
- On-target mutagenesis
- Chromosomal changes (translocation/inversion/deletion)
- *P53* mutation
- Immunogenicity of nucleases
- Germline modification

To ensure the safety of genome-editing technology, it is necessary to mitigate off-target effects. The design of sgRNA may be the most critical factor in mitigating the off-target effects of CRISPR/Cas. It is also important to use *in silico* analysis to select DNA sequences with few homologous sequences in other genomic regions; however, it must be noted that *in silico* analysis may not be able to predict all candidate off-target sites. A combination of *in silico* and *in vitro* analyses is useful in identifying candidate off-target sites, and it is very important to understand the frequency of off-target effects and their influence. 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.

In the case of *in vivo* genome editing, an analysis of the off-target effects of genome editing in animals as a non-clinical study is not appropriate because of species differences in the genome sequence between humans and animals. As part of the characterization studies of *in vivo* 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. To evaluate off-target effects on *in vivo* genome-editing products, *in vitro* analysis using a continuous cell line harboring many DNA mutations may not provide useful data. To assess the off-target effects of *in vivo* genome editing, analysis using primary cells is recommended, and induced pluripotent stem (iPS) cells or ES cell-derived functional

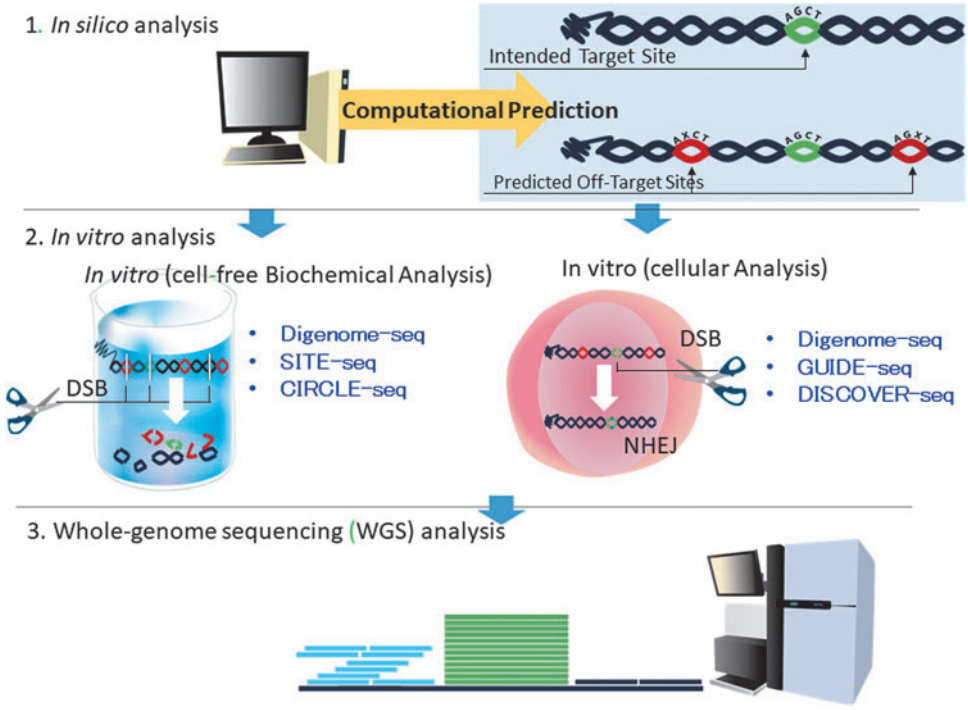

Figure 2. Analysis of off-target effects.

cells may also be useful. iPS and ES cell-derived cells are very promising tools for the assessment of off-target effects on human primary cultured cells that are not easily available.

Genome deletions/insertions of unintended DNA sequences and chromosomal translocations/inversions. It has been reported that large deletions (of several kb), and insertions and inversions of gene fragments during the DSB repair process may occur during genome editing (on-target mutagenesis).[54] The insertion in the target site of the genome DNA of a viral vector used for genome editing has also been reported.[55,56] This may occur because gene modifications by genome editing depend on the DSB-induced genome repair mechanism of cells in which the genome-repairing mechanism could not be controlled by genome-editing technology. The directionality of the genome editing how to modify or repair the target genome is not involved in the genome-editing tool.[54] Therefore, the target gene and/or its flanking genome sequence in cells/tissues as similar as possible to the actual target cells should be analyzed in detail. As mentioned earlier, the risk of chromosomal translocations and deletions after a DSB has been reported.[57–59] 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), and so on. However, it should be understood that these analyses also have certain limitations. For example, G-band analysis and mFISH can be applied to cells only in the metaphase stage. In G-band analysis, it is difficult to deal with many cells at once and detect a very small group of cells having chromosomal aberrations. mFISH is suitable for detecting 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, but CGH sensitivity is too low to detect DNA aberrations if 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.

Risk of DNA-repair gene mutations such as *p53* in genome-edited cells. Mutations of the *p53* tumor-suppressor gene in cells in which a gene modification was caused by HDR-based genome editing and an increase in HDR efficiency in cells knocked out for the *p53* gene have been reported.[60,61] These phenomena can be explained primarily by the fact that *p53* mutations increase resistance to cell death due to genome mutations. The occurrence of gene mutations related to genome repair, such as *p53* mutations, should be investigated in the introduction of HDR-derived genes.

Differences in the risk of cancer among target cells. From the point of view of intending to modify genomes, there are common concerns between genome-editing

technologies and current gene therapies using chromosomally integrated vectors such as retroviral and lentiviral vectors. 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 concerning risk associated with chromosomally integrated vectors was the induction of tumorigenicity in 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 has been reported to cause leukemia.[12] Currently, therefore, these gene therapies require long-term follow-up to monitor tumorigenesis. Nevertheless, to the best of our knowledge, there have been no reports 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 the insertion of a vector with a viral promotor/enhancer adjacent to a cancer-related chromosomal gene, which is thought to be the mechanism causing cancer associated with hematopoietic cell-based gene therapies.[62] Genome editing using neither a promoter nor an enhancer is unlikely to cause inserted mutations to promote cell proliferation. In particular, the direct transfection of a genome-editing tool in the form of a nuclease protein or its mRNA into cells is very unlikely to cause cancer induced by insertional mutagenesis. However, there are other concerns regarding such genome-editing tools, because genome editing may cause chromosomal translocations, deletions, and other aberrations accompanied by DSB. Chromosomal translocations may cause cancer chimeric proteins such as *Bcr-abl* or destroy tumor-suppressor genes.[63] Genome editing with homologous recombination may cause an increase in the 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 the destruction of tumor-suppressor genes has not yet been fully investigated. Based on reported experience with current gene therapies in which genes are integrated into cells, the risk of carcinogenesis appears to depend on the type of cell. Differentiated cells are likely to be more robust against the risk of carcinogenesis than undifferentiated or stem cells. In contrast, iPS/ES cells and hematopoietic cells have a higher risk of carcinogenesis than other somatic cells.

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 are administered *in vivo* and express these enzymes, the enzymes are recognized as heterologous antigens. The prevalence of pre-existing antibodies to Cas9 has been evaluated, and the prevalence of anti-SaCas9 and anti-SpCas9 antibodies has been reported to be 10% and 2.5%, respectively, in one study using 200 human serum samples[64]; similar results were also obtained in a study by Carsten *et al*.[65] Immunogenicity in humans may not be predicted from animal studies. Clinical trials should be designed taking into consideration the potential immune toxicity of genome-editing enzymes, that is, the immune response to these enzymes, including the attenuation of clinical effects and anaphylaxis.[66]

In vivo genome editing

Safety evaluation of modified target genes. Where there are safety concerns about the expression of modified target genes, a proof-of-concept study in model animals with the modified homologous gene may provide information about kinetics and safety related to the modification of the target gene. The results of this study should be taken into consideration together with data supporting the efficacy or performance of the gene therapy in question. Careful interpretation of the results obtained in this study due to differences in species.

Targeting and modification efficiency of genome-editing enzymes. In *in vivo* genome editing, targeting in the tissues/cells to be modified is important.[67] It is necessary to characterize the biodistribution of a genome-editing tool or enzyme expressed by the tool *in vivo* to understand its distribution not only to targeted cells/tissues but also to nontargeted cells/tissues. The persistence of the genome-editing enzyme in both desired and undesired tissues/cells should also be elucidated. In particular, when a biodistribution 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 considerations ''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 efficiency have been actively explored. For example, homology-independent targeted integration is a method in which 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. This technique allows efficient *in vivo* genome editing.[68] In addition, the introduction of CRISPR/Cas using AAV to express CRISPR/Cas for a prolonged period of time has been reported to enable efficient genome editing of nondividing cells.[69] However, the long-term expression of genome-editing enzymes such as CRISPR/Cas increases the risk of either off-target effects or undesirable gene modifications at the target sequence. It should be noted that off-target effects and on-target mutagenesis of *in vivo* genome editing, if they occur, are difficult to remove, unlike *ex vivo* genome editing.

Other factors. 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 of the assessment of their potential risks using these analyses. Clinical design should take into account both the identified potential risks and the potential usefulness of each technology or product under consideration.

IMPORTANT ISSUES IN CLINICAL TRIALS (INCLUDING LONG-TERM FOLLOW-UP)

Since genome-editing technologies are intended to modify target genes permanently, genome editing requires long-term follow-up of patients because these technologies have risks similar to those involved in current gene therapy products using a chromosomally integrated vector. Genome editing, which is used to delete or insert genes at specific sites, could be safer than current gene therapies involving random gene insertions. However, undesired genome modifications accompanied by off-target effects or on-target mutagenesis cannot be excluded. Furthermore, genome editing using homologous recombination may increase the 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, an appropriate follow-up period should be established according to each risk.[70]

The length of the follow-up period should be established based on the specific genome-editing technology used in each case (*e.g.*, gene modification through the direct introduction of a protein or introduction/modification using a viral vector), including the type of target cells, and the targeted gene. Among experiences with current gene-therapy products, the application of genome editing to hematopoietic stem cells in particular has been associated with a high risk of adverse events.[37] Long-term follow-up plans are strongly recommended, including periodic examinations.

In vivo genome editing should be considered to cause gene modification in off-target tissues/cells, especially germline cells. When there is a risk of gene modification in germline cells, measures to prevent that 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.[71] 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.

COMPARISON OF THE PMDA WHITE PAPER WITH DATA FROM OTHER REGULATORY AGENCIES

Genome-editing technologies have been progressing rapidly, and national regulatory agencies (NRAs) have published several viewpoints about genome-editing products instead of formal guidelines. Table 3 provides a comparison of the European Medicines Agency (EMA) and US Food and Drug Administration (FDA) viewpoints with those of the Japanese PMDA white paper. The FDA does not define genome-editing products, but the definitions given by the EMA and PMDA are similar. All NRAs assume that genome-editing products include not only viral vectors and plasmids but also mRNA and protein-based products.

Off-target effects, on-target mutations due to DSB, chromosomal abnormalities, and immune reactions to genome-editing enzymes are pointed out by all three organizations as important issues related to safety. Currently there is no gold standard for identifying off-target

Table 3. *Comparison of regulatory stance for genome-editing products for gene therapy*

	US FDA	*EU EMA*	*Japan MHLW/PMDA*
Relevant guidelines, etc.	Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs) (2020.1).	Guideline on the quality, nonclinical and clinical aspects of gene therapy medicinal products (2018.3)	Guidelines for Gene Therapy Clinical Research (2019, 2)
	Long-Term Follow-Up After Administration of Human Gene Therapy Products (Draft, 2020.1)	Guideline on quality, nonclinical and clinical aspects of medicinal products containing genetically modified cells (Draft, 2018.7)	Science Board: Reflection paper for genome-editing products (2020, 2)
Genome editing	No description on tools for genome editing	Products consisting of recombinant nucleic acid as an active ingredient and other components, and causing control, repair, replacement, insertion, or deletion of DNA sequences in humans	Modification of human genes of specific target DNA sequences and administration of genetically modified cells
Products including genome editing and raw materials	Gene therapy includes viral vectors and plasmids, as well as genome editing using mRNA	Vectors (including mRNA) coding for enzymes used for gene modification, enzyme proteins for gene modification, nucleic acid for genome editing, nucleic acid templates to be knocked in, and cells to be modified	Viral vectors, plasmids, mRNA, or proteins that modify the specific DNA sequences, nucleic acid, etc.

modification. Orthogonal methods are used to identify potential off-target genome alteration and to quantify percentage of modification at a site. Not all genome modifications lead to deleterious biological consequences; however, assessment is limited due to lack of sensitive functional assays *in vitro* and *in vivo*. Unfortunately, animal models are of limited value for identifying and evaluating off-targets as the products are human genome specific. Importantly, since genome-editing therapy causes permanent genome changes, to evaluate potential tumorigenicity that may be caused by undesired genome changes or chromosomal abnormalities, long-term clinical follow-up should depend on the results of the preclinical characterization of the relevant genome-editing products.

Many new genome-editing technologies such as dead-Cas and based-Editing are still being developed. These new technologies also involve the risk of off-target effects, and studies on these effects and on undesired mutations are underway. Furthermore, a new approach to modifying the histone around target genes is being explored.[24] It is not clear whether NRAs consider these technologies to be included in genome editing. It is clear that genome-editing technologies will continue to be developed one after another, and it is, therefore, difficult to determine what the focus of future genome editing might be. The characteristics and properties of genome editing must be revisited as necessary as the various technologies advance.

SUMMARY

This document summarizes recent discussions by experts in gene therapy and genome editing in Japan about the development of gene therapy products using genome-editing technology. We hope that these documents will help companies and researchers to develop new genome-editing-based gene therapy approaches, and assist reviewers in conducting regulatory reviews of genome-editing products. However, genome-editing technologies are advancing rapidly, and a great variety of technologies are emerging. This document should be revised as necessary to reflect technological advances.

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