Historic Overview of Genetic Engineering Technologies for Human Gene Therapy

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Abstract

The concepts of gene therapy were initially introduced during the 1960s. Since the early 1990s, more than 1900 clinical trials have been conducted for the treatment of genetic diseases and cancers mainly using viral vectors. Although a variety of methods have also been performed for the treatment of malignant gliomas, it has been difficult to target invasive glioma cells. To overcome this problem, immortalized neural stem cell (NSC) and a nonlytic, amphotropic retroviral replicating vector (RRV) have attracted attention for gene delivery to invasive glioma. Recently, genome editing technology targeting insertions at site-specific locations has advanced; in particular, the clustered regularly interspaced palindromic repeats/CRISPR-associated-9 (CRISPR/Cas9) has been developed. Since 2015, more than 30 clinical trials have been conducted using genome editing technologies, and the results have shown the potential to achieve positive patient outcomes. Gene therapy using CRISPR technologies for the treatment of a wide range of diseases is expected to continuously advance well into the future.

Keywords: gene therapy, genome editing, ZFN, TALEN, CRISPR/Cas9

Introduction

Gene therapy is a therapeutic strategy using genetic engineering techniques to treat various diseases.¹,² In the early 1960s, gene therapy first progressed with the development of recombinant DNA (rDNA) technology,¹ and was further developed using various genetic engineering tools, such as viral vectors.³⁻⁵ More than 1900 clinical trials have been conducted with gene therapeutic approaches since the early 1990s. In these procedures, DNA is randomly inserted into the host genome using conventional genetic engineering tools. In the 2000s, genome editing tools, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the recently established clustered regularly interspaced palindromic repeats/CRISPR-associated-9 (CRISPR/Cas9) technologies, were developed, which induce genome modifications at specific target sites.⁵ Genome editing tools are efficient for intentional genetic engineering, which has led to the development of novel treatment strategies for a wide range of diseases, such as genetic diseases and cancers. Therefore, gene therapy has again became a major focus of medical research. However, because gene therapy involves changing the genetic background, it raises important ethical concerns. In this article, we review the brief history of gene therapy and the development of genetic engineering technologies.

History of Genetic Engineering Technologies

Ethical issues

In 1968, the initial proof-of-concept of virus-mediated gene transfer was made by Rogers et al.⁶ who showed that foreign genetic material could be transferred into cells by viruses. In the first human gene therapy experiment, Shope papilloma virus was transduced into two patients with genetic arginase deficiency, because Rogers et al. hypothesized that the Shope papilloma virus genome contained a gene that encodes arginase. However, this gene therapy produced little improvement in the arginase levels in the patients.⁷ Sequencing of the Shope papilloma virus genome revealed that the virus genome did not contain an arginase gene.⁷

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This experiment prompted public concerns about the risks and ethical issues of gene therapy. In 1972, Friedman et al. proposed ethical standards for the clinical application of gene therapy to prevent premature application in human. However, in 1980, genetic engineering was unethically performed in patients with thalassemia without the approval of the institutional review board. The patients’ bone marrow cells were harvested and returned into their bone marrow after transduction with the plasmid DNA containing an integrated β-globin gene. This treatment showed no effects, and the experiments were regarded as morally dubious. The gene therapy report of the President’s Commission in the United States, Splicing Life, emphasized the distinction between somatic and germline genome editing in humans, and between medical treatment and non-medical enhancement.

An altered gene inserted into sperm or egg cells (germ cells) would lead to changes not only in the individual receiving the treatment but also in their future offspring. Interventions aimed at enhancing “normal” people also are problematic because they might lead to attempts to make “perfect” human beings.

Beginning of gene therapy using viral vector

In 1980, only nonviral methods, such as microinjection and calcium-phosphate precipitation, were used for gene delivery. Nonviral methods showed some advantages compared with viral methods, such as large-scale production and low host immunogenicity. However, nonviral methods yielded lower levels of transfection and gene expression, resulting in limited therapeutic efficacy. In 1989, the rDNA Advisory Committee of the National Institutes of Health proposed the first guidelines for the clinical trials of gene therapy. In 1990, retroviral infection, which is highly dependent on host cell cycle status, was first performed for the transduction of the neomycin resistance marker gene into tumor-infiltrating lymphocytes that were obtained from patients with metastatic melanoma. Then, the lymphocytes were cultured in vitro and returned to the patients’ bodies. The first Food and Drug Administration (FDA)-approved gene therapy using a retroviral vector was performed by Anderson et al. in 1990; the adenosine deaminase (ADA) gene was transduced into the white blood cells of a patient with ADA deficiency, resulting in temporary improvements in her immunity.  

First severe complications

A recombinant adenoviral (AV) vector was developed after advances in the use of the retroviral vector. In 1999, a clinical trial was performed for ornithine transcarbamylase (OTC) deficiency. A ubiquitous DNA AV vector (Ad5) containing the OTC gene was delivered into the patient. Four days after administration, the patient died from multiple organ failure that was caused by a cytokine storm. In 1999, of the 20 patients enrolled in two trials for severe combined immunodeficiency (SCID)-X1, T-cell leukemia was observed in five patients at 2–5.5 years after the treatment. Hematopoietic stem cells with a conventional, amphotropic, murine leukemia virus-based vector and a gibbon-ape leukemia virus-pseudotyped retrovirus were used for gene transduction in those trials. Although four patients fully recovered after the treatment, one patient died because oncogene activation was mediated by viral insertion.

Development of viral vectors

Viral vectors continued to be crucial components in the manufacture of cell and gene therapy. Adeno-associated viral (AAV) vectors were applied for many genetic diseases including Leber’s Congenital Amaurosis (LCA), and reverse lipoprotein lipase deficiency (LPLD). In 2008, remarkable success was reported for LCA type II in phase I/II clinical trials. LCA is a rare hereditary retinal degeneration disorder caused by mutations in the RPE65 gene (Retinoid Isomerohydrolase RPE65), which is highly expressed in the retinal pigment epithelium and encodes retinoid isomerase. These trials confirm that RPE65 could be delivered into retinal pigment epithelial cells using recombinant AAV2/2 vectors, resulting in clinical benefits without adverse events. Recently, the FDA approved voretigene neparvovec-rzyl (Luxturna, Spark Therapeutics, Philadelphia, PA, USA) for patients with LCA type II. Alipogene tiparvovec Glybera (uniQure, Lexington, MA, USA) is the first gene-therapy-based drug to reverse LPLD to be approved in Europe in 2012. The AAV1 vector delivers an intact LPL gene to the muscle cells. To date, more than 200 clinical trials have been performed using AAV vectors for several genetic diseases, including spinal muscular atrophy, retinal dystrophy, and hemophilia.

Retrovirus is still one of the mainstays of gene therapeutic approaches. Strimvelis (GlaxoSmithKline, London, UK) is an FDA-approved drug consisting of an autologous CD34 (+)-enriched cell population that includes a gammaretrovirus containing the ADA gene that was used as the first ex-vivo stem cell gene therapy in patients with SCID because of ADA deficiency. Subsequently, retroviral vectors were often used for other genetic diseases, including X-SCID.

Lentivirus belongs to a family of viruses that are responsible for diseases, such as acquired immunodeficiency syndrome caused by the human immunodeficiency virus (HIV) that causes infection by inserting DNA into the genome of their host cells. The lentivirus can infect non-dividing cells;
Table 1 History of gene therapy

| Year | History of GT |
|------|---------------|
| 1962 | Successful transformation of human cells with genomic DNA was achieved. |
| 1970 | Treatment strategy using viral vectors was developed. |
| 1972 | The concept of GT was established. Technologies using recombinant DNA were developed. |
| 1974 | Advisory committee was established for recombinant DNA |
| 1980 | Unapproved GT was performed. |
| 1981 | Retroviral vector was developed. |
| 1983 | Non-replicating retroviral vector was developed. |
| 1986 | Guideline of GT was established. |
| 1989 | A marker gene was first transduced into patient TILs using a retroviral vector. |

Table 2 Characteristics of genome editing technologies

| Characteristics | ZFNs | TALENs | CRISPR/Cas9 |
|----------------|------|--------|-------------|
| **Length of recognized DNA target** | 9–18bp | 30–40bp | 22bp + PAM sequence |
| **DNA recognition** | Multimeric protein-DNA interaction | Protein-DNA interaction | RNA-DNA interaction |
| **Nuclease design** | Difficult | Feasible | Easy |
| **Cost** | High | Moderate | Low |
| **Success rate of nuclease design** | Low | High | High |
| **Potential off-target effects** | Yes | Yes | Yes |
| **Specificity** | Moderate | High | Moderate |
| **Sensitivity to DNA methylation** | Not known | Sensitive to CpG methylation | Not sensitive to CpG methylation |

ADA: adenosine deaminase, ALD: adrenoleukodystrophy, B-ALL: B cell acute lymphoblastic leukemia, CAR: chimeric antigen receptor-modified, DLBCL: diffuse large B-cell lymphoma, GT: gene therapy, LCA: Leber's congenital amaurosis, LPL: lipoprotein lipase deficiency, OTC: ornithine transcarbamylase, SCID: severe combined immunodeficiency, SMA: spinal muscular atrophy, TCR: T cell receptor, TIL: tumor infiltrating lymphocyte.

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CRISPR/Cas9: clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins 9, PAM: protospacer adjacent motif, TALENs: transcription activator-like effector nucleases, ZFNs: zinc finger nucleases.
therefore, it has a wider range of potential applications. Successful treatment of the patients with X-linked adrenoleukodystrophy was demonstrated using a lentiviral vector with the deficient peroxisomal adenosine triphosphate–binding cassette D1.25 Despite the use of a lentiviral vector with an internal viral long terminal repeat, no oncogene activation was observed.25

A timeline showing the history of scientific progress in gene therapy is highlighted in Table 1.

Gene Therapeutic Strategies for Brain Tumor
A variety of studies were performed to apply gene therapy to malignant tumors. The concept of gene therapy for tumors is different from that for genetic diseases, in which new genes are added to a patient’s cells to replace missing or malfunctioning genes. In malignant tumors, the breakthrough in gene therapeutic strategy involved designing suicide gene therapy,26 which was first applied for malignant glioma in 1992.26,27 The first clinical study was performed on 15 patients with malignant gliomas by Ram et al (phase I/II).27 Stereotactic intratumoral injections of murine fibroblasts producing a replication-deficient retrovirus vector with a suicide gene (herpes simplex virus-thymidine kinase [HSV-TK]) achieved anti-tumor activity in four patients through bystander killing effects.27 Subsequently, various types of therapeutic genes have been used to treat malignant glioma. Suicide genes (cytosine deaminase [CD]), genes for immunomodulatory cytokines (interferon [IFN]-β, interleukin [IL]-12, granulocyte-macrophage colony-stimulating factor [GM-CSF]), and genes for reprogramming (p53, and phosphatase and tensin homolog deleted from chromosome [PTEN]) have been applied to the treatment of malignant glioma using viral vectors.28,29

Recently, a nonlytic, amphotropic retroviral replicating vector (RRV) and immortalized human neural stem cell (NSC) line were used for gene delivery to invasive glioma.30–32 In 2012, a nonlytic, amphotropic RRV called Toca 511 was developed for the delivery of a suicide gene (CD) to tumors.32 A tumor-selective Toca 511 combined with a prodrug (Toca FC) was evaluated in patients with recurrent high-grade glioma in phase I clinical trial.30 The complete response rate was 11.3% in 53 patients.30 In addition, the sub-analysis of this clinical trial revealed that the objective response was 21.7% in the 23-patient phase III eligible subgroup.30 However, in the recent phase III trial, treatment with Toca 511 and Toca FC did not improve overall survival compared with standard therapy in patients with recurrent high-grade glioma. A further combinational treatment strategy using programmed cell-death ligand 1 (PD-L1) checkpoint blockade delivered by TOCA-511 was evaluated in experimental models, which may lead to future clinical application.34
Since 2010, intracranial administration of allogeneic NSCs containing CD gene (HB1.F3. CD) has been performed by a team at City of Hope. Autopsy specimens indicate the HB1.F3. CD migrates toward invaded tumor areas, suggesting a high tumor-trophic migratory capacity of NSCs.31 No severe toxicities were observed in the trial. Generally, it is difficult
Gene Therapy and Genome Editing Technology

Gene Therapy and Genome Editing Technology

Recent advances in genetic engineering technologies have enabled the introduction of therapeutic genes into human cells, thereby offering potential treatments for various diseases. This review focuses on in vivo and ex vivo gene therapy strategies, new genetic engineering tools, and critical issues in genome editing.

In vivo and ex vivo strategies of gene therapy

In vivo strategy
- Treatment gene
- Transduction
- Injection
- Viral vector
- Ribosome

Ex vivo strategy
- Harvest
- Injection
- Genomic editing
- Culture
- Viral vector
- Nanoparticle

New Genetic Engineering Technologies for Gene Therapy

Genetic engineering technologies using viral vectors to randomly insert therapeutic genes into a host genome have been developed. However, these methods can lead to insertional mutagenesis and oncogene activation. Therefore, new technology to intentionally insert therapeutic genes at site-specific locations is needed. Genome editing is a genetic engineering method that allows for precise and efficient manipulation of the genome.

Genome editing tools

ZFNs are fusions of the nonspecific DNA cleavage domain of the Fok I restriction endonuclease and zinc-finger proteins that lead to DNA double-strand breaks (DSBs). Zinc-finger domains recognize a trinucleotide DNA sequence. The design and selection of zinc-finger arrays is difficult and time-consuming.

TALENs are fusions of the Fok I cleavage domain and DNA-binding domains derived from TALE proteins. TALEs have multiple 33–35 amino acid repeat domains that recognize a single base pair, leading to targeted DSBs.

CRISPR/Cas9 technology uses Cas9 nuclease and two RNAs (CRISPR RNA [crRNA] and trans-activating CRISPR RNA [tracrRNA]). The crRNA/tracrRNA complex (gRNA) induces the Cas9 nuclease and cleaves DNA upstream of a protospacer-adjacent motif (PAM, 5’-NGG-3’ for S. pyogenes) (Fig. 1). Currently, Cas9 from S. pyogenes (SpCas9) is the most popular tool for genome editing.

Critical issues in genome editing

Several studies have demonstrated the off-target effects of Cas9/gRNA complexes. It is important to carefully evaluate the potential off-target effects of genome editing technologies to ensure their safe and effective use.

Fig. 2 In vivo and ex vivo strategies of gene therapy. In vivo and ex vivo gene transfer strategies are shown. For in vivo gene transfer, genetic materials containing therapeutic genes, such as viral vectors, nanoparticles, and ribosomes, are delivered directly to the patient, and genetic modification occurs in situ. For ex vivo gene transfer, the harvested cells are modified by the appropriate gene delivery tools in vitro (e.g., recombinant viruses genome editing technologies). The modified cells are then delivered back to the patient via autologous or allogeneic transplantation after the evaluation of off-target effects.
| Trial number (Phase) | Disease | Target gene | Technology | Vector | Start date  |
|---------------------|---------|-------------|------------|--------|-------------|
| NCT04244656 (I)    | Multiple myeloma | BCMA        | CRISPR/Cas9 | CAR-T   | Jan. 2020   |
| NCT04037566 (I)    | Leukemia or Lymphoma | XYF19      | CRISPR/Cas9 | CAR-T   | Aug. 2019   |
| NCT04035434 (I/II) | B-Cell malignancies | N/A        | CRISPR/Cas9 | CAR-T   | Jul. 2019   |
| NCT03728322 (I)    | β-thalassemia | HBB         | CRISPR/Cas9 | iHSCs  | Jan. 2019   |
| NCT03745287 (I/II) | Sickle cell disease | BCL11A    | CRISPR/Cas9 | CD34+ hematopoietic stem cells | Nov. 2018 |
| NCT03747965 (I)    | Multiple solid tumors | PD-1       | CRISPR/Cas9 | CAR-T   | Nov. 2018   |
| NCT03655678 (I/II) | β-thalassemia | BCL11A     | CRISPR/Cas9 | CD34+ hematopoietic stem cells | Sep. 2018 |
| NCT03399448 (I)    | Multiple myeloma, Melanoma, Synovial sarcoma, Myxoid/Round cell Liposarcoma | PD-1, TCR | CRISPR/Cas9 | T cell | Sep. 2018   |
| NCT03538613 (I/II) | Gastrointestinal epithelial cancer | CISH       | CRISPR/Cas9 | CAR-T   | May. 2018   |
| NCT0399967 (I/II)  | B cell lymphoma | N/A        | CRISPR/Cas9 | CAR-T   | Jan. 2018   |
| NCT03545815 (I)    | Multiple solid tumors | PD-1 and TCR | CRISPR/Cas9 | CAR-T   | Jan. 2018   |
| NCT03057912 (I)    | HPV-related cervical intraepithelial neoplasia I | HPV 16 and 18 E7 oncogene | CRISPR/Cas9, TALEN | Plasmid | Jan. 2018 |
| NCT03166878 (I/II) | B cell lymphoma | TCR and B2M | CRISPR/Cas9 | CAR-T   | Jun. 2017   |
| NCT03164315 (N/A)  | HIV | CCR5        | CRISPR/Cas9 | CD34+ cell | May. 2017 |
| NCT03164315 (N/A)  | HIV | CCR5        | CRISPR/Cas9 | Hematopoietic stem cells | May. 2017 |
| NCT03081715 (N/A)  | Esophageal cancer | PD-1       | CRISPR/Cas9 | T cell | Mar. 2017 |
| NCT03044743 (I/II) | Epstein-Barr virus associated malignancies | PD-1 | CRISPR/Cas9 | T cell | Apr. 2017 |
| NCT02867345 (I)    | Prostate cancer | PD-1       | CRISPR/Cas9 | T cell | Nov. 2016 |
| NCT02867332 (I)    | Renal cell carcinoma | PD-1 | CRISPR/Cas9 | T cell | Nov. 2016 |
| NCT02863913 (I)    | Bladder cancer | PD-1       | CRISPR/Cas9 | T cell | Sep. 2016 |
| NCT02793856 (I)    | Non-small cell lung cancer | PD-1 | CRISPR/Cas9 | T cell | Aug. 2016 |
| NCT04150497 (I)    | B-cell acute lymphoblastic leukemia | N/A | TALEN | CAR-T | Oct. 2019 |
| NCT03226470 (I)    | Cervical precancerous lesions | HPV16 E6 and E7 DNA | TALEN | T27 and T512 | Jan. 2018 |
| NCT03190278 (I)    | Acute myeloid leukemia | N/A | TALEN | CAR-T | Jun. 2017 |
| NCT03653247 (I/II) | Sickle cell disease | BCL11A | ZFN | Hematopoietic stem cell | Jun. 2019 |
| NCT00842634 (I)    | HIV | CCR5 | ZFN | T cell | Jan. 2019 |
| NCT03432364 (I/II) | β-thalassemia | BCL11A | ZFN | Hematopoietic stem cells | Mar. 2018 |
| NCT03041324 (I/II) | MPS II | ALB | ZFN | AAV | May. 2017 |
| NCT02702115 (I/II) | MPS I | ALB | ZFN | AAV | May. 2017 |
| NCT02800369 (I)    | Cervical precancerous lesions | HPV16 and 18 E7 oncogene | ZFN | N/A | Dec. 2016 |
| NCT02695160 (I)    | Hemophilia B | ALB | ZFN | AAV | Nov. 2016 |
| NCT02500849 (I)    | HIV | CCR5 | ZFN | Hematopoietic stem cells | Jul. 2015 |
| NCT01252641 (I)    | HIV | CCR5 | ZFN | T cell | Nov. 2010 |

AAV: adeno-associated virus, CAR: chimeric antigen receptor, CRISPR/Cas9: clustered regularly interspaced short palindromic repeats/CRISPR-associated 9 proteins, HIV: human immunodeficiency virus, HPV: human papillomavirus, MPS: mucopolysaccharidosis, N/A: not available, PD-1: programmed cell death-1, TALEN: transcription activator-like effector nucleases, ZFN: zinc finger nucleases.
to select unique target sites without closely homologous sequences, resulting in minimum off-target effects.\textsuperscript{42} Additionally, other CRISPR/Cas9 gene editing tools were developed to mitigate off-target effects, including gRNA modifications (slightly truncated gRNAs with shorter regions of target complementarity <20 nucleotides)\textsuperscript{43} and SpCas9 variants, such as Cas9 paired nickases (a Cas9 nickase mutant or dimeric Cas9 proteins combined with pairs of gRNAs).\textsuperscript{44} The type I CRISPR-mediated distinct DNA cleavage (CRISPR/Cas3 system) was developed recently in Japan to decrease the risk of off-target effects. Cas3 triggered long-range deletions upstream of the PAM (5′-ARG).\textsuperscript{45}

A confirmatory screening of off-target effects is necessary for ensuring the safe application of genome editing technologies.\textsuperscript{46} Although off-target mutations in the genome, including the noncoding region, can be evaluated using whole genome sequencing, this method is expensive and time-consuming. With the development of unbiased genome-wide cell-based methods, GUIDE-seq (genome-wide, unbiased identification of DSBs enabled by sequencing)\textsuperscript{47} and BLESS (direct \textit{in situ} breaks labeling, enrichment on streptavidin; next-generation sequencing)\textsuperscript{48} were developed to detect off-target cleavage sites, and these methods do not require high sequencing read counts.

**Applications of Genome Editing Technologies**

Gene therapy has \textit{in vivo} and \textit{ex vivo} strategies. For the \textit{in vivo} strategy, vectors containing therapeutic genes are directly delivered into the patients, and genetic modification occurs \textit{in situ}. For the \textit{ex vivo} strategy, the harvested cells are modified by appropriate gene delivery tools \textit{in vitro} (e.g., recombinant viruses and genome editing technologies). The modified cells are then delivered back to the patient via autologous or allogeneic transplantation after the evaluation of off-target effects (Fig. 2).

HIV-resistant T cells were established by ZFN-mediated disruption of the C-C chemokine receptor (CCR) 5 coreceptor for HIV-I, which is being evaluated as an \textit{ex vivo} modification in early-stage clinical trials.\textsuperscript{49,50} Disruption of CCR5 using ZFNs was the first-in-human application of a genome editing tool. Regarding hematologic disorders, since 2016, clinical trials have attempted the knock-in of the factor IX gene using AAV/ZFN-mediated genome editing approach for patients with hemophilia B.\textsuperscript{51}

In addition to these promising ongoing clinical trials for genetic diseases, CRISPR/Cas9 and TALEN technologies have improved the effect of cancer immunotherapy using genome-engineered T cells. Engineered T cells express synthetic receptors (chimeric antigen receptors, CARs) that can recognize epitopes on tumor cells. The FDA approved two CD19-targeting CAR-T-cell products for B-cell acute lymphoblastic leukemia and diffuse large B-cell lymphoma.\textsuperscript{52,53} Engineered CARs target many other antigens of blood cancers, including CD30 in Hodgkin’s lymphoma as well as CD33, CD123, and FLT3 of acute myeloid leukemia.\textsuperscript{54} Recent research has shown that Cas9-mediated PD-1 disruption in the CAR-T cells improved the anti-tumor effect observed in \textit{in vitro} and \textit{in vivo} experimental models, leading to the performance of a clinical trial.\textsuperscript{55,56} All other ongoing clinical trials using genome-editing technologies are highlighted in Table 3.

**Future Direction**

Gene therapy has advanced treatments for patients with congenital diseases and cancers throughout recent decades by optimizing various types of vectors and the introduction of new techniques including genome editing tools. The CRISPR/Cas9 system is considered one of the most powerful tools for genetic engineering because of its high efficiency, low cost, and ease of use. CRISPR technologies have progressed and are expected to continuously advance. Although there are still many challenging obstacles to overcome to achieve safe clinical application, these methods provide the possibility of treatment for a wide variety of human diseases.

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**Conflicts of Interest Disclosure**

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