Therapeutic applications of gene editing in chronic liver diseases: an update

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INTRODUCTION

The liver is a major organ to maintain homeostasis by promoting detoxification and lipid and protein metabolism. Liver dysfunction results in toxin accumulation, organ damage and the development of pathological conditions. Various liver diseases are caused by gene mutations. Liver transplantation was considered as the only therapeutic option for liver diseases. However, liver transplantation is associated with several limitations, including shortage of liver donors and immune rejection. The advent of gene editing methods has enabled the repair of pathological mutations. Thus, gene editing technology will replace liver transplantation as a therapeutic strategy for liver diseases.

The first successful gene therapy in humans was reported approximately 30 years ago. The peripheral blood T lymphocytes transduced with a retrovirus encoding ADA have been used in the treatment of patients with adenosine deaminase (ADA) deficiency. This study demonstrated the benefits of gene therapy and its therapeutic role in genetic diseases, including inherited liver disorders. Over 100 types of liver-related diseases are caused by mutations of a single gene. Hence, gene therapy represents a potential alternative to liver transplantation for treating these disorders.

In the last two decades, various gene editing tools including meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9 system) have been developed and these tools promote double-strand breaks (DSBs) in the target gene. Subsequently, DSBs activate DNA repair responses including non-homologous end joining (NHEJ), leads to the random insertions and deletions (indels) at the site of cleavage and the repair of pathological mutations. Homology-directed repair (HDR) can change the DNA at the cleavage location in the presence of a homologous DNA template. However, the efficiency of HDR is lower than that of NHEJ owing to the frequency of unwanted indels, especially in non-dividing cells. Therefore, alternative strategies are needed to repair point mutations without introducing DSBs.

Komor et al. fused CRISPR/Cas9 and a guide RNA and cyti-
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Fig. 1. Timeline of the scientific progress in gene therapy. Purple boxes indicate human trials, and important events, such as human trials and winning Nobel prize. ADA-SCID: adenosine deaminase-severe combined immunodeficiency, crRNAs: CRISPR RNAs, trRNA (tracrRNA): trans-activating CRISPR RNA, sgRNA: single-guide RNA.

Table 1. Information about base and prime editors

| Editor | Functions | Composition | Hurdles | Ref. |
|--------|-----------|-------------|---------|------|
| CBEs   | C·G to T·A| Cytosine deaminase d/nCas9 (dead (d) or nickase (n)) UGI sgRNA | Cas9-mediated off target Unwanted ssDNA deamination | (5, 6) |
| ABEs   | A·T to G·C| Adenine deaminase d/nCas9 (dead (d) or nickase (n)) sgRNA | Cas9-mediated off target Random deamination | (5, 6) |
| PEs    | Insertion & deletion to any SNV | Reverse transcriptase nCas9 (nickase (n)) pegRNA | Off-target editing Immune response | (7, 8) |

CBEs: cytosine base editors, ABEs: adenine base editors, PEs: prime editors, C: cytosine, G: guanine, T: thymine, A: adenine, UGI: uracil glycosylase inhibitor, sgRNA: single-guide RNA, SNV: single nucleotide variants, pegRNA: prime-editing guide RNA.

dine deaminase to convert specific cytidines to uridines, which resulted in a C>T replacement without DSBs. Additionally, there are two classes of DNA base editor: (i) cytosine base editors (CBEs) consist of a dead (d) or a nickase (n) Cas9 (d/nCas9) protein fused to cytosine deaminase and UGI (uracil glycosylase inhibitor) and sgRNA (single guide RNA) to covert C·G into T·A base pairs, (ii) adenine base editors (ABEs) consist of d/nCas9 protein fused to adenine deaminase and sgRNA to covert A·T into G·C base pairs (Table 1) (5, 6). Emmanuelle Charpentier and Jennifer Doudna, who are the pioneers of the CRISPR system, were awarded the 2020 Nobel Prize in chemistry (Fig. 1).

A new method known as “prime editing” has been developed to fix single nucleotide polymorphism (SNP) without DSBs. Prime editors (PEs) require an engineered reverse transcriptase fused to nCas9 and a prime editing guide RNA (pegRNA) that can induce both insertions and deletions in all single nucleotide variants (SNVs) of human cells (7). Prime editors exhibit enormous editing capacities, but the limitations include off-target editing, poor immune response and few delivery options (Table 1) (8).

In this review, we summarize gene mutations related to liver diseases in the descending order of their prevalence and discuss previous approaches to repair these mutations using gene editing tools (Table 2). Successful recent applications of ex vivo and in vivo gene editing strategies in the livers of mice, monkeys and human patients are discussed.

GENE EDITING IN CHRONIC LIVER DISEASES

Alpha-1 antitrypsin deficiency (AATD)

AATD is one of the most common liver disorder and an autosomal recessive metabolic disease with a high prevalence in Scandinavia and North America. The disease results from c.1096G>A mutation in SERPINA1, which encodes alpha-1 antitrypsin (AAT). The mutation causes Glu342Lys substitution (also called Z mutation), which decreases the levels of AAT by 70-85% (9). In addition to the most general clinical symptom of chronic obstructive pulmonary disease (COPD), AATD is associated with hepatic dysfunction that normally presents with high serum aminotransferase levels, hyperbilirubinemia and jaundice. AATD-related lung disease is managed via AAT injection, while hepatic disease often requires liver transplantation (10).
Smith et al. used the CRISPR/Cas9 system to repair the Z mutation in hiPSCs derived from patients with AATD, which demonstrated the promise of the CRISPR/Cas9 system for allele-specific genome editing (11). CRISPR/Cas9 repaired the Glu342Lys mutation in the PiZ mouse model for human AATD (Glu342Lys mutation in SERPINA1) back to Lys342Glu (12). In both neonatal and adult mice, in vivo gene editing partially recovered physiological levels of serum AAT although therapeutic levels were not achieved (13). Other therapeutic approaches for AATD involve knocking out the mutated SERPINA1. Bjursell et al. repaired human mutant SERPINA1 in the liver of a PiZ mouse using CRISPR/Cas9 system. Disruption of the human transgene leads to a decline in hepatic and plasma hAAT levels, liver fibrosis and hepatic protein aggregation (14). Similar results were also obtained in the PiZ mouse by targeting the exon 2 of the mutated human SERPINA1 with CRISPR/Cas9, which resulted in a 98% reduction in AAT expression in hepatocytes. To alleviate lung symptoms in patients with AAT, a dual recombinant adeno-associated virus (rAAV) system was delivered to repair the E342K substitution directly at the intersection of exon 22 and intron 22 in HA-hiPSCs (18). These inversions using HA-derived or HB-derived iPSCs. The two inversions in hiPSCs were repaired using CRISPR/Cas9 or TALENs (19, 20). The efficiency of reversion was up to 6.7% and 7.4% in the engineered hiPSCs. The engineered cells expressed F8 levels in vitro compared to non-transplanted HA mice (19).

Some strategies involve the insertion of wild-type SERPINA1 to increase the levels of AAT. One approach integrated wild-type human SERPINA1 into C57BL/6j mouse liver using the CRISPR/Cas9 system targeting the ROSA26, often referred to as a "safe harbor" locus, without any side effects associated with transgene insertion; the newly inserted gene exhibits a stable expression. This knock-in approach of SERPINA1 resulted in long-term increase of serum AAT in wild-type mice (16). Another approach integrated an AAV vector into the NSG-PiZ mouse model, which is derived from PiZ and immune-deficient (NSG) mouse strains. The AAV vector containing wild-type AAT and miRNAs targeting the pathogenic allele was inserted into the hepatocytes of NSG-PiZ mice, resulting in the upregulation of wild-type AAT levels and downregulation of mutant protein levels (17).

### Hemophilia

Hemophilia caused by mutations in F8 gene encoding coagulation factor VIII (FVIII) or F9 gene encoding coagulation factor IX (FIX), leading to hemophilia A (HA) or hemophilia B (HB) is an X-linked bleeding disorder. Various genetic mutations including insertions, inversions, point mutations, and large deletions in the F8 locus result in HA. Almost 50% of serious HA cases are caused by inversion breaking in intron 1 or 22 and in the upstream of correlated homologous sequences of F8 gene (18).

Initial attempts to repair mutations via genome editing targeted these inversions using HA-derived or HB-derived iPSCs. The two inversions in hiPSCs were repaired using CRISPR/Cas9 or TALENs (19, 20). The efficiency of reversion was up to 6.7% and 7.4% in the engineered hiPSCs. The engineered cells expressed F8 levels in vitro compared to non-transplanted HA mice (19).

Another approach for the repair of inversion was used TALENs to insert the exon 23-26 fragment of wild-type F8 cDNA correctly at the intersection of exon 22 and intron 22 in HA-hiPSCs via HDR. The mRNA and protein levels of F8 were rescued in the engineered hiPSC-derived endothelial cells (21). Several studies reported general strategies to repair all genetic variants

### Table 2. Gene editing methods to treat liver diseases

| Liver disease                  | Prevalence     | Model                          | Target gene | Mutation | Method               | Ref. |
|--------------------------------|----------------|-------------------------------|-------------|----------|----------------------|------|
| α1 antitrypsin deficiency (AATD)| 1:1,500-7,000  | iPSCs from patients PiZ mouse | SERPINA1    | Glu342Lys, Ser53Phe, His334Asp | ZFN-piggyBac, Transposon CRISPR/Cas9, Promoterless rAAV | (9-7) |
| Hemophilia A (HA)              | 1:5,000        | hiPSCs from patients engineered hiPSCs, HACD4 null mice | F8          | Deletions, insertions, inversions, and point mutations | CRISPR/Cas9, TALENs: ZFNs, Promoterless rAAV | (18-29) |
| Phenylketonuria (PKU)          | 1:10,000-15,000| COS-7 cells                  | PAH         | c.1222C>T (p.Arg408Trp) | SNP, FokI-dCas9 system CRISPR-Cas9 | (30-35) |
| Wilson’s disease (WD)          | 1:30,000       | WD mouse                     | ATP7B       | Loss-of-function | AAV containing WT CRISPR/Cas9 | (36-38) |
| Ornithine transcarbamylase (OTC) | 1:70,000     | spt4th mouse                  | OTC         | Loss-of-function | CRISPR/Cas9 | (39-41) |
| Tyrosinemia type 1 (HT1)       | 1:100,000      | fab-/- mice                  | FAH/HPD     | Loss-of-function | Promoterless rAAV CRISPR/Cas9, saCas9, Nme-Cas9 Cas9 nickases CRISPR-Cas9 | (42-53) |
| Arginase-1 deficiency          | 1:1,000,000    | Induced mouse model           | ARG1        | Deletions in exon 7 and 8 | TALEN | (54-56) |

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in patients with HA by nuclease-mediated insertion of wild-type F8 cDNA fragment into the F8 locus, Alb locus or H11 safe harbor (22). Interestingly, a B-domain deleted form of F8 under the control of EF1α promoter into HA-hiPSCs using CRISPR/Cas9 were increased F8 activity in knocked-in hiPSC-derived endothelial cells (23). Also, the F8 activity increased in the F8-encoding gene inserted into the human H11 safe harbor locus in HA-hiPSCs harboring a deleted or inverted F8-encoding gene using CRISPR/Cas9 (22). This strategy may represent a potential therapeutic approach in patients with HA. The site-specific integration of F8 into Alb locus validated the possibility of these general approaches in vivo. In this strategy, HA/CD4 null mice were treated with ZFN pairs, which were separately packaged into rAAV8 including intron 1 of the Alb-encoding gene and a donor rAAV encoding a docked structure of hF8. The hF8 activity was increased by 37% (24).

A similar approach was used in HB, which is an ideal disease for liver-directed genome editing strategy due to marked upregulation of hF9 activity. The cDNA of F9 exons 2-8 flanked by a splicing a poly-A and acceptor signal were transduced with rAAV8-ZFNs into mice and the levels of hF9 were stably upregulated for up to 1 year. A similar strategy was adopted by delivering ZFNs mRNAs using lipid nanoparticles (LNPs), which are not limited by the pre-existing neutralizing antibodies (25).

In 2018, the first patient treated with SB-FIX (Clinical Trial: NCT02695160), which is ZEN-regulated genome editing transformed by rAAV inserted the wild-type of Factor IX cDNA into the Alb locus. Barzel et al., used the same mAlb locus via rAAV-mediated HR without the nucleases. In this strategy, the F9 plasma level was upregulated and the coagulation time reverted to physiological levels in F9 deficient mice-treated full length F9 cDNA (26).

Alternative general knock-in strategies adopted for HB mice models entailed transgene insertion into F9, AAV51, or Rosa26 loci (27). F9 cDNA encoding exons 2-8 were inserted into intron 1 of F9 via rAAV8-packaged ZFNs, which promoted HR, upregulated the F9 levels, and markedly shortened activated partial thromboplastin time. This approach was effective in both neonatal and adult hF9/HB mice. However, the expression of F9 in neonatal mice was 5-fold higher than in adult hF9/ HB mice, which can be attributed to the decreased loss of rAAV (28). Similar strategies involved the insertion of a full-length F9 sequence into HB-derived hiPSCs with AAV51 and Rosa26 loci in a juvenile model of HB using CRISPR/Cas9. F9 transcript expression and hFIX activity were upregulated not only in F9- AAV51-hiPSCs-derived hepatocytes but also in F9-Rosa26-juvenile R333Q mice with hemophilia (27, 29).

Phenylketonuria (PKU)

PKU is caused by mutations in PAH, which encodes phenylalanine hydroxylase (PAH). PAH regulates the catalysis of hydroxylation of phenylalanine in tyrosine. The decline of PAH activity is increased in phenylalanine in blood, which leads to neurotoxic effects. If untreated, PKU can impair cognitive development and other symptoms, including autism, eczematous rashes, motor deficits, and seizures (30). The main treatment for PKU is dietary phenylalanine restriction. However, patients often exhibit limited improvements. Other potential strategies treat with tetrahydrobiopterin to induce the enzyme activity including PAH or use the antagonists of phenylalanine uptake at the blood-brain barrier (BBB) (31). To overcome the poor outcomes of these currently used treatments, an alternative therapeutic method is needed to repair mutations in PAH.

Patients with PKU have mutations involving both alleles of PAH. The repair of only a single allele enhances PAH activity to alleviate symptoms of PKU. The missense mutations of PAH are over 50% of the PAH mutations and the c.1222C>T (p.Arg408Trp) is the most common mutation in severe PKU (32). To repair the c.1222C>T mutation used the CRISPR system with a deactivated Cas9 (dCas9) and a guided RNA for FokI nuclease to repair this mutation in a c.1222C>T mutant-expressing Cos7 cell line. The mutation was repaired in approximately 27% of treated cell clones and PAH activity was partially rescued. Furthermore, no off-target alterations were detected (33). Another recent study attempted to repair PAH pathogenic variant (c.835T>C; p.F263S) in homozygous Pahenu2 mouse carrying homozygous missense mutation (34). Villiger et al. used CRISPR with cytidine deaminase base editors packed in rAAV, and achieved an mRNA correction rate of up to 63% and recovered physiological levels of phenylalanine in the blood of adult mice. Additionally, growth retardation of Pahenu2 mice was mitigated in corrected heterozygous mice (35). This results suggest that base editors for gene correction are possible in adults, specifically in treating hepatic tissue of decreased proliferative ability.

Wilson’s disease (WD)

WD is a rare genetic disorder caused by loss-of-function mutations in the copper-transporting P-type ATPase-encoding gene (ATP7B). ATP7B encodes a protein that transports copper out of the liver. Therefore, WD is characterized by excessive hepatic accumulation of copper, which leads to systemic toxicity. If untreated, WD progresses to liver disease, brain injury, and premature death. Patients often develop liver dysfunction, fatigue, and yellow discoloration of the skin (36). Currently, WD is treated via liver transplantation, which restores biliary copper excretion and normalizes copper level in circulation, but requires life-long immunosuppression (37).

Murillo et al. used AAV to repair ATP7B in the WD mouse model. A single injection resulted in normalized plasma holoceruloplasmin, increased biliary copper excretion, and decreased hepatic copper levels after 6 months. Interestingly, hepatocytes from AAV-transduced WD mice exhibited higher copper-excreting ability than those derived from wild-type mice, suggesting enhanced gene editing efficiency through AAV injection (38).

Orotate transcarbamylase (OTC) deficiency

OTC deficiency is a rare X-linked genetic disorder caused by
absence of the OTC enzyme, in which plays the role in elimination of nitrogen, which results in the form of ammonia (hyperammonemia) via the urea cycle. The accumulation of ammonia caused by the lack of the OTC enzyme moves to central nervous system through the blood and leads to the symptoms with OTC deficiency. The treatment of the disease involves the treatment with ammonia scavengers, such as sodium benzoate and dietary protein restriction. The transplantation of liver is considered in cases of hyperammonemia (39).

One study repaired OTC mutation in an OTC deficiency mouse model using rAAV-mediated CRISPR/Cas9. Spf<sup>ab</sup> mouse, which harbors a G>A point mutation at the splice site located at the end of exon 4 in OTC, exhibits impaired mRNA splicing and decreased OTC levels (40). The authors injected rAAV containing CRISPR/Cas9 components into newborn and adult spf<sup>ab</sup> mice, which induced HDR and repaired the mutation in approximately 10% of hepatocytes in newborns. Additionally, the survival of mice was improved with a high-protein diet, which causes hyperammonemia. However, the gene correction rate in adult mice was markedly low and the toxicity was increased significantly repopulated the mouse liver. Additionally, the blood tyrosine levels decreased to physiological levels (49, 50). Ex vivo gene editing facilitates selection of accurately edited cells. Some studies have attempted to silence Hpd using Cas9 and base editors. One study used murine leukemia virus (MLV)-like particles loaded with Cas9-sgRNA ribonucleoproteins (Nano-blades) (51). Another study used Neisseria meningitides Cas9 (NmeCas9) and sgRNA packed in AAV and Rossidis et al. finally used base editor technique to generate a nonsense mutation in Hpd<sup>newborn</sup> mice (52). The in utero treatment recovered the lethal phenotype and the liver function in Fah<sup>−/−</sup> mice (53).

Arginase-1 deficiency (argininemia)

Arginase-1 deficiency is a genetic disorder associated with urea metabolism in which the hydrolysis of arginine to urea and ornithine is impaired due to mutations in ARG1. Similar to OTC deficiency management, the clinical management of arginase-1 deficiency involves dietary restrictions, treatment with nitrogen scavengers, and enzyme replacement therapies (54).

Sin et al. generated a mouse model of arginase-1 deficiency via Cre-loxP-regulated deletion of exons 7 and 8 of Arg1. Then, the authors tried to repair the genetic defect in iPSC-derived mouse hepatocytes and macrophages using the CRISPR/Cas9 system combined with the piggyBac technique. Gene repair was successful in iPSCs. However, differentiated hepatocytes did not restore the expression of arginase-1 markedly. Interestingly, the expression of arginase-1 significantly recovered in differentiated macrophages derived from the same iPSCs (55). And also, TALEN-regulated recombination of deleted exons was completed in iPSCs derived from the murine model. The edited cells differentiated into hepatocyte like cells, which were transferred to the liver of the arginase-1 deficient mouse. However, the expression of arginase-1 was not restored owing to the poor proliferation of hepatocytes (56).
RECENT SUCCESSES IN LIVER GENE EDITING OF MOUSE, MONKEY AND HUMAN PATIENTS USING EX VIVO AND IN VIVO METHODS

Ex vivo gene editing
Recently, we demonstrated the efficacy of an ex vivo gene editing method targeting liver (57). The hepatocytes were extracted from the HT1 mouse model to generate chemically derived hepatic progenitors (CdHs) (58). CdHs were treated with adenine base editors (ABEs) and prime editing components to repair FAH. The engineered cells, which were injected back into HT1 mice through the tail vein, proliferated in the liver. HT1 mice injected with the engineered CdHs exhibited marked alleviation of hepatic dysfunction when compared with control HT1 mice (Fig. 2, right).

Our finding indicated that genetic liver disease can be treated using ex vivo gene editing strategies. Ex vivo gene editing has several advantages. First, ex vivo editing does not elicit pathological immune response against the edited cells, which are derived from the patient. Additionally, in vitro gene editing can facilitate the screening and elimination of cells with unexpected edits and only cells with the desired edited gene can be injected into patients, which can further ensure safety. However, ex vivo approaches require highly advanced facilities with specialized protocols and experienced researchers.

In vivo gene editing
Several successful in vivo gene therapies involving monkeys and humans have been reported recently. Instead of using viral vectors, lipid nanoparticles (LNPs) conjugated with apolipoprotein E can directly target the hepatocytes, which can significantly improve the safety of gene therapy. Further, the efficacy of mRNA vaccines during the COVID-19 pandemic indicated that the safety and accuracy of gene therapy can be further improved using Cas mRNA instead of Cas gene or Cas protein. The administration of one or two doses of LNP-packed Cas9 or base editor mRNA and CRISPR guide RNA represents successful gene therapy strategy. Two recent studies demonstrated that transiently knock-out of gene related to cardiovascular disease was efficient in non-human primates (59, 60). Highly expressed proprotein convertase subtilisin/kexin type 9 (PCSK9) in the liver plays a role in cholesterol homeostasis and the interaction between PCSK9 and low-density lipoprotein (LDL) receptor induces endocytosis and degradation of the receptor, which results in reduction of LDL cholesterol from the blood. Gain-of-function variants in PCSK9 relate to familial hypercholesterolemia.

A recent study reported that a single administration of NLTA-2001, an in vivo gene-editing therapeutic agent, decreases the level of serum transthyretin (TTR) in patients, indicating potent therapeutic efficacy (61). The sustained reduction of TTR levels is supposed to alleviate disease symptoms. This finding provides strong evidence supporting the clinical application of gene editing. TTR amyloidosis caused by the accumulation of misfolded TTR proteins is formed the amyloid fibrils in cardiomyocytes and nerves, which subsequently leads to amyloid polyneuropathy and cardiomyopathy. A recent study by Gillmore et al. indicated that strategies for gene therapy can be upgraded from ex vivo to in vivo approaches (Fig. 2, left) (61).

CONCLUSIONS AND FUTURE PERSPECTIVES
Gene editing components can be easily delivered to the liver intravenously since liver metabolizes all foreign particles. Recent studies have demonstrated that the direct delivery of LNPs conjugated with apolipoprotein E to the hepatocytes was markedly
safer than the AAV-mediated delivery. Hence, LNPs enable in vivo gene editing to treat various liver diseases. Additionally, ex vivo gene editing approaches using patient cells have yielded promising results. The application of the combination of in vivo and ex vivo gene editing approaches represents a potent therapeutic strategy against genetic liver diseases.

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CONFLICTS OF INTEREST
The authors have no conflicting interests.

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