EBioMedicine xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Paper

# Therapeutic Genome Editing With CRISPR/Cas9 in a Humanized Mouse Model Ameliorates α1-antitrypsin Deficiency Phenotype

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ARTICLE INFO

*Article history:*
Received 15 November 2017
Received in revised form 15 February 2018
Accepted 16 February 2018
Available online xxxx

*Keywords:*
α1-antitrypsin deficiency
CRISPR/Cas9 gene editing
Liver fibrosis
Protein aggregation

ABSTRACT

α1-antitrypsin (AAT) is a circulating serine protease inhibitor secreted from the liver and important in preventing proteolytic neutrophil elastase associated tissue damage, primarily in lungs. In humans, AAT is encoded by the *SERPINA1* (*hSERPINA1*) gene in which a point mutation (commonly referred to as PiZ) causes aggregation of the miss-folded protein in hepatocytes resulting in subsequent liver damage. In an attempt to rescue the pathologic liver phenotype of a mouse model of human AAT deficiency (AATD), we used adenovirus to deliver Cas9 and a guide-RNA (gRNA) molecule targeting *hSERPINA1*. Our single dose therapeutic gene editing approach completely reverted the phenotype associated with the PiZ mutation, including circulating transaminase and human AAT (hAAT) protein levels, liver fibrosis and protein aggregation. Furthermore, liver histology was significantly improved regarding inflammation and overall morphology in *hSERPINA1* gene edited PiZ mice. Genomic analysis confirmed significant disruption to the *hSERPINA1* transgene resulting in a reduction of hAAT protein levels and quantitative mRNA analysis showed a reduction in fibrosis and hepatocyte proliferation as a result of editing. Our findings indicate that therapeutic gene editing in hepatocytes is possible in an AATD mouse model.


## 1. Introduction

α1-antitrypsin (AAT) is an abundant circulating serine protease inhibitor, primarily secreted by hepatocytes, which protects against protease related lung connective tissue damage (Janciauskiene et al., 2011). In humans, homozygous inheritance of the Z-type mutant allele of AAT (PiZ) can result in AAT deficiency (AATD), which is a rare genetic condition resulting from mutations in the human *SERPINA1* (*hSERPINA1*) gene. The PiZ mutation in the *hSERPINA1* gene results in a single amino acid substitution (Glu342Lys), which causes protein miss-folding and aggregated protein retention in hepatocytes (Lomas et al., 1992; Ekeowaa et al., 2010). Around 10–20% of PiZZ homozygous AATD patients have an increased risk for clinical liver disease, including cirrhosis and hepatocellular carcinoma, due to mutant AAT protein aggregation and accumulation (Teckman and Lindblad, 2006). In addition to liver disease, AAT release from hepatocytes is impaired, resulting in lower circulating levels of AAT and consequently impaired lung function. Targeting a defective gene in order to cure inherited human disease is an attractive therapeutic option and the stable disruption of *SERPINA1* variants in vivo, at the genomic or mRNA level, is a promising strategy to reduce levels of miss-folded AAT protein and ameliorate pathological findings in the liver.

A PiZ transgenic mouse model expresses the human *Z*-AAT protein at high levels and displays pathology associated with AATD in humans, including AAT protein aggregation and ER retention, liver fibrosis and hepatocellular carcinoma (Carlson et al., 1989; Qu et al., 1996; Teckman and Perlmutter, 1996; Wu et al., 1994; Marcus et al., 2010). The PiZ mouse has been used extensively in pre-clinical AATD research including hepatic repopulation (Ding et al., 2011) and in short interfering RNA (siRNA) and antisense oligonucleotide (ASO) approaches, which resulted in reduced Z protein production and improved liver health (Cruz et al., 2007 and Guo et al., 2015).

In this study, we explored whether targeting the *hSERPINA1* gene, rather than its mRNA, can be used as an alternative therapeutic approach requiring only a single treatment. The CRISPR/Cas9 technology has been successfully used in mouse models for correcting the dystrophin gene mutation (Long et al., 2014; Zhang et al., 2017), correction of a *Fah* mutation in hepatocytes (Yin et al., 2014) and for excision of the HIV-1 provirus (Yin et al., 2017). CRISPR/Cas9 introduces a DNA double strand break (DSB) at predefined loci determined by a highly specific guide RNA molecule (gRNA), and is a technology which has

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https://doi.org/10.1016/j.ebiom.2018.02.015

the potential to give rise to new classes of treatments for a wide range of diseases. We designed and used a gRNA specific for *hSERPINA1* expressed in the liver of PiZ mice with the aim of disrupting the gene and reversing the disease phenotype associated with the expression of the human PiZ allele. Our therapeutic gene editing approach led to a reversal of the PiZ phenotype, which included reduced circulating transaminases and Z-AAT levels, liver fibrosis and protein aggregation. Furthermore, liver histology was significantly improved in terms of inflammation and overall morphology in gene edited PiZ mice. Genomic and molecular evaluation of gene edited PiZ mice revealed major disruption of *hSERPINA1* transgene sequences and reduced expression of liver fibrosis-related and proliferation-related markers.

## 2. Methods

### 2.1. Animal Care and Experimentation

All animal experiments were approved by the Gothenburg Ethics Committee for Experimental Animals. PiZ mice were generated as described previously (Carlson et al., 1989) and were kindly provided by Jeff Teckman of St. Louis University, USA. Experimental animals were generated by breeding heterozygous *PiZ* mice to C57Bl/6 N mice (Charles River). Control animals used throughout the experiments were wild type littermates of the PiZ mice. Mice were housed in a temperature controlled room (21 °C) with a 12:12 h light-dark cycle (dawn: 5.30 am, lights on: 6.00 am, dusk: 5.30 pm, lights off: 6 pm) and controlled humidity (45–55%). They had access to a normal chow diet (R36, Lactamin AB, Stockholm, Sweden) and water ad libitum and were checked daily.

Baseline plasma and tissue sampling was performed at 8 weeks of age. At 9 weeks of age female PiZ mice and female wild type littermates were injected intravenously with an amount of replication-deficient Type-5 adenovirus (deleted in the E1 and E3 regions) corresponding to $1.4 \times 10^9$ PFU of either the control Ad-CMV-eGFP-CBh-FLAG-spCas9 virus ($n = 4$,catalog number 1904, Vector Biolabs, Malvern, PA, USA) or the targeting Ad-U6-*SERPINA1*-gRNA-CBh-FLAG-spCas9 virus (n = 4, custom order, construct name: "Ad-SerpinA-gRNA-CBh-spCas9", Vector Biolabs, Malvern, PA, USA; gRNA sequence TGCTGACCATCGACAAGAA). Blood plasma was analyzed one week prior to viral dosing and at 4, 6 and 9 weeks post-dosing through a panel of plasma chemistry parameters (human AAT, ALT and AST). At termination, mice were euthanized under isoflurane anesthesia, blood samples collected by intra-cardiac puncture in EDTA coated tubes, and livers removed, weighed and used for subsequent gene expression, protein expression and histological analyses. All tissues for histological analysis were preserved in fixatives and sectioned at 5 μm prior to staining.

### 2.2. hSERPINA1 Guide RNA Selection

Guide RNA sequences were designed using online software tools. Surveyor nuclease assay was used to detect indels introduced by four separate *hSERPINA1* specific gRNAs. Surveyor nuclease assay using the Transgenomic SURVEYOR mutation detection kit for standard gel electrophoresis was used according to the manufacturer's instructions (Integrated DNA Technologies, Stokie, IL, USA). The human retinal pigment epithelial cell line ARPE-19, maintained in DMEM/F12 supplemented with 10% FBS, was used to transfect a plasmid construct expressing Cas9 and *hSERPINA1* specific gRNAs.

### 2.3. Droplet Digital PCR

Genomic DNA was isolated from mouse liver obtained from 4 *hSERPINA1* gRNA treated mice and 4 control treated mice by incubating approximately 10 mg liver overnight in a shaking incubator at 56 °C in lysis buffer containing 50 mM Tris at pH 8 (15504-020, Invitrogen), 25 mM EDTA at pH 8 (AM9260G, ThermoFisher), 100 mM NaCl (S7653, Sigma-Aldrich), 1% SDS (862010, Sigma-Aldrich) and 0.05 mg/ml proteinase K (P6556, Sigma-Aldrich). The DNA was extracted using isopropanol, the pellet was washed with 70% ethanol and dried before being resuspended in ultrapure RNase and DNase free water (10977-035, Invitrogen). The DNA concentrations were measured and solutions of 3 ng/μl prepared for all samples. A FAM-labeled ddPCR-assay for the *hSERPINA1* gene was designed using the BioRAD website tool. The exact sequence of the primers and probe is proprietary (custom assay number 10042958, BioRAD). A Mastermix was prepared using a final concentration of 1× ddPCR Supermix for Probes, no dUPT (186-3024, BioRad), 1× FAM-labeled human *SERPINA1*-assay (custom assay 10042958, BioRAD), 1× AP3B1-HEX labeled mouse reference assay (dHsaCP1000001, BioRAD) and 1/40 *Hae*III (15205016, Invitrogen). 20 μl Mastermix per well to be analyzed was prepared in ultrapure RNase and DNase free water (10977–035, Invitrogen). After adding 5 μl DNA at 3 ng/μl to the 20 μl Mastermix in the semi-skirted 96-well plate (30129504, Eppendorf) followed by careful mixing, the plate was sealed (18114040, BioRAD) using a PX1™ PCR Plate Sealer (Bio-Rad, cat no. 181-4000). An automated Droplet Generator (BioRAD) was used to generate droplets in a new semi-skirted 96-well PCR plate (30129504, Eppendorf). The PCR plate was sealed (18114040, BioRAD) using a PX1™ PCR Plate Sealer (Bio-Rad, cat no. 181-4000). After sealing, the PCR plate was placed in a C1000 Touch™ Thermal Cycler (Bio-Rad, cat no. 185-1197) for PCR amplification, as detailed in Supplementary data Table 2. The droplet reading was performed with the QX 100 Droplet reader (Bio-Rad, cat no. 186-3001) using ddPCR™ Droplet Reader Oil (Bio-Rad, cat no. 186-3004). To explore the performance of the assay, an annealing (and extension) temperature gradient from 55 to 65 °C was tested to identify the temperature giving the largest separation between the four droplet populations (SERPINA1-positive, AP3B1-positive, positive for both targets, and empty droplets). Annealing temperatures of 55.0–59.0 °C worked equally well, and 57.0 °C was used in the sharp run.

Data acquisition and analysis was performed using the software QuantaSoft (Bio-Rad) and the "CNV2" program (two copies of the reference gene AP3B1 exist in the mouse genome). The fluorescence amplitude threshold was set manually as the midpoint between the average fluorescence amplitude of the four droplet clusters (SERPINA1-positive, AP3B1-positive, positive for both targets, and empty droplets). The same threshold was applied to all the wells of the ddPCR plate.

### 2.4. Quantitative Real-time PCR

RNA was extracted from 50 mg mouse liver using Qiagen RNeasy mini columns (74,104, Qiagen) in a Qiacube instrument (Qiagen) after first lysing the tissue in the presence of RLT-buffer (Qiagen) containing 20 mM dithiothreitol (43816, Sigma-Aldrich) and stainless steel beads (69989, Qiagen) in a Tissue lyser II instrument (5 min shaking at 25 Hz/s), following the instructions from the supplier. A DNase treatment step was included. Total RNA (2.5 μg) was transcribed using the High Capacity cDNA Reverse Transcription kit (4368813, Life Technologies). The cDNA was diluted 10-fold, and 12 ng was used in a total qPCR reaction volume of 10 μl. For gene expression assays used, see Supplementary table 1. The qPCR-reactions were set up in triplicate for each sample and target in 384-well format using TaqMan® Gene Expression Master Mix containing the FAM-dye reporter (4369016, ThermoFisher) and PPIA as the internal control. The Quantstudio 7 Flex instrument (Life technologies) was used to run the qPCR selecting the procedure for standard reagents. After retrieving the Ct-values, relative expression levels were determined using the $2^{-\Delta\Delta Ct}$-method (Livak et al., 2001).

### 2.5. Targeted Deep Sequencing

Off-target sites (up to 4 mismatches) for the *hSERPINA1* gRNA in mouse genome were identified. Primers for target site and 16 potential off-target sites were designed using Primer-Blast (Ye et al., 2012) and ordered with NGS linkers. PCR products for on-target and selected off-

Please cite this article as: Bjursell, M., et al., Therapeutic Genome Editing With CRISPR/Cas9 in a Humanized Mouse Model Ameliorates α1-antitrypsin Deficiency Phenotype, EBioMedicine (2018), https://doi.org/10.1016/j.ebiom.2018.02.015