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Preclinical evaluation of a clinical candidate AAV8 vector for ornithine transcarbamylase (OTC) deficiency reveals functional enzyme from each persisting vector genome

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ABSTRACT

Ornithine transcarbamylase deficiency (OTCD), the most common and severe urea cycle disorder, is an excellent model for developing liver-directed gene therapy. No curative therapy exists except for liver transplantation which is limited by available donors and carries significant risk of mortality and morbidity. Adeno-associated virus 8 (AAV8) has been shown to be the most efficient vector for liver-directed gene transfer and is currently being evaluated in a clinical trial for treating hemophilia B. In this study, we generated a clinical candidate vector for a proposed OTC gene therapy trial in humans based on a self-complementary AAV8 vector expressing codon-optimized human OTC (hOTCco) under the control of a liver-specific promoter. Codon-optimization dramatically improved the efficacy of OTC gene therapy. Supraphysiological expression levels and activity of hOTC were achieved in adult *spf*^{ash} mice following a single intravenous injection of hOTCco vector. Vector doses as low as 1×10^{10} genome copies (GC) achieved robust and sustained correction of the OTCD biomarker orotic aciduria and clinical protection against an ammonia challenge. Functional expression of hOTC in 40% of liver areas was found in mice treated with a low vector dose of 1×10^{9} GC. We suggest that the clinical candidate vector we have developed has the potential to achieve therapeutic effects in OTCD patients.

1. Introduction

Inborn errors of metabolism affecting the urea cycle can trigger hyperammonemia, a life threatening condition that often leads to irreversible cognitive impairment, coma and death [1,2]. The prevalence of urea cycle disorders is estimated to be at least 1 in 15,000 [3]. Patients with ornithine transcarbamylase deficiency (OTCD), an X-linked disorder, account for nearly half of all cases of inborn errors of urea synthesis, making it a compelling disorder for developing new therapies [4]. Current therapies for OTCD have numerous challenges [5–7]. Patients can be managed with a low protein diet combined with the use of medications that activate alternate nitrogen clearance pathways, however, this regimen does not prevent hyperammonemic crises [6,7]. Liver transplantation can cure OTCD, although it is limited by availability of donor livers, associated morbidity and mortality of the procedure, and the immunosuppressive drugs that are necessary to prevent rejection of the graft [8–11].

Vectors based on adeno-associated virus (AAV) have shown great potential for sustained expression of therapeutic transgenes. The main advantages of AAV are the attractive safety profiles and the possibility of generating long-term transgene expression without the requirement for chromosomal integration. Successful transduction of hepatocytes has been achieved in a number of animal species, including mice, dogs and non-human primates [12–15]. Among the AAV serotypes, AAV8 has been shown to be the most efficient for liver-directed gene transfer [16,17], and is currently being evaluated in a clinical trial for hemophilia B [18].

We and others have demonstrated the capacity of AAV-based gene therapy to restore protective levels of liver OTC enzyme activity with a single treatment in *spf*^{ash} mouse, a mouse model of OTCD [19–21]. Systemic delivery of AAV2/8 vectors expressing murine OTC (mOTC) under the control of liver-specific promoters achieved sustained correction. Modifications of the vector and/or transgene cassette, such as the use of a self-complementary (sc) AAV vector, incorporation of Kozak-like sequences or a post-transcriptional regulatory element, dramatically improved the potency of the vector [20,21]. More recently, Cunningham et al. showed that in *spf*^{ash} mice rendered completely deficient in OTC through vector-mediated expression of shRNA that the dose of OTC-expressing AAV vector required to prevent hyperammonemia was

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five-fold lower than that required to normalize the biomarker for OTCD, orotic aciduria [22].

Progression of a gene therapy product for OTC into the clinic eventually requires pre-clinical evaluation of a vector expressing the human OTC (hOTC) gene. However, previous studies using adenoviral vectors have illustrated the difficulties of expressing sufficient levels of active human OTC in OTCD mice [23,24]. Using chimeric OTC constructs, Ye et al. demonstrated that differences in the human and mouse amino-terminal leader peptides of OTC caused low activity of hOTC in *spf*^{ash} mice [25]. In the current study, we focused on generating an efficient clinical candidate AAV vector for OTC gene therapy in humans. We performed detailed evaluations of the kinetics, stability, and efficacy of the AAV vector in *spf*^{ash} mice.

2. Materials and methods

2.1. Codon optimization, vector construction and production

The initial codon optimization of human OTC cDNA was performed by GenScript using proprietary OptimumGene™ codon optimization technology (GenScript, Piscataway, NJ). The DNA sequences were examined and further modified to eliminate potential alternative reading frames from internal out of frame ATG with the size equal to or greater than 9 peptides. The final codon-optimized human OTC cDNA (hOTCco) was synthesized by GenScript. pAAVsc.TBG.hOTCwt and pAAVsc.TBG.hOTCco were constructed by replacing the mOTC coding sequencing with wild-type (WT) hOTC (hOTCwt) or hOTCco cDNA, respectively, in a plasmid derived from the previously described pAAVsc.TBG.mOTC1.3 with the intron disrupted [19–21]. The two vector preps (AAV2/8sc.TBG.hOTCwt and AAV2/8sc.TBG.hOTCco) used in the initial comparison study (described in Fig. 1) were purified by two rounds of cesium chloride gradient centrifugation, as previously described [17]. Vectors used in the rest of the study were produced by a scaled production method based on polyethylenimine (PEI) transfection and purified from supernatant or total lysate by iodixanol gradient centrifugation as described [26]. Genome titers [genome copies (GC)/ml] of AAV vectors were determined by real-time PCR using primer and probe sets targeting the TBG promoter (forward primer 5′-AAACTGCCAATTCCACTGCTG-3′, reverse primer 5′-CCATAGGCAAAAGCACCAAGA-3′, probe 6FAM–TTGGCCCAATAGTGAGAACTTTTTCCTGC–TAMRA), and using a linearized plasmid as the standard. The forward primer is located 400 bp downstream of the 5′ closed hairpin. Fagone et al. recently reported that the quantitative PCR (Q-PCR) method could significantly underestimate the titer of scAAV vectors, especially when the PCR primers were close to the closed hairpin of the scAAV vector [27]. We remeasured the titer of one lot of AAV2/8sc.TBG.hOTCco vector using a primer and probe set targeting the polyA region (1900 bp downstream of the 5′ closed hairpin), and the genome titer was 1.1-fold of the original titer, which was within the intra-assay error of Q-PCR.

2.2. Animal studies

All animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania. *Spf*^{ash} mice were maintained at the Animal Facility of the Translational Research Laboratories at the University of Pennsylvania as described previously [19]. Three to six months old *spf*^{ash} mice and their normal littermates were used in the studies. Vectors were administered by intravenous (i.v.) injection via the tail vein.

2.3. Measurement of urinary orotic acid

Urine samples were collected before and at various time points after vector treatment for orotic acid analysis as previously described [19].

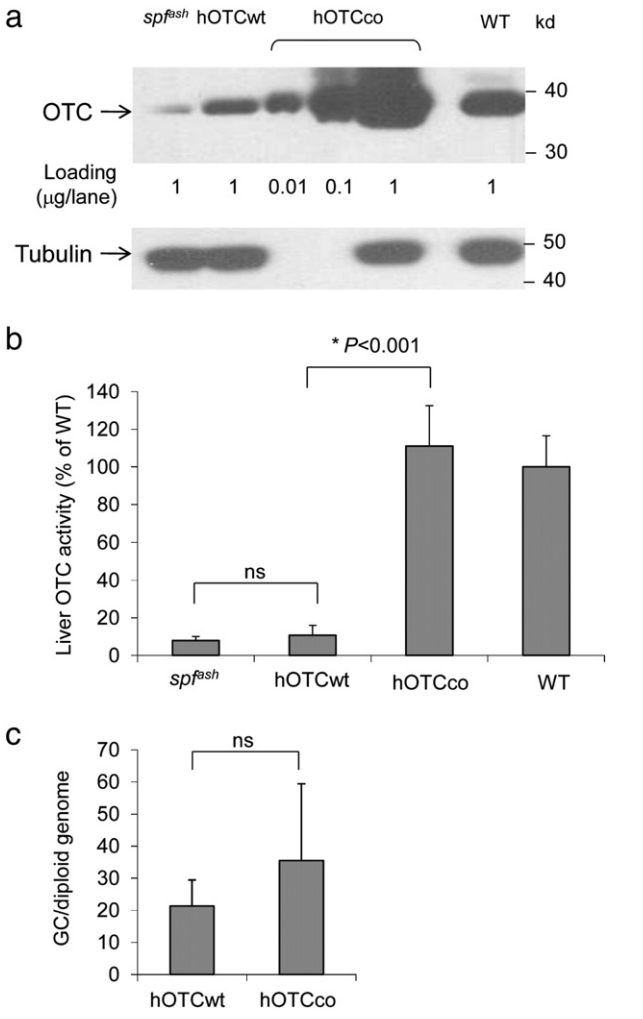

Fig. 1. Dramatic improvement of OTC expression levels and activity in liver by codon optimization of the hOTC gene. Adult male *spf*^{ash} mice were injected intravenously with 1×10^{11} GC of AAV2/8sc.TBG.hOTCwt or AAV2/8sc.TBG.hOTCco vectors. Fourteen days after vector treatment, liver was harvested. (a) Western analysis to detect OTC protein in the liver lysates from untreated and vector-treated *spf*^{ash} mice, and WT mouse. (b) Liver OTC activity following vector treatment. OTC activity levels are presented as percentages of the mean level in WT mice (n = 5; Mean ± S.D.). The OTC activities in mice treated with AAV2/8sc.TBG.hOTCwt (hOTCwt) were not statistically different (ns) from untreated *spf*^{ash} mice. (c) Vector genome copies in liver.*$P<0.001$, student *t* test.

2.4. OTC enzyme activity assay

OTC enzyme activity was measured using a liquid chromatography mass spectrometry stable isotope dilution method to detect the formation of citrulline normalized to [1,2,3,4,5-^{13}C5] citrulline (98% ^{13}C). The method is adapted from a previously developed assay for detection of N-acetylglutamate synthase activity [28]. Slivers of fresh frozen liver were weighed and briefly homogenized in buffer containing 10 mM HEPES, 0.5% Triton X-100, 2.0 mM EDTA and 0.5 mM DTT. Volume of homogenization buffer was adjusted to obtain 50 mg/ml tissue. Enzyme activity was measured using 250 μg liver tissue in 50 mM Tris-acetate, 4 mM ornithine, 5 mM carbamyl phosphate, pH 8.3. Enzyme activity was initiated with the addition of freshly prepared 50 mM carbamyl phosphate dissolved in 50 mM Tris-acetate pH 8.3, allowed to proceed for 5 min at 25 °C and quenched by addition of an equal volume of 5 mM ^{13}C5-citrulline in 30%TCA. Debris was separated by 5 min of microcentrifugation, and the supernatants were transferred to vials for mass spectroscopy. Ten microliter of sample was injected into an Agilent 1100 series LC–MS under isocratic conditions with a mobile phase of 93% solvent A (1 ml trifluoroacetic acid in 1 L water):7%