Review

Adeno-associated virus vector-based gene therapies for pediatric diseases

Kazuhiro Muramatsu\textsuperscript{1,2} and Shin-ichi Muramatsu\textsuperscript{2,3,*}

\textsuperscript{1} Department of Pediatrics, Jichi Medical University

\textsuperscript{2} Division of Neurological Gene Therapy, Center for Open Innovation, Jichi Medical University.

\textsuperscript{3} Center for Gene & Cell Therapy, The Institute of Medical Science, The University of Tokyo.

*Correspondence to: Shin-ichi Muramatsu, M.D., Ph.D,
Division of Neurological Gene Therapy, Jichi Medical University,
3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan.
Tel: +81-285-58-7543, E-mail: muramats@jichi.ac.jp

Conflict of interest statement

S.M. owns equity in a gene therapy company (Gene Therapy Research Institution) that commercializes the use of AAV vectors for gene therapy applications. Since the work in this manuscript may increase the value of these commercial holdings, S.M. has a potential conflict of interest.
Abstract

Gene therapy using adeno-associated virus (AAV) is a rapidly developing technology with widespread treatment potential. AAV2 vectors injected directly into the brain by stereotaxic brain surgery have shown good results in treating aromatic L-amino acid decarboxylase deficiency. Moreover, gene therapy using the AAV9 vector, which crosses the blood-brain barrier, has been performed in more than 2,000 patients worldwide as a disease-modifying therapy for spinal muscular atrophy. AAV vectors have been applied to the development of gene therapies for various pediatric diseases. Gene therapy trials for hemophilia and ornithine transcarbamylase deficiency are underway. Clinical trials are planned for glucose transporter I deficiency, Niemann-Pick disease type C, and spinocerebellar ataxia type 1. The genome of AAV vectors is located in the episome and is rarely integrated into chromosomes, making the vectors safe. However, serious adverse events such as hepatic failure and thrombotic microangiopathy have been reported, and ongoing studies are focusing on developing more efficient vectors to reduce required dosages.

Keywords: AAV vector, adeno-associated virus, gene therapy, pediatric disease

Running title: AAV vector gene therapies for pediatric diseases
1. Gene therapy advances in clinical applications

The concept of gene therapy in which nucleic acids are introduced into cells to restore their functions was proposed over 50 years ago.¹ Therapeutic gene can be delivered directly to the brain, liver, and muscles (in vivo) or to hematopoietic stem cells (HSCs) cultured outside the body and then returned to the body (ex vivo). In both cases, the virus is modified and used as a vector (carrier) to efficiently introduce the gene into the cells.² In in vivo gene therapy, adeno-associated virus (AAV)-derived vectors are primarily used,² and lentiviral vectors have been applied in ex vivo gene therapy.³ Developments in ex vivo gene therapies to manage neurological diseases include cell products for metachromatic leukodystrophy and adrenoleukodystrophy in which the causative genes, ARSA and ABCD1, respectively, were transduced into HSCs by lentiviral vectors, which has been approved by the European Medicines Agency.⁴ In these cases, microglia differentiated from the transduced HSCs are expected to enter the brain and exhibit enzymatic activity. However, it is not sufficient to control the long-term exacerbation of neurological symptoms, and the development of more potent gene therapy is desired. In this review, we describe the status of current research on in vivo gene therapies in children using AAV vectors including our work. (Figure 1)

2. AAV vector

AAVs are classified as belonging to the Paroviridae family and have a single-stranded DNA
genome of 4.7 kb in a 25 nm capsid. A large number of genotypes have been isolated, including AAV9, which crosses the blood-brain barrier. The genomes of AAV vectors that have entered cells are located in the nuclear episome and are rarely integrated into chromosomes, thus ensuring the safety of AAV vectors. In non-dividing cells such as neurons, the transduced genes are expressed for long periods without being lost. In fact, in a monkey model of Parkinson's disease, the enzyme gene introduced into the putamen was expressed for more than 15 years.

In vector construction, inverted terminal repeats (ITRs) are left at both ends of the genome, and the target therapeutic gene is inserted between the ITRs together with a promoter and a poly(A) sequence. In 2005, brain tissues of mice injected with type 8 AAV (AAV8) vectors via the tail vein showed that marker genes were expressed in neurons of the cerebral cortex, striatum, hippocampus, and cerebellum, indicating that the AAV8 vector penetrates the blood-brain barrier. Subsequently, it was reported that intravenous administration of type 9 AAV (AAV9) vector to newborn mice resulted in highly efficient gene transfer to neurons in the spinal cord, and AAV9 became a target of interest as a gene transfer vector that can penetrate the blood-brain barrier. Then after, it was shown that intravenous administration of the AAV9 vector can transduce neurons in a wide range of regions in adult animals, including mice, cats, pigs, and monkeys, and the application of gene therapy to neurological diseases has rapidly progressed. Currently, research is underway to develop more efficient AAV vectors by artificially modifying capsid proteins.
3. Aromatic L-amino acid decarboxylase deficiency

Gene therapy for aromatic L-amino acid decarboxylase (AADC) deficiency is a representative example of successful gene therapy. In this therapy, AAV vectors expressing the AADC gene were injected into the putamen by stereotaxic brain surgery. To date, this therapy has been performed in 30 patients in Taiwan, 10 patients in Japan, and 3 patients in Europe. A clinical trial in which AAV vectors are injected into the midbrain instead of the putamen has been started in the United States and Europe. In the midbrain approach, AAV vectors transduce neurons in the ventral tegmental area as well as the pars compacta of the substantia nigra. The AAV vectors, however, also transduce non-dopaminergic neurons that project to various brain areas. In addition, there is a risk of lethal hemorrhage following midbrain infusion. The method of introducing the AADC gene into the putamen was originally developed as gene therapy for Parkinson's disease. For Parkinson's disease, a method to simultaneously express genes for two enzymes necessary for L-dopa synthesis in addition to AADC has also been developed.

4. Spinal muscular atrophy

Spinal muscular atrophy (SMA) is caused by loss-of-function variants in the survival motor neuron 1 (SMN1) gene, resulting in degeneration and loss of spinal motor neurons (spinal cord anterior horn cells). In neonatal and infantile type I SMA, 90% of patients die before the age of
2 years unless ventilatory management is provided. Intravenous administration of an AAV9 vector expressing the SMN1 gene has demonstrated remarkable efficacy, including promoting independent walking. Antisense oligonucleotides that increase the expression of functional SMN protein by targeting the mRNA of the SMN2 gene, which differs by five nucleotides from SMN1, were previously approved as the first disease-modifying drug for this disease. However, because this drug intervention requires repeated intrathecal administration, gene therapy that can be effective with a single administration is becoming more popular. Despite the high cost of AAV9 (Onasemnogene abeparvovec) at approximately 2 million US dollars per patient, more than 2,000 patients have been treated worldwide by August 2022. The efficacy of Onasemnogene abeparvovec has been reported in patients who have not yet developed the disease. Therefore, extended newborn screening is important to initiate treatment at an early stage.

5. Lysosomal disease

Diseases caused by single enzyme deficiency have long been considered good candidates for gene therapy. Clinical trials using AAV vectors are underway mainly in the United States for mucopolysaccharidosis types I, II, IIIA, and IIIB, as well as for ceroid lipofuscinosis types 2, 3, and 6. In the case of mucopolysaccharidosis type I and II, genome editing has also been attempted and clinical trials have been initiated. Because hematopoietic stem cell
transplantation is a primary therapeutic option for mucopolysaccharidosis, *ex vivo* gene therapy using lentivirus is also under development.\textsuperscript{25,26} In Niemann-Pick type C (NPC1), which is caused by a genetic variant of lysosomal lipid transport protein, the therapeutic effects of various AAV vectors and promoters using animal models have been investigated. We showed the therapeutic effects on systemic organs such as the heart and liver as well as the central nervous system when the AAV vector expressing *NPC1* was injected into the lateral ventricle and cisterna magna\textsuperscript{27}. Based on the beneficial results, we are planning a clinical trial for NPC1.

6. Duchenne muscular dystrophy

Progressive muscular dystrophy caused by the deficiency of dystrophin protein has been difficult to treat with gene therapy using AAV vectors because of the large size of the gene. To address this limitation, micro-dystrophin and mini-dystrophin have been developed and are now used in clinical applications. A clinical trial using AAVrh74 (SRP-9001), in which micro-dystrophin is expressed by the skeletal and cardiac muscle-specific MHCK7 promoter, is underway in the United States.\textsuperscript{28} Following a single dose of $2.0 \times 10^{14}$ vector genome (vg)/kg AAV vector was administered via a vein in the lower limb, creatine kinase levels were reduced. Extensive expression and proper localization of micro-dystrophin were also confirmed after a 3-year follow-up. However, one death due to thrombotic microangiopathy was reported in a clinical trial in which a micro-dystrophin-expressing AAV9 vector (SGT-001) was administered.
at $2.0 \times 10^{14}$ vg/kg. In a trial of the AAV9 vector (PF-06939926) carrying mini-dystrophin, one death was reported at a dose of $2.0 \times 10^{14}$ vg/kg.

7. X-linked myotubular myopathy

X-linked myotubular myopathy (XLMTM) is an X-linked muscle disease characterized by hypotonia of the whole body including the facial muscles, respiratory distress, feeding difficulties, and intellectual disability from the neonatal period. In a clinical trial using the AAV8 vector (AT132) carrying human MTM1, good results were initially obtained at $1.0 \times 10^{14}$ vg/kg, including recovery of muscle strength, but three patients died at a higher dose of $3.0 \times 10^{14}$ vg/kg. Subsequently, one death occurred even at the lower dose of $1.0 \times 10^{14}$ vg/kg, and the study was discontinued. XLMTM is associated with severe hepatic injury such as hepatic peliosis, and death is often caused by bleeding from the liver. It is possible that the high dose of AAV may have caused injury to the liver, which was originally fragile, but detailed analysis results are not yet available.

8. Ornithine transcarbamylase deficiency

Many congenital metabolic disorders are caused by the dysfunction of liver enzymes. One of the most common disorders that lead to central nervous system disorders is ornithine transcarbamylase (OTC) deficiency, a loss of enzyme activity in the urea cycle. OTC deficiency is an X-linked disorder that primarily occurs in males and occasionally occurs in female carriers.
of the disease. The onset of feeding in the neonatal period causes fulminant hyperammonemia leading to central nervous system damage and requires continuous hemodialysis. Currently, the only curative treatment is living donor liver transplantation, but gene therapy is expected to be a potential alternative to this highly invasive treatment. Clinical trials using the AAV8 vector have been initiated mainly for adults (in some cases, patients over 12 years of age) (Table 1).32 We have developed an AAV vector that transduces human hepatocytes efficiently to facilitate treatment at an earlier stage.33

9. Hemophilia

Hemophilia is an inherited bleeding disorder caused by a genetic variant of blood coagulation factor VIII (hemophilia A) or factor IX (hemophilia B). Protein preparations of coagulation factors are used to treat hemorrhage, but their short half-life and frequent administration are problematic. Therefore, gene therapy using AAV vectors has been developed. (Table 1) In the beginning, AAV2 vectors were injected into the muscle, but they did not show sufficient efficacy. However, a gene therapy targeting the liver was developed, and a single administration of the AAV2 vector resulted in the long-term maintenance of coagulation factors in the blood and eliminated the need for administering coagulation factor preparations.34, 35 Furthermore, a method of inserting therapeutic genes into the target genome sequence via genome editing has
been developed to maintain the therapeutic effect even when hepatocytes divide and proliferate.\textsuperscript{36}

\section*{10. Spinocerebellar ataxia}

In Spinocerebellar ataxia type 1, levels of high molecular group box 1 (HMGB1), a DNA structural regulator protein, are decreased in neurons, leading to cerebellar ataxia and neuronal cell death via impaired DNA repair and transcription. We are planning a clinical trial in which an AAV vector expressing HMGB1 is injected into the cerebellum.\textsuperscript{37} In addition, in Spinocerebellar ataxia type 6, the CAG repeats in the coding region of the transcription factor alpha1ACT in the calcium voltage-gated channel subunit alpha1 A gene (CACNA1A) is abnormally elongated and disrupts neuronal cell function. We demonstrated that AAV vectors expressing miR-3191-5p selectively inhibited alpha1ACT mRNA translation and rescued from the degeneration of Purkinje cells in a mouse model.\textsuperscript{38}

\section*{11. Epilepsy}

In the absence of a clear link to a single gene variant, several methods have been investigated for effectively managing epilepsy, including induction of inhibitory synapses, suppression of neuronal hyperexcitability by gene transfer of inhibitory neurotransmitters, and repair of tissues by gene transfer of neurotrophic factors.\textsuperscript{39} Although AAV vectors can be delivered to neurons
in broad areas of the brain by intrathecal or intravascular administration,\textsuperscript{40} stereotaxic neurosurgery to inject the vectors around the epileptic focus would be a shortcut to clinical application.

11.1. Dravet syndrome

Dravet syndrome is caused by a dysfunction of voltage-gated sodium channel 1.1 (NaV 1.1). The SNCNIA gene encoding NaV 1.1 is approximately 6 kb in length and cannot be inserted into AAV vectors. Therefore, the gene for a short voltage-gated sodium channel accessory subunit (NaV β1) was loaded into an AAV vector and injected into the Scn1a\textsuperscript{+/-} mice, resulting in prolonged survival and reduced seizure frequency.\textsuperscript{41} In addition, in Scn1a\textsuperscript{+/-} mice, researchers have attempted to introduce dCAS9 bound to VP16, a transcription activator, together with a guide RNA using an AAV vector and initiate binding to the promoter region of NaV1.1α to enhance transcription.\textsuperscript{42, 43}

11.2. Glucose transporter 1 deficiency

In Glucose transporter 1 deficiency (GLUT1D), variants in the SLC2A1 gene impair glucose transport to brain tissues, resulting in epileptic seizures. Because patients with GLUT1D have intractable epilepsy and are forced to follow a high-fat, low-carbohydrate, low-protein diet for the rest of their lives, it is hoped that gene therapy will provide curative treatment. We have demonstrated that intrathecal administration of AAV vectors that express the SLC2A1 gene under the SLC2A1 core promoter transduced vascular endothelial cells and neurons in broad
areas of the pig brain.\textsuperscript{44}

\textbf{12. Wilson’s disease}

Wilson’s disease is an inherited disorder of copper metabolism associated with variants in $ATP7B$ gene. Gene therapy based on AAV vectors has been developed for Wilson's disease using mouse models.\textsuperscript{45,46} Addressing the relatively large size of $ATP7B$ cDNA, a dual AAV vector approach using split intein technology or mini-$ATP7B$, with four out of six metal binding domains deleted, was designed.\textsuperscript{47,48} Two phase I/II AAV-based clinical trials are currently ongoing (NCT04537377, NCT04884815).

\textbf{13. Adverse events caused by AAV vectors}

In the AAV9-based vector, Onasemnogene abeparvovec, for spinal muscular atrophy, even when prednisolone is administered prophylactically at 1 mg/kg, liver function tests (ALT, AST, bilirubin) are increased in many cases.\textsuperscript{49} In addition, severe thrombotic microangiopathy has been reported, although it is rare. The pathogenesis of these adverse events has not been precisely elucidated. An immune response to the capsid protein of AAV has been postulated. The dosage of onasemnogene abeparvovec is $1.1 \times 10^{14}$ vg/kg for patients under 2 years of age, and most patients weigh less than 10 kg. In the trials conducted in older children with Duchenne muscular dystrophy and XLMTM, the vector dose may be excessive. When the viral capsid load reaches a certain threshold, proliferation of capsid-specific activated T cells may cause
Intrathecal administration of AAV vectors can cause a neuronal loss in the dorsal root ganglia due to strong gene expression. In fact, a case of temporary sensory disturbance has been reported after gene therapy for amyotrophic lateral sclerosis. However, in most cases, it is presumed to be clinically unproblematic.

Most of the DNA introduced by AAV vectors is localized to episomes and is very unlikely to be incorporated into chromosomes. The increased risk of hepatocellular carcinoma reported in animals has not been demonstrated in humans. However, long-term follow-up is required, especially in children.

Despite these challenges, gene therapy with AAV vectors is expected to be clinically applicable as a condition-modifying approach to treating many pediatric diseases for which no effective treatment is available.

References

neurometabolic disorders (metachromatic leucodystrophy and adrenoleucodystrophy).

5. Wang D, Tai PWL, Gao G. Adeno-associated virus vector as a platform for gene therapy

of Dopamine-Synthesizing Enzymes 15 Years After Gene Transfer in a Primate Model of

7. Nakai H, Fuess S, Storm TA, Muramatsu S, Nara Y, Kay MA. Unrestricted hepatocyte

AAV9 preferentially targets neonatal neurons and adult astrocytes. *Nat Biotechnol*


and safety of eladocagene exuparvovec in patients with AADC deficiency. *Mol Ther*
2022;30:509–18.

improves motor and mental function of aromatic L-amino acid decarboxylase deficiency. 

*Brain* 2019;**142**:322–33.


325 6.
of mitochondrial DNA damage and extends the lifespan of mutant ataxin-1 knock-in mice. 


43. Yamagata T, Raveau M, Kobayashi K, Miyamoto H, Tatsukawa T, Ogiwara I, et al. CRISPR/dCas9-based Scn1a gene activation in inhibitory neurons ameliorates epileptic


**Figure 1 legend**

Various routes of administration in AAV vector-based gene therapy.

AADC, aromatic L-amino acid decarboxylase; CSF, cerebrospinal fluid; GLUT1, glucose transporter type 1; MPS, mucopolysaccharidosis; NCL, neuronal ceroid lipofuscinosis; OTC,
ornithine transcarbamylase; SCA, spinocerebellar ataxia; SMA, spinal muscular atrophy;

XLMTM, X-linked myotubular myopathy
<table>
<thead>
<tr>
<th>Disease</th>
<th>NCT number</th>
<th>Study Description</th>
<th>AAV</th>
<th>Vector Dose (vg/kg)</th>
<th>Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>05345171</td>
<td>DTX301</td>
<td>scAAV8OTC</td>
<td>n/a</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>05092685</td>
<td>HORACE</td>
<td>AAVLV03hOTC</td>
<td>6×10e11 (low), 2×10e12 (intermediate)</td>
<td>I/II</td>
</tr>
<tr>
<td>OTC Deficiency</td>
<td>04909346</td>
<td>OTC Deficiency, GSDIa, Wilson Disease</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>02991144</td>
<td>DTX301</td>
<td>scAAV8OTC</td>
<td>6×10e12 (high), 2×10e11 (low)</td>
<td>I/II</td>
</tr>
<tr>
<td></td>
<td>02576795</td>
<td>BMN-270-201</td>
<td>AAVS3-FIXco- Padua</td>
<td>6×10e11, 2×10e13, 6×10e13</td>
<td>I/II</td>
</tr>
<tr>
<td></td>
<td>03392974</td>
<td>BMN-270-302</td>
<td>AAV2/6- FVIII-BDD</td>
<td>4×10e13</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>03370913</td>
<td>BMN-270-301</td>
<td>AAVS3-FIXco- Padua</td>
<td>6×10e13</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>03520712</td>
<td>BMN-270-203</td>
<td>AAV8-FIXco-wt</td>
<td>6×10e13</td>
<td>I/II</td>
</tr>
<tr>
<td></td>
<td>03003533</td>
<td>SPK-8011-101</td>
<td>AAV8-FIXco-Padua</td>
<td>n/a</td>
<td>I/II</td>
</tr>
<tr>
<td>Hemophilia A</td>
<td>03432520</td>
<td>SPK-8011-LTFU</td>
<td>AAV-SPARK200-FVIII-BDD</td>
<td>5×10e11, 1×10e12, 2×10e12</td>
<td>I/II</td>
</tr>
<tr>
<td></td>
<td>03734588</td>
<td>SPK-8016-101</td>
<td>AAV2/HLP-FVIIIv3</td>
<td>n/a</td>
<td>I/II</td>
</tr>
<tr>
<td></td>
<td>03001830</td>
<td>GO-B</td>
<td>AAV2/6-FVIII-BDD</td>
<td>6×10e11, 2×10e12, 6×10e12</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>03061201</td>
<td>SB-525-1603</td>
<td>AAV2/6-FVIII-BDD</td>
<td>9×10e11, 2×10e12, 1×10e13, 3×10e13</td>
<td>I/II</td>
</tr>
<tr>
<td></td>
<td>03370172</td>
<td>BAX-888</td>
<td>AAV8-FIXco-BDD</td>
<td>n/a</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>01899299</td>
<td>BAY2599023(DTX201)</td>
<td>AAVhu37</td>
<td>n/a</td>
<td>I/II</td>
</tr>
<tr>
<td>Hemophilia B</td>
<td>00979238</td>
<td>n/a</td>
<td>scAAV2/8-LP1-hFIXco</td>
<td>2×10e11, 6×10e11, 2×10e12</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>01687608</td>
<td>AskiiBio009</td>
<td>AAV8-sc-TTR-FIXco-Padua</td>
<td>2×10e11, 1×10e12, 3×10e12</td>
<td>I/II</td>
</tr>
<tr>
<td></td>
<td>02396342</td>
<td>AMT-060-01</td>
<td>AAV5-FIXco-wt</td>
<td>5×10e12, 2×10e13</td>
<td>I/II</td>
</tr>
<tr>
<td></td>
<td>03489291</td>
<td>AMT-061-01</td>
<td>AAV5-FIXco-Padua</td>
<td>2×10e13</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>03569891</td>
<td>AMT-061-02</td>
<td>AAV5-FIXco-Padua</td>
<td>2×10e13</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>02484092</td>
<td>SPK-9001-101</td>
<td>AAV5-FIXco-Padua</td>
<td>5×10e11</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>03307980</td>
<td>SPK-9001-LTFU</td>
<td>AAV5-FIXco-Padua</td>
<td>5×10e11</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>03861273</td>
<td>BENEGENE-2</td>
<td>AAV Spark100 FIX Padua</td>
<td>n/a</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>03649444</td>
<td>FLT-180a</td>
<td>FLT180a</td>
<td>6×10e11, 2×10e12</td>
<td>I/II</td>
</tr>
</tbody>
</table>

Co, codon-optimized; FVIII-BDD, B-domain deleted FVIII; n/a, not applicable; sc, self-complementary; vg, vector genomes
• AADC deficiency
• MPS IIIA
• NCL 2
• Epilepsy

Stereotaxic surgery

• SMA
• Hemophilia A, B
• OTC deficiency
• MPS II, IIIA, IIIB, VI
• Muscular dystrophy
• XLMTM
• Wilson’s disease

Intra-venous injection

• SCA
• MPS I, II
• NCL 3, 6
• GLUT1 deficiency
• Niemann-Pick C

Intra-CSF infusion