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Adeno-associated virus vectors for human gene therapy

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Abstract

Adeno-associated virus (AAV) is a small, non-enveloped virus that contains a single-stranded DNA genome. It was the first gene therapy drug approved in the Western world in November 2012 to treat patients with lipoprotein lipase deficiency. AAV made history and put human gene therapy in the forefront again. More than four decades of research on AAV vector biology and human gene therapy has generated a huge amount of valuable information. Over 100 AAV serotypes

and variants have been isolated and at least partially characterized. A number of them have been used for preclinical studies in a variety of animal models. Several AAV vector production platforms, especially the baculovirus-based system have been established for commercial-scale AAV vector production. AAV purification technologies such as density gradient centrifugation, column chromatography, or a combination, have been well developed. More than 117 clinical trials have been conducted with AAV vectors. Although there are still challenges down the road, such as cross-species variation in vector tissue tropism and gene transfer efficiency, pre-existing humoral immunity to AAV capsids and vector dose-dependent toxicity in patients, the gene therapy community is forging ahead with cautious optimism. In this review I will focus on the properties and applications of commonly used AAV serotypes and variants, and the technologies for AAV vector production and purification. I will also discuss the advancement of several promising gene therapy clinical trials.

Key words: Adeno-associated virus; Adeno-associated virus production and purification; Clinical trials; Gene therapy; Baculovirus

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Core tip: Adeno-associated virus (AAV) has become the first gene therapy drug approved by the Western world and spurred huge excitement in the gene therapy field. The gene therapy community is forging ahead with cautious optimism despite some challenges down the road. A battery of more than 100 AAV serotypes and variants are available and AAV production and purification technologies have become well established. Several clinical trials with AAV vectors have yielded exciting results. This paper will give you the information needed to understand the current development of the gene therapy field with AAV vectors.

Chen H. Adeno-associated virus vectors for human gene therapy. *World J Med Genet* 2015; 5(3): 28-45 Available from: URL: <http://www.wjgnet.com/2220-3184/full/v5/i3/28.htm> DOI: <http://dx.doi.org/10.5496/wjmg.v5.i3.28>

INTRODUCTION

With the approval of first adeno-associated virus (AAV)-based gene therapy drug, Glybera, to treat lipoprotein lipase deficiency (LPLD) by the European Union on November 2, 2012^[1,2], human gene therapy entered a new era. It has been a long march from the first discovery of the AAV in the 1960's, to the final approval of the first AAV-based gene therapy drug. The once-abandoned gene therapy field has now become a hotbed, with 11 different companies raising at least \$618 million from venture capitalists and equity markets since the beginning of 2013. Top venture capital firms are among their backers, and some of the industry's top talent is being attracted to what was once seen as a lost cause. The iShares Nasdaq Biotechnology Index is up 65% in 12 mo^[3]. Basic research on AAV biology, vectorology, and gene therapy, since the first discovery of AAV, has generated much valuable information. More than 100 AAV serotypes and variants have been isolated and characterized. Some of them have been used for preclinical studies in a variety of animal models. Several AAV vector production technologies, especially the baculovirus-based technology have been established for commercial scale AAV vector production. AAV purification methods with density gradient centrifugation, column chromatography, or a combination of both, have been well developed towards commercialization. More than 117 clinical trials have been conducted with AAV vectors and yielded a vast amount of valuable information regarding the safety, efficacy, dosage, toxicity, immune response, biodistribution, and tropism of a few key AAV vectors, such as AAV2, and some phase I / II clinical trials have yielded promising data. The gene therapy community is forging ahead with cautious optimism, although there are still challenges down the road, such as cross-species variation in vector tissue tropism and gene transfer efficiency, pre-existing humoral immunity to AAV capsids and vector dose-dependent toxicity in patients. In this review I will focus on the properties and applications of several commonly used AAV serotypes and variants, and the technologies for AAV vector production and purification. I will also discuss the advancement of several promising gene therapy clinical trials utilizing AAV vectors.

AAV SEROTYPES AND VARIANTS

AAV is a single-stranded DNA virus of the *Dependovirus* genus of the *parvovirus* family with a genome size of about 5000 nucleotides. Its shell is about 25 nm in

diameter, and is composed of 60 viral protein subunits arranged on a T = 1 icosahedral lattice^[4]. The AAV genome encodes three open reading frames (ORFs), rep, cap, and AAP, flanked with inverted terminal repeats (ITRs) (Figure 1). AAV enters host cells *via* specific receptors on the cell surface. Once inside the cell, AAV uncoats and releases its genome, which is transported into the nucleus. The AAV genome integrates into the host chromosome 19 AAVS1 site^[5,6] when no helper virus is present, or it replicates to produce progeny when a helper virus, such as adenovirus or herpes virus, is present. To make an AAV vector, the rep and cap sequences are removed and replaced with an expression cassette containing the target gene. When the AAV vector containing the target gene, together with the rep and cap sequences provided *in trans*, and herpes simplex virus (adenovirus, HSV, or baculovirus, etc.) are introduced into host cells under proper conditions, AAV vectors will be produced (Figure 2). Since the discovery of AAV in the 1960's, now there have been over 100 AAV serotypes and variants isolated from adenovirus stocks or from human/nonhuman primates tissues and even some other mammals^[7-11]. With their diverse tissue tropism, transduction efficiency and immunological profiles, these AAV vectors can be used to target various tissues for a variety of applications. The properties and applications of the commonly used AAV serotypes and variants are summarized in Table 1.

AAV1

AAV1 has a genome size of 4718 nucleotides and exhibits high homology with those of other AAV serotypes. It appears that AAV6 was actually generated through homologous recombination between AAV1 and AAV2. Studies show that sera from nonhuman primates with neutralizing antibodies (NAB) against AAV1 are more common than those from humans, whereas sera from humans with NAB to AAV2 are more common than those from nonhuman primates. AAV1 was more efficient for muscle^[12], whereas AAV2 transduced liver more efficiently. High titers of NAB were detected for each vector administered to murine skeletal muscle, which prevented re-administering the same serotype but did not substantially cross-neutralize the other serotype. In the context of liver-directed gene transfer, similar results were observed except for a significant, though incomplete, neutralization of AAV1 from a previous treatment with AAV2^[13]. Point mutations on the AAV1 capsid (S663A, S669A, and K137R) increased its transduction efficiency both *in vitro* and *in vivo* up to 6-fold^[14]. Swapping the amino acids of AAV2 VP1 from 350 to 736 with the corresponding VP1 region of AAV1 gave rise to a hybrid vector that exhibited very similar properties to AAV1 in muscle both *in vitro* and *in vivo*. Analyses of smaller regions of the AAV1 VP1 amino acid sequence corresponding to the AAV2 capsid protein from additional mutants indicated that a small region of VP1 amino acids (from 350 to 430) functions as a major determinant of tissue tropism.

Table 1 Properties and applications of the commonly used adeno-associated virus serotypes and variants

AAV serotypes and variants	Target tissues	Preclinical applications	Clinical applications	Ref.
AAV1	Muscle, heart	Charcot-Marie-Tooth Neuropathy, congestive heart failure, Duchenne muscular dystrophy	Glybera, a drug for lipoprotein lipase deficiency, congenital heart failure	[1,2,11,15-17,19-21]
AAV2	Liver, eye	In vitro assays, various animal model studies	Cystic fibrosis; hemophilia B, Leber's congenital amaurosis, Parkinson's disease; Canavan disease	[11,26-36]
AAV3A, 3B	Liver cancer	Liver cancer		[40]
AAV4	Ependyma, astrocyte, retinal pigmented epithelium	Mucopolysaccharidosis type VII, Familial amyotrophic lateral sclerosis, RPE65-deficient vision loss	RPE65-deficient disease	[42,47]
AAV5	Sensor neuron, airway epithelia, Dendritic cells	Globoid cell leukodystrophy; human immunodeficiency	-	[49-52]
AAV6	Airway epithelia, skeletal muscle, Dendritic cells, pancreatic beta cells	Duchenne muscular dystrophy	-	[53-56]
AAV7	Skeletal muscle, liver, central nervous system	-	-	[11,58-60]
AAV8	Liver, skeletal and cardiac muscle	Hemophilia A, familial hypercholesterolemia, glycogen storage disease type II	Hemophilia B	[11,62-66,143]
AAV9	Cardiac muscle, central nervous system	Heart failure, central nervous system disorders	Spinal muscular atrophy	[70-75]
AAVShH10	Müller cells	Retinitis pigmentosa	-	[76,77]
AAV7m8	Vitreous humor	Retinoschisis, Leber's congenital amaurosis	-	[79,80]
AAVDJ	Liver, kidney, cervix, retina, ovary, skin, fibroblast, lung	-	-	[81]

AAV: Adeno-associated virus.

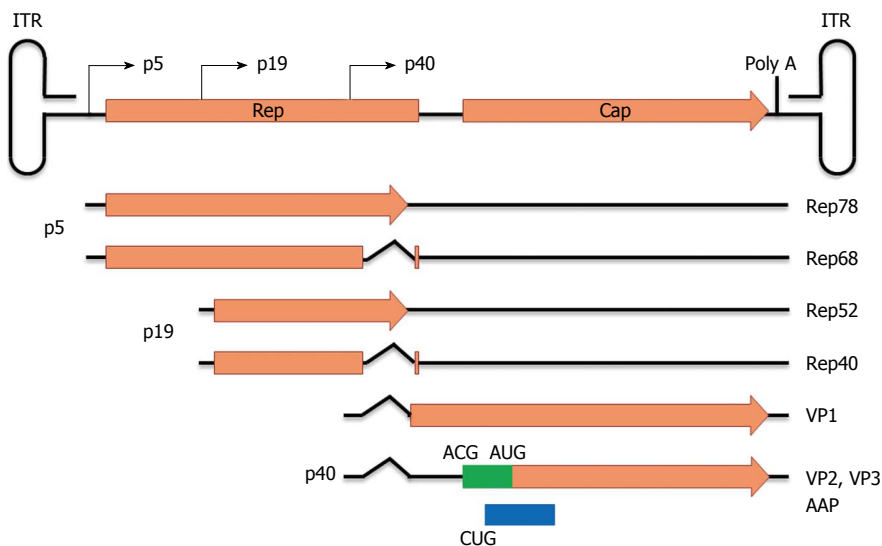


Figure 1 Diagram of adeno-associated virus genome depicting the inverted terminal repeats, promoters, polyadenylation sequence, and mRNAs coding for rep, cap, and assembly-activating protein proteins. The rep open reading frames codes for four rep proteins (Rep78, Rep68, Rep52, and Rep40) that are synthesized from mRNAs transcribed from the p5 and p19 promoters. Rep78 and Rep68 have site-specific endonuclease, DNA helicase, and ATPase activities that are required for adeno-associated virus (AAV) DNA replication^[148-150]. Rep52 and Rep40 contain helicase activity and are required for packaging AAV DNA into the capsids^[151]. VP1, VP2, and VP3 are synthesized from mRNA transcribed from the p40 promoter. To main a 1:1:10 ratio of VP1:VP2:VP3 for virus particle assembly, AAV uses an alternative splicing mechanism for VP1 and a less efficient start codon (ACG) for VP2 to lower their protein levels, yet keeps high efficiency start codon for VP3^[152]. The N-terminal sequence present in VP1 contains a phospholipase A2 domain that is required for AAV infectivity^[114,115,153]. In addition, the VP2/VP3 mRNA codes for an assembly-activating protein (AAP) from a weak CTG start codon but in a different reading frame^[154]. AAP facilitates nuclear import of the major VP3 capsid protein and promotes assembly and maturation of the capsid, but AAP is not present in the mature capsid. ITR: Inverted terminal repeat; Poly A: Polyadenylation.

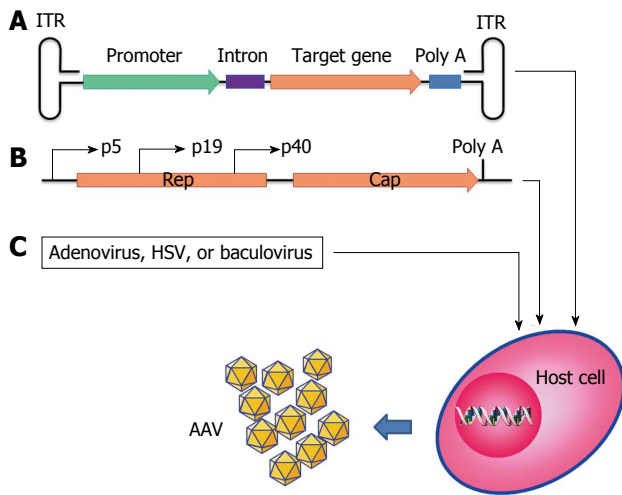


Figure 2 Diagram of adeno-associated virus vector production. A: The expression cassette containing promoter, intron, target gene, and polyadenylation sequence is flanked with adeno-associated virus (AAV) inverted terminal repeats (ITRs); B: AAV rep and cap sequences without ITRs are provided in trans; C: helper virus can be adenovirus, herpes simplex virus (HSV), or baculovirus depending on the production system used. Once these three components are introduced into a host cell under proper conditions, AAV vectors will be produced.

Additional analysis demonstrated that both the major antigenic determinants and the heparin binding domain in the AAV capsid region were not required for efficient transduction of muscle by AAV1^[15]. Due to its high efficiency in muscle transduction, AAV1 vectors have been used to study disease models such as Charcot-Marie-Tooth Neuropathy^[16], congestive heart failure^[17], Duchenne muscular dystrophy^[18], etc., and in clinical trials to treat congenital heart failure^[19-21]. The first gene therapy drug (Glybera) approved by the Western world to treat LPLD is based on AAV1^[1,2].

AAV2

AAV2 is the most thoroughly characterized serotype. It has a genome size of 4675 nucleotides and contains ITRs of 145 nucleotides, the first 125 nucleotides of which form a palindromic sequence^[22]. Nearly all serotypes of AAV vectors use the AAV2 ITRs for AAV manufacture^[23]. AAV2 requires heparan sulfate proteoglycan (HSPG) for cell attachment^[24]. Among all the AAV serotypes discovered, AAV2 has the best transduction efficiency in cell culture^[12] and therefore is the best tool for *in vitro* studies. The transduction efficiency of AAV2 vectors can be improved dramatically by point mutations (Y730F and Y444F) on the viral capsid^[25]. Even though stable transgene expression mainly results from extra-chromosomal vector genomes in the liver, a series of studies has shown that vector genomes integrate into host chromosomes of hepatocytes at a low frequency preferentially into genes that are expressed in the liver^[26].

All previous gene therapy studies in animal models and clinical trials were undertaken with AAV2 vectors. The earliest clinical trials with AAV2 vectors were for

monogenic disorders. The first trial involved airway delivery (nasal, endobronchial, sinus and aerosol inhalation) of AAV2 vectors carrying cystic fibrosis transmembrane conductance regulator (AAV2-*CFTR*) in cystic fibrosis patients with mild lung disease^[27]. The second set of trials involved intramuscular and hepatic delivery of AAV2 vectors carrying factor IX gene in patients with hemophilia B^[28]. AAV2 vectors have also been utilized in clinical trials for ocular diseases^[29-31], and diseases involved the central nervous system^[32-36].

AAV3

There are two subtypes of AAV3 and they were designated as AAV3A and AAV3B. They differ by only 16 nucleotides or 6 amino acids^[37,38]. AAV3 has a genome size of 4726 nucleotides and has an overall sequence homology of 82% with AAV2. At the amino acid level AAV3 has a homology of 88% with the nonstructural (rep) proteins and 87% with the capsid proteins of AAV2. The major differences between AAV3A and AAV2 are that AAV3A lacks a typical TATA-box sequence at p40 promoter but contains the consensus binding sequence within the upstream region of the p5 promoter for adenovirus-related transcription factor E4F. These results imply that AAV3 contains serologically distinct structural proteins and its viral propagation may be controlled at the transcription level by different gene regulatory elements^[37]. AAV3 requires HSPG for cell attachment^[39]. AAV3 vectors transduce human liver cancer cells extremely efficiently because they utilize human hepatocyte growth factor receptor (hHGFR) as a cellular co-receptor for viral entry and these cells express high levels of hHGFR. Both extracellular and intracellular kinase domains of hHGFR are required for AAV3 vector entry and AAV3-mediated transgene expression. The host cell proteasome machinery is responsible for AAV3 vector degradation and the transduction efficiency of AAV3 vectors is greatly improved with surface-exposed tyrosine (Y) to phenylalanine (F) mutations such as Y701F, Y705F or Y731F. AAV3 vectors with combined mutations such as Y705 + 731F show significant higher transduction efficiency than each of the single mutants in liver cancer cells *in vitro*. Direct intra-tumoral injection of AAV3 vectors in immune-deficient mouse xenograft models also result in high transduction efficiency of human liver tumor cells *in vivo*. The optimized AAV3 vectors carrying tyrosine-mutations result in increased efficiency of transduction following both intra-tumoral and tail-vein injections *in vivo* and AAV3 vectors carrying proapoptotic genes may be useful for gene therapy of human liver cancer^[40].

AAV4

AAV4 has a genome size of 4767 nucleotides in length and contains an expanded p5 promoter region compared to AAV2 and AAV3^[41]. The rep gene product of AAV4 shows greater than 90% homology to the rep products of AAV2 and AAV3, with none of the changes

occurring in regions that had previously been shown to affect the known functions of Rep68 or Rep78. Most of the differences in the capsid proteins were thought to be located on the outer surface of the virus capsid. AAV4 can transduce human, monkey, and rat cells. A series of experiments including comparison of transduction efficiencies in a number of cell lines, competition co-transduction, and the effect of trypsin on transduction efficiency all suggest that the cellular receptor for AAV4 is different from that of AAV2^[441].

AAV4 transduces ependyma with high efficiency when injected into the striata or lateral ventricles of adult mice^[42]. AAV4 also efficiently transduces type B astrocytes in the subventricular zone, and glia overlying the rostral migratory stream neural tube^[43]. AAV4 vectors harboring a beta-glucuronidase gene administered unilaterally into the lateral ventricle mediated global functional and pathological improvements in the mucopolysaccharidosis type VII murine model that was caused by beta-glucuronidase deficiency^[44]. AAV4 vectors carrying insulin-like growth factor-1 or vascular endothelial growth factor-165 genes delivered in the cellular components of the ventricular system including the ependymal cell layer, choroid plexus [the primary cerebrospinal fluid (CSF)-producing cells of the central nervous system (CNS)] and spinal cord central canal lead to trophic factor delivery throughout the CNS, delayed motor decline and a significant extension of survival in SOD1 (G93A) transgenic mice^[45]. AAV4 vectors containing RPE65 gene delivered by subretinal injection into RPE65^{-/-} purebred Briard dogs restore functional vision in the treated eye, with the untreated contralateral eye serving as an internal control^[46]. A phase I / II clinical trial was conducted to assess the safety and efficiency of one subretinal injection with AAV4.rpe65. hrep65 vectors in the worse eye of patients with rpe65^{-/-} retinal dystrophy^[47].

AAV5

AAV5 has a genome size of 4642 nucleotides and is different from other parvovirus serotypes according to serological and DNA hybridization data^[48]. Its DNA genome is similar to that of AAV2 in length and genetic organization. The AAV5 *rep* gene is 67% homologous to the *rep* gene of AAV2, with changes mainly occurring in the carboxyl and amino termini. The AAV5 ITRs are also different from the ITRs of other AAV serotypes. Though the Rep DNA binding site and the characteristic hairpin structure of AAV5 ITRs are retained, there is no consensus terminal resolution site. These differences in the ITR structures and the Rep proteins lead to the failure of cross-packaging between AAV2 and AAV5 as indicated by the inability to produce recombinant AAV particles. Analysis of alignment between the cap ORFs of AAV5 and other serotypes identifies both variable and conserved regions which could affect viral particle stability and tissue tropism. The failure of soluble heparin to inhibit AAV5 and the comparison of transduction efficiencies between AAV5 and AAV2 in a

variety of cells lines show that AAV5 may use a different mechanism of uptake from AAV2^[48].

A comparative *in-vivo* study with vectors based on AAV1, 2, 3, 4, 5, 6, and 8, and lentivirus (LV) indicates that AAV5 is the most efficient vector for transducing sensory neurons^[49]. Even though AAV1, AAV5, and AAV6 all showed the most transduction of neurons two weeks after injection into the dorsal root ganglia (DRG), the time course of GFP expression from these three vectors studied from 1 to 12 wk after injection indicates that overall AAV5 was the most effective serotype, followed by AAV1. These two serotypes exhibited increasing rates of neuronal transduction at later time points, leading to over 90% of DRG neurons GFP⁺ at 12 wk with some injections of AAV5^[49]. When delivered to the neocortex, hippocampus and cerebellum of twitcher mice, AAV5 carrying the galactocerebroside cDNA was effectively dispersed along the neuraxis of CNS as far as the lumbar spinal cord, and reduced the accumulation of psychosine in the CNS of *Twitcher* mice. Most importantly, the treated *Twitcher* mice were protected from loss of oligodendrocytes and Purkinje cells, axonopathy and marked gliosis, and had significantly improved neuromotor function and prolonged lifespan^[50].

When delivered at low multiplicity of infection to the apical surface of differentiated airway epithelia, AAV5 was 50-fold more efficient than AAV2 to mediate gene transfer. In transferring beta-galactosidase cDNA to murine airway and alveolar epithelia *in vivo*, AAV5 was also more efficient than AAV2, indicating that AAV5 vectors are good for mediating gene transfer to human and murine airway epithelia^[51]. In addition, AAV5 vectors show a higher tropism for both mouse and human dendritic cells than did AAV1, AAV2, AAV7, and AAV8 vectors^[52]. Scientists at Virovek created a chimeric version of AAV5, named AAV5.2, by replacing the phospholipase A2 domain of AAV5 with that of AAV2. When both are produced in insect cells, the chimeric AAV5.2 shows much higher transduction efficiency than wild type AAV5 in a number of cell lines *in vitro* (unpublished data).

AAV6

AAV6 has a genome size of 4683 nucleotides in length and was isolated as a contaminant in a laboratory adenovirus stock, which appears to be related to AAV1 by sequence analysis^[38]. The two ITR's of AAV6 have different sequences, with the right repeat having a unique sequence and the left repeat identical to that of AAV2. Further analysis of the variable region of the cap gene revealed that AAV6 was 96% identical to AAV1 in that region, with only one amino acid change out of the 139-amino-acid translated sequence (a substitution of lysine-to-glutamate at position 531 of AAV6 VP1). Because of the 99% DNA sequence homology between AAV1 and AAV6, and the identical sequence of the first 508 nucleotides between AAV6 and AAV2, it was speculated that AAV6 may be derived from recombination between AAV1 and AAV2^[13].

In-vivo studies show that AAV6 vectors are much more efficient than AAV2 in transducing epithelial cells in small and large airways, with as much transduction as 80% in some airways. This result indicates that AAV6 may have considerable advantages over AAV2 for gene therapy of lung diseases such as cystic fibrosis^[53]. In addition, AAV6 exhibited body-wide transduction of the entire skeletal musculature through a single intravenous dose^[54] and was used for gene therapy studies of Duchenne muscular dystrophy in mice^[55]. Most of AAV vectors have poor transduction efficiency in blood cells. However, AAV6 with mutations on its surface-exposed serine (S) and threonine (T) residues (T492V + S663V) can efficiently transduce monocyte-derived dendritic cells^[56], indicating its potential uses in dendritic cell gene therapy. AAV6 shows the best transduction efficiency in pancreatic beta-cells among AAV1, AAV2, AAV5, and AAV8 serotypes tested in this study. Nearly the entire islet population was gene transferred but with unique gene transfer efficiency and patterns when different delivery methods and vectors were used. Remarkably, localized gene delivery coupled with an insulin promoter allowed robust but specific gene expression in the beta-cells^[57].

AAV7

AAV7 was isolated from rhesus monkeys and has a genome size of 4721 nucleotides^[58]. Antisera generated to the other serotypes are not able to neutralize AAV7. AAV7 neutralizing antibodies are not common in human serum and low in activity when present. *In vivo* studies in mice indicate that AAV7 can transduce skeletal muscle at similar efficiency to AAV1^[11]. While in mouse liver AAV8 out-performs AAV7, in nonhuman primate liver, expression from AAV7 vector stabilized at higher levels than AAV8, indicating that AAV7 should be considered a preferred vector for gene transfer in the primate liver^[58]. In nonhuman primates, AAV7 can direct as efficiently as AAV9 a robust and widespread cellular transduction in the central nervous system and other peripheral neural structures^[59]. AAV7 has also been used to target neurons within the basal and lateral amygdala area and shows a trend toward having the highest efficiency of transduction^[60].

AAV8

AAV8 was isolated from rhesus monkeys and its rep and cap coding region was fully sequenced but the rest of the genome has not been analyzed. The rep and cap sequences of AAV8 are 88% homologous to AAV7 and 82% homologous to AAV2 in nucleotides^[11]. Between AAV8 and AAV2, the most significant structural differences are located at protrusions surrounding the 2-, 3-, and 5-fold axes on the capsid surface. Amino acid residues on those axes were reported to control antibody recognition and transduction efficiency for AAV2. Furthermore, comparing the amino acids on capsid surfaces between AAV8 and AAV2 revealed that the distribution of basic charge for AAV8 at the region

corresponding to AAV2 heparin sulfate receptor binding motif was reduced. This results were consistent with the observation that AAV8 is a non-heparin-binding phenotype^[61].

AAV8 exhibits remarkably greater transduction efficiency in liver than those of other serotypes^[11]. This high transduction efficiency in liver and low cross-reactivity to antibodies against other human AAVs have led to great efforts in developing AAV8 as a viral vector for gene therapy of liver-targeted applications. Using AAV8 in mouse models as a gene therapy vector for long-term correction of hemophilia A, familial hypercholesterolemia, and glycogen storage disease type II has been reported^[62-64]. AAV8 has also been used successfully in a canine model for liver-targeted gene therapy^[65]. In mice and hamsters, AAV8 has been shown to be able to cross efficiently the barrier of blood-vessel to gain systemic gene transfer in both cardiac and skeletal muscles^[66]. In mouse model, AAV8 has also been used successfully to target the pancreas^[57]. However, recent data indicate that AAV8 vectors, which are very effective in many animal models, transduced human hepatocytes rather poorly, approximately 20-fold less, when compared to its transduction efficiency in mouse hepatocytes^[67].

Scientists at Virovek created a chimeric version of AAV8, named AAV8.2, by replacing the phospholipase A2 domain of AAV8 with that of AAV2. When both are produced in insect cells, the chimeric AAV8.2 shows much higher transduction efficiency than wild type AAV8 both *in vitro* and *in vivo* (unpublished data).

AAV9

AAV9 was isolated from human tissues and its genome was not fully sequenced except the rep and cap coding sequences^[68]. AAV9 capsid differs from AAV4 in nine variable surface regions (VR- I to -IX), but differs AAV2 and AAV8 in only three (VR- I , VR- II , and VR-IV). The difference in VR- I region modifies the raised region of the capsid surface between the 2-fold and 5-fold depressions. The difference in VR-IV produces smaller 3-fold protrusions in AAV9 that are less "pointed" than AAV2 and AAV8. Remarkably, residues in the VRs of AAV9 have been identified as important determinants of cellular tropism and transduction and distinguish AAV9's antigenic diversity from AAV2^[69].

AAV9 was reported to provide global cardiac gene transfer stable for up to 1 year in mouse or rat that was superior to other serotypes such as AAV1, AAV6, AAV7, and AAV8^[70]. AAV9 transduced myocardium 5- to 10-fold higher than AAV8, resulting in over 80% cardiomyocyte transduction after tail vein injection in mice^[71]. In large animal model such as pigs, treatment with AAV9 carrying the small calcium-binding protein S100A1 prevented and reversed functional and structural changes by restoring cardiac S100A1 protein levels. AAV9-S100A1 treatment normalized cardiomyocyte Ca²⁺ cycling, sarcoplasmic reticulum calcium handling, and energy homeostasis^[72].

When delivered systemically and intra-cerebrospinally, AAV9 has also been reported to obtain widespread gene delivery to the CNS. Delivering AAV9-GFP in the cisterna magna of both newborns and young cats indicated that high levels of motor neurons (MNs) from the cervical (84% ± 5%) to the lumbar (99% ± 1%) spinal cord were transduced, which demonstrates that the age at CSF delivery does not affect significantly AAV9 tropism for MNs^[73]. AAV9 can transduce brain's antigen-presenting cells and trigger a full immune response that mediates significant brain pathology, depending on the transgene immunogenicity. These observations raise concerns that foreign-proteins expressed at certain level may be able to trigger both humoral and cell-mediated responses, which may complicate preclinical toxicology studies^[74].

A clinical trial of AAV9 vectors carrying human spinal muscular atrophy (*SMN*) gene has been initiated in early 2014 for treatment of SMN in infants^[75].

AAVShH10

AAVShH10 is derived from a shuffled library closely related to AAV6 and is capable of efficient, selective Müller cell infection through intra-vitreous injection. Remarkably, AAVShH10 exhibits significantly improved transduction efficiency relative to AAV2 (> 60%) and AAV6^[76]. AAV-ShH10 has been employed to overexpress GDNF from Müller cells and thereby significantly slow the rate of retinal degeneration in a rat model of autosomal dominant *Retinitis Pigmentosa*^[77]. AAVShH10 delivery through intra-vitreous injection can transduce Müller cell in a significantly different pattern in Dp71-null mice with a compromised blood-retinal barrier (BRB), indicating that there are changes in viral cell-surface receptors as well as differences in the permeability of the inner limiting membrane in this mouse line. However, the compromised BRB of the Dp71-null mice does not lead to virus leakage into the bloodstream when the virus is injected intra-vitreally - an important consideration for AAV-mediated retinal gene therapy^[78].

AAV7m8

AAV7m8 was isolated from a mixture of three libraries that went through several rounds of *in vivo*-directed capsid evolution. AAV7m8 is a genetic variant of AAV2, with a peptide inserted on its heparin-binding site^[79]. AAV7m8 is able to transport the gene payload to the outer retina after injection into the eye's easily accessible vitreous humor and mediates widespread gene expression to the outer retina, which rescued the disease phenotypes of Leber's congenital amaurosis and X-linked Retinoschisis in corresponding mouse models. In addition, AAV7m8 is able to transduce primate photoreceptors when delivered *via* the vitreous, expanding its therapeutic promise^[79]. AAV7m8 encoding channel rhodopsin under the ON bipolar cell-specific promoter mediates long-term gene expression restricted to ON-bipolar cells after intra-vitreous administration. Channel rhodopsin expression in the ON-bipolar cells

leads to restoration of ON and OFF responses at the retinal and cortical levels. Moreover, light-induced locomotor behavior is restored in treated blind mice^[80].

AAVDJ

AAVDJ is a chimera derived from AAV2, AAV8, and AAV9, differentiated by 60 amino acids from its closest natural relative AAV2 in the capsid. It was isolated through an adapted DNA family shuffling technology. AAVDJ outperformed eight standard AAV serotypes (AAV1, 2, 3, 4, 5, 6, 8, and 9) in cultures of 10 cell lines and greatly surpasses AAV2 in livers of IVIG-immunized and naïve mice^[81].

Self-complementary AAV genomes

Native AAV packages single-stranded genomes^[82,83] and requires host-cell factors to synthesize the complementary strand before transcription can be initiated. However, when the single-stranded genome is less than half wild-type size, AAV can package either two copies, or dimeric inverted repeat DNA molecules^[84]. These dimeric inverted repeat DNA molecules can spontaneously anneal and form self-complementary molecules once uncoated inside the host-cell^[85]. Packaging of self-complementary AAV (scAAV) or sometimes called double-stranded AAV (dsAAV) genomes, can be made more efficient by deleting the terminal resolution site (*trs*) or the D-sequence (the packaging signal) together with the *trs* from one AAV terminal repeat^[86,87]. The important trade-off for scAAV vectors is the loss of half the coding capacity. However, small protein-coding genes (up to 55 kd), and any currently available RNA-based therapy can be accommodated.

These scAAV vectors exhibit fast onset and enhanced AAV transduction efficiency and have been widely used in many gene therapy studies. Wang *et al*^[87] reported that scAAV vectors dramatically improved transduction efficiency in more than 20 cell lines of human, monkeys and rodent origins and accelerated long-term transduction *in vivo* in mice when delivered *via* intramuscular or tail vein injections. Nathwani *et al*^[88] reported a 20-fold improvement in hFIX expression from scAAV in mice over comparable ssAAV vectors. It has been reported that a single intravenous injection of scAAV9 vectors carrying U7ex23 (small nuclear RNAs) in the utrophin/dystrophin double-knockout (dKO) mouse restored the dystrophin expression to near-normal levels in all muscles examined, including the heart. This resulted in a considerable improvement of their muscle function and dystrophic pathology as well as a remarkable extension of the dKO mice lifespan^[89]. Yang *et al*^[90] reported that expression of miRNA from scAAV inhibited the replication of cell culture-propagated HCV (HCVcc) by 98%, and resulted in up to 93% gene silencing of RLuc-HCV reporter plasmids in mouse liver, indicating the combination of an AAV vector delivery system and exploitation of the endogenous RNAi pathway is a potentially viable alternative to current HCV treatment regimens.

AAV VECTOR PRODUCTION

There are several technologies available for production of AAV vectors. These include transient plasmid transfection, adenovirus infection, stable-cell lines harboring AAV helper functions, HSV infection/transfection, and baculovirus infection technologies. All these technologies have the common elements for AAV vector manufacturing: (1) target gene flanked by ITRs, which in most cases are derived from AAV2; (2) AAV rep and cap genes provided *in trans*, in which the *rep* gene is derived from AAV2 while the cap gene can be of any serotypes; and (3) helper functions from adenovirus, HSV, or baculovirus. When these three components are introduced into a host cell under proper conditions, AAV vectors will be produced (Figure 2). Each of the technologies has unique properties to suit specific applications.

Plasmid transfection of mammalian cells

Initially, AAV vectors were produced by infection of mammalian cells with a wild type adenovirus and a recombinant adenovirus carrying AAV rep and cap genes followed by transfection with plasmid carrying the target gene flanked by AAV2 ITRs^[91]. This method produced large quantities of adenoviruses that had to be removed. Although purification removes most of the contaminating adenoviruses, and heat treatment inactivated the remainder, the AAV vector preparations were still contaminated with adenovirus proteins capable of causing host immune response. In order to eliminate the adenovirus from AAV vector production, two groups at about the same time reported the use of adenovirus-free system to produce AAV vectors^[92,93]. They used three plasmids, one harboring adenovirus VA, E2A and E4 genes, the second harboring AAV rep and cap genes, and the third harboring target gene flanked by two AAV2 ITRs. After transfection of these plasmids mediated by calcium phosphate into HEK-293 cells, which contained stably integrated adenovirus E1 genes, AAV vectors were produced free of adenovirus. Later, the method was stream-lined to contain only two plasmids, one with the target gene flanked by two AAV2 ITRs and the other with AAV rep and cap genes as well as the required adenovirus helper genes. AAV vectors were produced by transfecting both plasmids into HEK-293 cells^[94]. The advantages of plasmid transfection, whether with two or three plasmids, to produce AAV vectors are that it requires substantially less time and is fairly easy to perform compared with other systems such as recombinant HSV or baculovirus systems. It can produce small-scale AAV vectors enough for *in-vitro* assays and small animal studies and is still widely used by many academic labs. The disadvantages are that it is difficult to scale up due to the inherent property of adherent cells and use of animal serum, which is not favored by Food and Drug Administration. Lock *et al*^[95] reported that typical yield of AAV vector production from forty 15-cm plates is

about 1 to 2×10^{13} vg.

In order to increase production scale, HEK-293 cells were adapted to suspension culture and transfection was performed with polyethylenimine to produce AAV vectors. The AAV vector production yields range from 5×10^{12} vg/L in serum-containing suspension culture^[96] to 2.85×10^{13} vg/L in serum-free suspension culture^[97].

Stable-cell line harboring AAV helper functions

One of the methods to produce AAV vectors is to employ cell line stably harboring AAV helper genes. In some cases, only AAV rep/cap genes were stably integrated into mammalian cells and AAV vectors were produced upon infection of the cell lines with a wild-type adenovirus followed by transfection with AAV vector plasmid or infection with a second adenovirus carrying target gene flanked by two AAV ITRs^[98-103]. In one case, both AAV rep/cap genes and AAV vector plasmid were stably integrated into the cell line for AAV vector production^[99]. AAV production yield with stable cell line system was up to 1×10^4 vg/cell. Stable cell line systems require adenovirus co-infection for AAV vector production, which is not a desirable feature and poses downstream challenges for AAV purification. In addition, stable cell lines tend to lose integrated genes after frequent passages and AAV production yields tends to decrease with the increase of cell passage number. Another drawback is that it takes several months to establish and characterize a stable cell line.

HSV-based systems

The first generation HSV-based method for AAV vector production was developed by Conway *et al*^[104], which depended on an amplicon system. The AAV2 rep and cap genes and their native p5, p19, and p40 promoters were cloned into a plasmid that carries the HSV origin of replication and packaging signal. To produce HSV viruses carrying the AAV2 rep and cap genes, Vero cells with either wild-type HSV DNA or infected with wild-type HSV were transfected with the resulting pHSV-RC plasmid. The missing *trans* helper genes required for HSV amplicon DNA replication and packaging into HSV particles were provided by the wild type HSV. During this process, HSV particles generated both from amplicon and wild-type HSV sources were further amplified through serial infection passages. Finally, HSV-RC stocks were used to produce rAAV vectors by infecting either proviral cell lines that contained an integrated rAAV-2 genome or cells transfected with a rAAV2 plasmid or infected with rAAV2. This amplicon system has disadvantages such as the requirement of three components (HSV-RC, wild type HSV, and rAAV), the undesirable safety concern of wild-type HSV, and the dominant amplification of wild type HSV that is toxic to the producer cells. It was reported in the same study that use of a mutant HSV instead of wild type HSV produced higher titer of rAAV because of its lower cytotoxicity.

In order to increase AAV production yield, a second

generation of HSV-based system was developed in which the AAV rep/cap gene was cloned into one replication-deficient HSV and the gene of interest flanked by ITRs was cloned into a second replication-deficient HSV^[105]. Upon dual infection of HEK-293 cells with these two rHSV vectors, AAV vectors were produced with yields as high as 1.55×10^{12} vg/flask with 1×10^7 cells. This system was further scaled up in cell factories to produce AAV-AAT in serotypes 1 and 9. This method was able to produce more than 8.5×10^{13} AAV1 vg (8.5×10^4 vg/cell) from one cell factory^[106]. The problem with this system is that the production of high-titer and infectious replication-deficient HSV vectors is very challenging due to the fact that: (1) the production efficiency and the profile of product safety are usually inversely correlated, since rendering HSV vectors replication incompetent by genetic deletions also typically reduces rHSV yield; and (2) HSV particles are very sensitive to production and processing conditions such as temperature, shear, solvents, and detergents and can easily be inactivated during manipulation.

Baculovirus-based systems

Production of AAV vectors in insect cells was first pioneered by Urabe *et al*^[107]. In this system, the AAV2 rep78 was cloned under control of a deleted version of baculovirus early promoter (E1) and rep52 under control of p10 promoter in a head-to-head orientation. The AAV2 capsid gene was cloned under control of baculovirus polyhedrin (polh) promoter and the VP1 start codon ATG was mutated into ACG to diminish the translation efficiency so that the ribosome machinery can scan down to next low efficiency ACG for VP2 expression and then scan further down to the start codon ATG of VP3 for highly efficient expression^[107]. The production yield of this system has been reported up to 5×10^4 vg/cell. Though the AAV production yield is increased compared to plasmid transfection systems, there are two flaws with this system: (1) the rep78 sequence contains 100% of rep52 sequence, which renders the rep containing baculovirus unstable due to homologous recombination between rep78 and rep52 as demonstrated by Kohbrenner *et al*^[108]; and (2) the VP1 level is lower than normal due to the ATG-to-ACG mutation, which results in less infectious AAV vectors.

In order to make this system more stable, Smith *et al*^[109] mutated the rep78 start codon ATG into ACG and subsequent nine in-frame ATGs into non-start codons, but retained the start codon ATG for rep52. This modification enabled the expression of both rep78 and rep52 from a single rep78 coding sequence and made the baculovirus more stable. However, the VP1 retained the same suboptimal ACG start codon, which resulted in suboptimal VP1 expression. This modified system produced AAV vectors up to 7×10^4 vg/cell. Several research groups reported that the AAV vectors produced in insect cells with suboptimal VP1 expression were less infectious than that produced in mammalian cells and that increasing the VP1 expression improved

the infectivity of AAV vectors^[108,110-112]. Urabe *et al*^[111] also reported in their patent (US 8163543 B2) that increasing VP1 expression levels improved AAV vector infectivity.

Researchers from both UniQure and NIH scaled up this system into 200 L bioreactor using the baculovirus-infected insect cells method^[113]. By using conditions established with small-scale cultures, AAV was produced in larger volume cultures. Consistent AAV yields were attained in cultures ranging from 10 to 200 L. Based on the final yield, each cell produced 18000 ± 6800 particles of purified AAV in 10-, 20-, 100-, and 200-L cultures. Thus, with an average cell density of 4.32×10^6 cells/mL, $\geq 10^{16}$ purified AAV particles are produced from 100 to 200 L. The downstream process resulted in about 20% recovery estimated from comparing the quantities of capsid protein antigen in the crude bioreactor material and in the final, purified product.

Researchers at Genethon reported a modified baculovirus system for AAV vector production (patent application WO 2013/014294 A2). They made two versions of baculoviruses, one with the *cathepsin*, *chitinase*, and *p10* gene disrupted, and the other with the *cathepsin*, *chitinase*, *p26*, *p10*, and *p24* genes disrupted. The AAV rep2/cap8 cassette and the murine embryonic alkaline phosphatase (mSEAP) reporter gene flanked by AAV2 ITRs were respectively cloned into the polyhedrin region. Their results showed that, even though the disruption of *cathepsin*, *chitinase*, *p26*, *p10*, and *p24* genes did not improve the AAV production yield, it indeed improved the infectivity of the AAV vectors 2- to 4-fold due to the reduction of AAV capsid protein degradation possibly caused by the protease *cathepsin*. The AAV vector production yield with this modified system ranges from 1.31×10^{11} vg/mL (or 1.31×10^{14} vg/L) to 2.09×10^{11} vg/mL (or 2.09×10^{14} vg/L).

In our laboratory we made two important modifications to the baculovirus-based system: (1) an artificial intron harboring the *polh* promoter was inserted into the AAV rep78 coding sequence at the p19 promoter region such that both the rep78 and the rep52 can be expressed from a single rep coding sequence; and (2) the same artificial intron containing the *polh* promoter was inserted into the AAV VP1 coding sequence upstream of the VP2 start codon such that all three capsid proteins (VP1, VP2, and VP3) can be expressed from a single capsid gene without the need to mutate the VP1 start codon ATG into suboptimal start codons^[112]. This is the only baculovirus-based system with VP1 coding sequence that retains the authentic optimal ATG start codon. These modifications not only make the baculovirus more stable due to the elimination of rep sequence repeats, but also restore the infectivity of AAV vectors produced in insect cells because of the optimal VP1 expression level. It is well known that the VP1 protein contains a phospholipase A2 domain required for AAV infectivity and that decreased level of VP1 protein in the virus particle renders the virus less infective^[114-116].

We made additional improvement to our baculovirus-based system in order to produce AAV vectors carrying toxic genes at a normal level^[117]. Recombinant viruses carrying toxic genes such as *diphtheria* toxin, *Pseudomonas* exotoxin, ricin, and barnase are extremely difficult to produce since trace amount of toxin expression can kill the producer cells. We exploited the difference in intron splicing machineries between insect and mammalian cells. By inserting a mammalian intron that is not recognized by insect cells to disrupt the ORF of the toxin gene carried by the recombinant virus, we are able to abolish toxin expression during virus production but restore expression once the recombinant virus is introduced into mammalian cells. In this improved system, recombinant baculovirus carrying the toxic gene can be produced at normal levels. By using this recombinant baculovirus harboring the intron-interrupted toxin gene, we are able to produce AAV vectors up to 1.81×10^{15} vg purified from each liter of culture, 10- to 100-fold higher than with other AAV production systems. Recently we performed a 25-L production run in the Wave Bioreactor 20/50EH system, and obtained 3.50×10^{16} vg of total purified AAV6 vectors and the yield was independently verified by a third party (unpublished data).

AAV manufacturing technologies have sufficiently advanced such that we now have a robust system to produce AAV vectors with yields that exceed 1×10^{15} vg/L, or 1×10^{18} vg from 1000-L bioreactor, which will be able to meet the demand of treating ten thousand patients at a dosage of, say, 1×10^{14} vg per patient.

AAV PURIFICATION

There are several methodologies to purify AAV vectors from cell cultures. They include density gradient ultracentrifugation, column chromatography, and chloroform extraction/polyethylene glycol (PEG) precipitation partitioning. Virus particle purification by density gradient ultracentrifugation with cesium chloride (CsCl) has been used for more than 50 years^[118,119]. When subjected to a strong centrifugal field, CsCl in solution forms a density gradient and viruses that are centrifuged to equilibrium in CsCl are separated from contaminants and collected in bands based on their buoyant densities. The history of chromatography spans from the mid-19th century to the 21st century. Column chromatography is a well-established method for efficient and scalable purification of biomolecules and has been used for AAV vector purification^[120-124]. Chloroform extraction/PEG precipitation partitioning for AAV vector purification is rather new and not widely used yet.

Density gradient ultracentrifugation

The common strategy for AAV purification through ultracentrifugation starts with infected cell lysis and DNA digestion. The cell lysate is cleared by centrifugation to remove cell debris and applied to a discontinuous CsCl step-gradient with 1.3 g CsCl/mL on the top and

1.5 g CsCl/mL on the bottom. Since the AAV particles have a buoyant density of 1.4 g/mL, they are able to be separated from protein contaminants after the first round of centrifugation, and collected as a single band in the middle of the gradient. The harvested AAV band is then mixed with 1.4 g CsCl/mL and subjected to a second round of isopycnic gradient ultracentrifugation. Since they all have the same buoyant density, this method can be used to purify all different serotypes of AAV vectors. In our lab, we have used this method to purify many serotypes of AAV vectors ranging from 1×10^{13} vg to 3×10^{16} vg per production run and obtained satisfactory results. The advantage of this method is its versatility because this one process can be used for any serotype. The recovery rate is generally more than 70% and purity is more than 98% as judged by SDS-PAGE gel (Figure 3).

Although some researchers reported that CsCl has deleterious effect on AAV vector infectivity^[121,125], we performed a side-by-side comparison between CsCl and iodixanol purified AAV1-GFP vectors and have not seen any difference in infectivity (unpublished data). Ayuso *et al*^[126] optimized the CsCl protocol by incorporating differential precipitation of AAV particles with polyethylene glycol and produced AAV vectors in higher yield and markedly higher vector purity, which correlating with better transduction efficiency detected with several AAV serotypes in multiple tissues and species. In fact, the Center for Cellular and Molecular Therapeutics at the Children Hospital of Philadelphia (CHOP) is using a combination method to purify AAV vectors under cGMP conditions in which a column chromatography is used to capture AAV and CsCl ultracentrifugation is used to separate the empty from the full AAV particles^[127], which indicate that AAV vectors purified by CsCl ultracentrifugation method are acceptable for clinical usage.

Iodixanol, an X-ray contrast compound, can be used as a density gradient medium in place of CsCl. Zolotukhin *et al*^[125] reported the use of iodixanol medium combined with chromatography for AAV vector purification and obtained over 50% recovery with 99% purity. Hermens *et al*^[128] reported the use of iodixanol to replace CsCl for purification of AAV vectors and shortened the centrifugation period to 3 h with reproducible concentration and purity of AAV vector stocks. In our laboratory, we used iodixanol to replace CsCl for AAV purification and obtained similar recovery rates and purity as with CsCl.

Column chromatography

The modes of column chromatography suitable for AAV purification include affinity, ion exchange, gel filtration and hydrophobic interaction. Several optimized chromatographic steps are required to obtain virus of high yield and purity. Optimal AAV purification protocols generally include two chromatographic steps or a combination of chromatography with ultracentrifugation/filtration. Because different AAV serotypes have different compositions on the surface of viral particles,

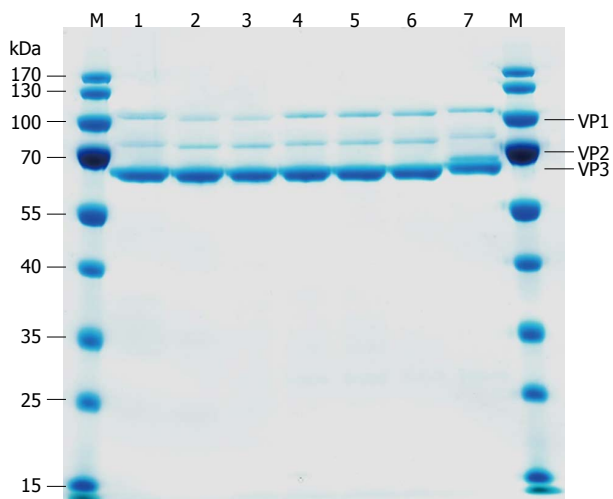


Figure 3 SimplyBlue SafeStaining of purified adeno-associated virus vectors on SDS-PAGE gel. Seven purified lots of adeno-associated virus (AAV) vectors at the amount of $1e + 11$ vg per lane were loaded on a 10% Tris-glycine gel. The gel was stained with SimplyBlue SafeStain Kit (Invitrogen). Lanes 1, 4-6: AAV9; lanes 2 and 3: AAV5; lane 7: AAV8.2. AAV capsid proteins VP1, VP2, and VP3 are indicated; M: Protein ladders.

specific resins should be used for purification. Heparin-based affinity column chromatography has been used for AAV2 vector purification due to the fact that AAV2 uses heparin sulfate proteoglycan as its receptor^[24]. Gao *et al*^[121] reported the use of a fully closed two-column chromatography system to purify AAV vectors. Yields of AAV vectors purified by this method are high, potency is increased, and the purity of column-purified preparations is substantially improved. Brument *et al*^[129] developed a two-step chromatography protocol on the basis of using ion exchange resins. Average recovery rate is 33%. *In-vitro* and *in-vivo* data demonstrated that this protocol, which does not need any pre-purification of the cell lysate, can be used to obtain highly pure AAV2 and AAV5 stocks. AVB resin has also been used to purify AAV vectors^[109]. Based on published data, on average the recovery rate of AAV purification with column chromatography is around 30%, which is substantially lower than the recovery rate (70%) of CsCl method used in our laboratory. In addition, commonly used column chromatography methods cannot remove empty AAV capsids from the fully packaged virus particles. Though Qu *et al*^[130] succeeded in using ion-exchange chromatography alone to separate empty and full particles from a semi-purified mixture of partially purified AAV, co-author Dr. Wright at CHOP has adopted a combinational method of column chromatography to capture AAV followed by CsCl gradient centrifugation, which allows his group to separate empty from full particles for their cGMP material purification^[127].

Chloroform extraction, PEG precipitation and partitioning

Alternative methods for AAV vector purification have also been developed. Wu *et al*^[131] reported chloroform treatment, PEG/NaCl precipitation and a final chloroform

extraction to purify AAV vectors and obtained greater than 95% purity. The whole procedure can be performed in 4 h without using ultracentrifugation or chromatography equipment. Another method was reported by Guo *et al*^[132] in which AAV vectors from culture media and cleared cell lysate were precipitated with PEG8000/NaCl, and the pellet was resuspended in Hepes buffer, followed by chloroform extraction and PEG/salt partitioning. AAV vectors were purified and showed infective in both *in vitro* and *in vivo* studies. However, these methods have not been widely used yet.

CLINICAL TRIALS

To date, there have been over 2076 gene therapy clinical trials worldwide, in which approximately 5.9% (over 127 clinical trials) have used AAV vectors^[133]. Two general delivery methods have been employed to treat diseases with AAV vectors. Local delivery (surgical injection) method is used to treat diseases that affect specific organs. Systemic delivery (intravenous injection) method is used to treat diseases that affect all cells, such as lysosome storage diseases^[134], and muscular dystrophies^[54]. Many eye diseases, for example, are treated with either intra vitreal injection by primarily affecting retinal neurons or sub-retinal injection by placing virus in contact with the photoreceptor and retinal pigmented epithelial (rpe) layers of the eye^[135]. Local delivery of AAV into the heart has been used to treat cardiac diseases^[136]. Similarly, delivering AAV into the target region with stereotactic surgery can be used to treat some neurodegenerative diseases that primarily affect a particular region of the brain (striatum), such as Parkinson's disease^[137,138]. As a result, many promising data have been obtained from Phase 1 and Phase 2 clinical trials for a number of diseases in recent years.

Leber's congenital amaurosis

Leber's congenital amaurosis is an inherited retinal disease that causes severe visual impairment in infancy or early childhood. Three groups of investigators reported the use of AAV2 to treat homozygous recessive *rpe65* deficiency successfully in their clinical trials. The *rpe65* codes for a protein that is responsible for regenerating 11-*cis* retinal in the retinal pigmented epithelial cell layer of the eye, and lacking this *rpe65* protein the patient is essentially blind in low light. In these three independent phase 1 clinical trials, each patient was injected sub-retinally into one of the eyes with the rAAV-*rpe65* vector^[29-31]. Studies of gene expression in the portion of the treated eye demonstrated that virtually 100% of the remaining photoreceptor cells were corrected and remarkable recovery of vision was seen in these patients^[139]. The positive results from these *rpe65* clinical trials indicate that essentially any recessive genetic defect in the eye with loss-of function should be able to be corrected. Studies for a variety of other eye diseases with genetic defects, as well as diseases such as macular degeneration are now underway.

Hemophilia B

Hemophilia B is a rare bleeding disorder in which blood doesn't clot normally due to mutations in the gene for coagulation factor IX. A few clinical trials have been conducted for this factor IX deficiency. Patients lacking factor IX, a serum protein that is an essential component of the blood clotting cascade, experience increased episodes of bleeding in response to mild trauma or spontaneous hemorrhage in joints and muscle. The two initial hemophilia B phase I/II clinical trials, injecting AAV2 with a factor IX cDNA to skeletal muscle or liver, exhibited no serious adverse events^[28,140,141]. Even though the muscle trial did not achieve a therapeutic level of factor IX in the circulation, long-term expression of clotting factor was detected on muscle biopsies taken up to 3 years after vector injection. AAV delivery to liver *via* the hepatic artery determined a therapeutic dose, which agreed closely with the doses predicted by studies in hemophilic dogs. However, the expression of factor IX in the treated patients lasted for only a period of weeks, followed by a gradual decrease in factor IX levels accompanied by a self-limited, asymptomatic rise and fall of liver enzymes^[142]. The loss of expression was associated with a cytopathic T cell response to AAV capsid protein but not the transgene. More recently, a new phase I clinical trial for hemophilia B was conducted. This time AAV8 vectors carrying a codon-optimized, self-complementary factor IX cassette were used^[143]. The AAV8 vectors were delivered intravenously and dose-dependent and stable expression of therapeutic levels of factor IX in serum at middle and high vector doses were observed. Expression was stable over 6 mo of follow-up, and several patients no longer found it necessary to infuse factor IX protein. Similar to earlier hemophilia B trials, some patients appeared to mount an inflammatory response, as determined by increased levels of serum alanine aminotransferase. After a short course of an immune modulator (prednisolone), these patients recovered a normal enzyme profile and retained therapeutic levels of factor IX after immunosuppression was stopped.

Congestive heart failure

In 2007, the first clinical trial for heart failure was launched in the United States^[20,21]. This phase 1/2 multicenter trial was designed into two parts to evaluate the safety and the biological effects of AAV1.SERCA2a intracoronary delivered into patients with advanced heart failure. In part 1 of the trial, a satisfied safety profile was observed in the 12-mo follow-up with these patients^[20,21]. Several patients showed improvement as measured by biomarker (two patients), functional (four patients), symptomatic (five patients), and LV function/remodeling (six patients) parameters. These results indicated that treatment with AAV1. SERCA2a provides quantitative biological benefit.

Thirty-nine patients with advanced heart failure were enrolled in part 2 of the trial and randomly divided into four groups, in which three groups received

intracoronary AAV1.SERCA2a (low dose: 6e + 11 DRP, middle dose: 3e + 12 DRP, and high dose: 1e + 13 DRP) and one group placebo^[144]. Over six months of the trial, patients' symptoms including Minnesota Living With Heart Failure Questionnaire (MLWHFQ) and New York Heart Association (NYHA) score, echocardiographic measures, NT proBNP levels, and functional status [six-minute walk test (6MWT) and VO₂ max], were evaluated. Based on the above end-points, clinical outcomes and concordant trends among groups and patients were compared to determine the success of treatment. At the group and individual patient levels, the high-dose group met the pre-specified criteria for success. Patients treated with AAV1. SERCA2a showed improvement or stabilization in MLWHFQ and NYHA scores, NT proBNP, 6MWT, and VO₂ max levels, and LV end-systolic volumes at 12 mo when compared with patients treated with placebo. In the placebo group over a one-year period, cumulative recurrent cardiovascular events (myocardial infarction, cardiac transplantation, LV assist device insertion, heart failure admission, and death) increased. The patients treated in the high dose group continued to perform significantly better at 12 mo when compared with patients in the rest of groups by showing no increase of adverse events, disease-related events, laboratory abnormalities, or arrhythmias. Though the patient groups with low- and middle-dose of AAV1. SERCA2a had decreased recurrent cardiovascular events for the first six months, they had events that were similar to placebo group from 6 to 12 mo^[144].

Parkinson's disease

Since 2003, a total of nine clinical trials have been conducted for gene therapy of Parkinson's disease (PD) with AAV vectors^[145]. PD is a chronic and progressive neurodegenerative disease that is most widely diagnosed for the profound degeneration of mid-brain dopamine nigrostriatal neurons linked to serious motor symptoms. In the hope of preventing neurodegeneration and increasing dopamine neuron synapses, Bartus *et al*^[137] used AAV2 to deliver a neurotrophic factor, neurturin, to striatal tissue of PD patients. Though they observed some evidence of improvement, they did not reach their primary therapeutic end points due to the lack of sufficient nigral neurons to show a significant effect. Christine *et al*^[32] overexpressed aromatic amino acid decarboxylase, the final enzyme in the dopamine synthetic pathway and also observed some improvement clinically and demonstrated clearly continuous gene expression over time.

Alzheimer disease

Alzheimer's disease (AD) is a neurodegenerative disorder. Though the cause and progression of AD is not fully understood, it is well recognized that the function and survival of basal forebrain cholinergic neurons that are vulnerable in AD can be enhanced by nerve growth factor (NGF). Encouraging clinical trial results have come from a study in which AAV2 vectors carrying human NGF

gene was used to treat AD through stereotactic surgical delivery in the hippocampus^[146,147]. The results indicated that^[147] AAV2-NGF was safe and well-tolerated for 2 years. No evidence of accelerated decline was observed through positron emission tomographic imaging and neuropsychological testing. Long-term, targeted, gene-mediated NGF expression and bioactivity were confirmed in the brain autopsy tissues. This clinical trial provides important evidence that bilateral stereotactic administration of AAV2-NGF to the nucleus basalis of Meynert is feasible, well-tolerated, and able to produce long-term, biologically active NGF expression, supporting the initiation of an ongoing multicenter, double-blind, sham-surgery-controlled trial.

CONCLUSION

Human gene therapy has advanced into a new stage where more and more investments will fuel more research and clinical trials. With a battery of AAV serotypes and variants and a series of well-established production and purification methods available to use, researchers and clinicians will be able to accelerate progress in the field. Though there are still some challenges ahead, more gene therapy drugs with AAV vectors are on the horizon.

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Value of predictive bioinformatics in inherited metabolic diseases

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Abstract

Typically, inherited metabolic diseases arise from point mutations in genes encoding metabolic enzymes. Although some of these mutations directly affect amino acid residues in the active sites of these enzymes,

the majority do not. It is now well accepted that the majority of these disease-associated mutations exert their effects through alteration of protein stability, which causes a reduction in enzymatic activity. This finding suggests a way to predict the severity of newly discovered mutations. *In silico* prediction of the effects of amino acid sequence alterations on protein stability often correlates with disease severity. However, no stability prediction tool is perfect and, in general, better results are obtained if the predictions from a variety of tools are combined and then interpreted. In addition to predicted alterations to stability, the degree of conservation of a particular residue can also be a factor which needs to be taken into account: alterations to highly conserved residues are more likely to be associated with severe forms of the disease. The approach has been successfully applied in a variety of inherited metabolic diseases, but further improvements are necessary to enable robust translation into clinically useful tools.

Key words: Genetic disease; Metabolism; *In silico* method; Protein stability; Disease-associated mutation

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Core tip: Bioinformatics and other *in silico* methods are increasingly being used to predict the severity of disease-associated mutations in inherited metabolic diseases. In general, severity correlates with altered protein stability and the best predictions occur when a variety of tools are applied.

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INTRODUCTION

Inherited metabolic diseases result from mutations in the genes encoding enzymes involved in intermediary metabolism. Well characterised examples include galactosemia, lysosomal storage diseases and phenylketonuria. Typically these diseases manifest with effects at the whole organism level, despite their origins at the metabolic pathway level. Physical and cognitive disabilities are associated with many inherited metabolic diseases. While individual diseases are generally rare, the cumulative effect of many of these diseases has a significant effect on societies and economies^[1-4]. Furthermore, the burdens on patients, their families and their immediate communities can be devastating since many of these diseases result in progressive deterioration of the patient resulting, in some cases, in death in childhood or early adulthood. Very few of these diseases have effective therapies (*i.e.*, treatments which restore normal, or near-normal, functioning to the patient). One barrier to the development of therapies is the rareness of the diseases: there is limited incentive to the development of drugs or other treatments which would only be applicable to a small number of patients worldwide^[2,5,6]. Where therapies do exist, they tend to be extremely expensive, often exceeding United States \$100000 per patient per year (for example, see^[7,8]).

Biochemical studies on the underlying molecular pathology of a range of inherited metabolic diseases have revealed some common themes. In particular, mutations associated with these diseases often cause changes which destabilise the corresponding protein (for examples, see^[9-15]). Very few disease-associated mutations directly affect the residues in the active site of the enzyme; the majority affect residues elsewhere in the protein. A common molecular mechanism of disease causation is that the altered amino acid residue causes a global reduction in the enzyme's stability resulting in reduced catalytic activity^[16]. The loss of stability can also be associated with reduced affinity for essential cofactors or increased aggregation of the partially folded protein. It is, of course, the loss of enzymatic activity which commonly leads to disease, for example by reducing the amount of product made or causing a build-up of toxic intermediates. In other cases the accumulation of aggregated protein results in a breakdown of cellular homeostasis. Nevertheless, partial protein misfolding lies at the base of these problems and is the fundamental cause of the disease in these cases.

It is also apparent that, in many inherited metabolic diseases, there is a range of possible symptoms. This is particularly stark in diseases like type III galactosemia and mevalonate kinase deficiency. In these diseases the manifestations range from near-normal physiology with some alterations in blood chemistry to highly disabling, life-threatening conditions^[17,18]. The experience of each patient will be determined by his/her genetic background, lifestyle and environment. Critical elements

include the patient's diet, activity levels and access to good quality medical care. However, the most important factor in determining the severity of symptoms is normally the exact mutation(s) that the patient has. Most inherited metabolic diseases are not caused by one, single mutation. The majority have many possible mutations which are associated with the disease. For example, there are almost 250 mutations in galactose 1-phosphate uridylyltransferase which are associated with type I galactosemia^[19,20]. Since these mutations alter different amino acid residues, it follows that they will have different effects on the protein. Some will have relatively minor effects on the protein's overall structure and stability whereas others may render the protein essentially non-functional.

Novel, disease-associated mutations continue to be discovered. Indeed, with the decreasing price of whole exome sequencing, we should expect that the rate of discovery of novel mutations will increase in the next few years^[21,22]. In some inherited metabolic diseases problems are apparent within a few days of birth; however, in other cases, babies are born with near normal physiology but progressively decline over the following years. Given the range of possible severities associated with some inherited metabolic diseases it is a challenge to physicians and scientists to predict the likely symptoms of an individual patient and to plan treatment accordingly. This is particularly the case for newly discovered disease-associated mutations.

THE CHALLENGE OF PREDICTION

The link between protein stability and severity of disease suggests a way in which predictions might be made. There are a variety of software packages, many freely available online, which claim to predict the stability of proteins (Table 1). In theory if a range of disease-associated variant proteins are compared to the wild-type in one of these packages then the greater the predicted instability, the more severe the disease is likely to be. In practice the situation is more complex. No prediction software is 100% accurate and different packages can give different results for the same variant. This problem can be overcome by using a variety of different programs and aggregating the results together to obtain a consensus. However, problems still remain. In some diseases, both decreases and increases in stability can be associated with disease (for examples see^[9,23]). Indeed, it appears that as well as an optimum structure for activity, proteins also require an optimal degree of flexibility and stability. Protein flexibility is inextricably linked to ligand binding and catalysis^[24]. Thus, increased stability can lead to a more rigidified, less flexible protein which is less able to bind substrates and catalyse the reaction.

While protein stability is undoubtedly a key factor in disease causation, there are other factors which need to be considered. Alterations in key residues involved in binding substrates or in the chemistry of catalysis will

Table 1 Examples of freely available, online tools for predicting the properties of variant proteins

Category	Name	Weblink	Ref.
Structural analysis	YASARA energy minimisation	www.yasara.org/minimizationserver.htm	[29]
	LS-SNP	ls-snp.icm.jhu.edu/ls-snp-pdb/main	[30]
Stability prediction	GETAREA	curie.utmb.edu/getarea.html	[31]
	I-Mutant 3.0	gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi	[32,33]
	mCSM	bleoberis.bioc.cam.ac.uk/mcsm/	[34]
	SDM score	mordred.bioc.cam.ac.uk/~sdm/sdm.php	[35,36]
	Mupro	mupro.proteomics.ics.uci.edu	[37]
	iStable	predictor.nchu.edu.tw/iStable/	[38]
	PredictSNP 1.0	loschmidt.chemi.muni.cz/predictsnp/	[39]
	Meta-SNP	snps.biofold.org/meta-snp/	[40]
	KD4V	decryphon.igbmc.fr/kd4v	[41]
	Fold-X	foldx.crg.es	[42]
	PoPMuSiC	dezyme.com/	[43]
	CUPSAT	cupsat.tu-bs.de	[44,45]
	GETAREA	curie.utmb.edu/getarea.html	[31]
	Binding affinity changes	BeAtMuSiC	babylone.ulb.ac.be/beatmusic
Aggregation tendency, amyloid formation and chaperone binding	TANGO	tango.crg.es/	[47]
	WALTZ	www.switchlab.org/bioinformatics/waltz	[48]
Sequence conservation	LIMBO	www.switchlab.org/bioinformatics/limbo	[49]
	Clustal Omega	www.ebi.ac.uk/Tools/msa/clustalo/	[50]
	Scorecons	www.ebi.ac.uk/thornton-srv/databases/cgi-bin/valdar/scorecons_server.pl	[51]
	SIFT	sift.jcvi.org/	[52]
	PROVEAN	provean.jcvi.org/index.php	[53]
	LS-SNP	ls-snp.icm.jhu.edu/ls-snp-pdb/	[30]
	SNPs and GO	snps.biofold.org/snps-and-go/pages/help.html	[54]
	PANTHER	www.pantherdb.org/tools/csnpscoreForm.jsp	[55]
	GenMAPP	www.genmapp.org	[56]
	PolyPhen 2	genetics.bwh.harvard.edu/pph2/	[57,58]
	nsSNP Analyzer	snpanalyzer.uthsc.edu	[59]
FI mutation assessor	mutationassessor.org/v1	[60]	
YALE MU2A	krauthammerlab.med.yale.edu/mu2a	[61]	

lead to direct loss of activity. A failure to interact with cellular chaperones may impede folding. Disruptions to other protein-protein interactions may also affect the enzyme's function. Residues involved in catalysis and protein-protein interactions are generally well conserved through evolution. Therefore, we might expect that mutations which alter highly conserved residues might also lead to more severe forms of disease. Therefore, many predictions incorporate measures of sequence conservation and propensity to interact with cellular chaperones (Table 1). Overall, it is accepted that the best predictions will result from using a variety of different software packages which address different aspects of the protein's structure and function^[25-27]. Furthermore, any output requires intelligent and critical analysis by the users.

APPLICATION TO INHERITED METABOLIC DISEASES

These approaches have been employed in a number of inherited metabolic diseases (Table 2). Typically, a set of known mutations and their associated protein variants are identified from the literature and classified according to their association with different severities of the disease. Other information from the literature is required

- most importantly an experimental demonstration that protein misfolding is an important factor in disease causation. Using the known variants, a range of prediction tools are applied and the combination which best predicts the known outcomes are then selected. This can then be applied to uncharacterised mutations or to polymorphisms identified through genome and exome sequencing projects. In general, the severity of disease correlates with the predicted loss of stability of the protein. The degree of conservation of the residue(s) affected is also important in some conditions (Table 2). Most studies employ a range of different prediction tools and aggregate results together to make informed predictions (for example see^[28]).

CONCLUSION AND FUTURE PERSPECTIVES

To date, no prediction protocol has achieved complete accuracy and it is unlikely that physicians would be confident to rely on them to guide treatment of their patients. In addition, the prediction protocols published so far mostly require extensive bioinformatics analysis using a number of different tools on separate websites. Ideally these would be integrated into a single web-based package which enabled the user to submit a

Table 2 Examples of bioinformatics based predictions of the severity of variants associated with inherited metabolic diseases

Disease	Protein	Comments	Ref.
Alkaptonuria	Homogentisate 1,2-dioxygenase	Combining a variety of computational approaches gave rise to the most accurate predictions	[62]
Apparent mineralocorticoid excess	11 β HSD2	The predicted degree of structural change in the enzyme correlates with disease severity	[63]
Fabry disease	GLA	A purpose built program designed to detect protein instability outperformed existing, generic tools	[64]
Fabry disease	GLA	A purpose built web interface allows prediction of a patient's responsiveness to pharmacological chaperone therapy	[65]
Gaucher disease	GBA	Slightly different results were obtained with different programs; however, 22 out of 47 variants were predicted to be harmful by all seven programs used	[28]
Glucose 6-phosphate dehydrogenase deficiency	G6PDH	A combination of prediction tools suggested that protein stability is an important factor in this disease; novel potentially disease-associated variants were identified	[66]
Hyperargininemia	ARG1	Mutations affect residues in the active site, or protein stability, or quaternary structure	[67]
MODY 2	GCK	Variations which decrease protein stability and/or occur in highly conserved regions of the protein are associated with disease	[68]
Niemann-pick disease type C	NPC1 and NPC2	The majority of disease-associated variants were predicted to be less stable than wild-type	[69]
Phenylketonuria	PAH	Protein stability predicted to be most important factor in disease causation	[10]
Pyruvate kinase deficiency	PK1 and PK2	A combination of prediction tools suggested that protein stability is an important factor in this disease; novel potentially disease-associated variants were identified	[66]
Type I galactosemia	GALT	Main predicted effect is the loss of stability of GALT	[70]
Type III galactosemia	GALE	Effects on protein stability and degree of sequence conservation combined were required for good predictions	[71]

11 β HSD2: 11 β -hydroxysteroid dehydrogenase type 2; GLA: α -galactosidase A; GBA: Glucocerebrosidase; G6PDH: Glucose 6-phosphate dehydrogenase; ARG1: Arginase 1; GCK: Glucokinase; PAH: Phenylalanine hydroxylase; PK1 and PK2: Pyruvate kinase isoforms 1 and 2; GALT: Galactose 1-phosphate uridylyltransferase; GALE: UDP-galactose 4'-epimerase; MODY 2: Maturity-onset diabetes of the young, type 2.

novel variant and receive a prediction. This would require the software to submit the new variant to the various online tools and integrate the responses into a single prediction. This, in turn, requires the software tools to remain "live" and at the same, stable url - something which cannot be guaranteed when each tool is provided by different organisations. One solution would be to use the source code for the prediction tools directly, but this is not always freely available.

Nevertheless carefully designed prediction methods can already provide some guidance in the assessment of novel mutations and have also had some success in classifying mutations identified through genome and exome sequencing projects. Interestingly, these tend to suggest that there are a number of mutations in the human population which were not previously associated with disease. In the case of type III galactosemia, several of these were predicted to be associated with severe forms of the disease. Further experimental testing will be required to see if these predictions are valid. In conclusion, the best predictions will be made when a variety of tools are used and where they are supported by strong experimental and clinical studies. It is highly unlikely that bioinformatics predictions will completely supplant experimental studies; however, with continuing improvements in the tools used they have the potential to be a useful tool for both clinicians and scientists who work on inherited metabolic diseases.

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