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REVIEW

Cell mediated gene therapy: A guide for doctors in the clinic

Moon Jong Noh, Ogden Copeland, Michael O'Mara, Kwan Hee Lee

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Author contributions: Noh MJ wrote most of this paper and gave input on the molecular aspect of TG-C; Copeland O reviewed and wrote the Food and Drug Administration related aspects; O'Mara M wrote the manufacturing part; Lee KH selected the review papers, decided the contents of the paper.

Conflict-of-interest: All of the authors are employees of TissueGene Inc. TissueGene Inc. will pay the fees for publication and editing of this paper. TissueGene Inc. owns the patent rights to protect the commercialization of TG-C.

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Abstract

The recent approval of gene therapy products in Europe and Asia and the upsurge of gene therapy products in clinical trials signal the rebound of this technology not only for many orphan diseases but also for non-life threatening diseases. Following the success of induced pluripotent stem (iPS) cells in research, other modified *ex vivo* gene therapies are also knocking on the door of the clinic. Historically, gene therapy has experienced many ups and downs and still faces many challenges. During the past 10 years, many new ideas have been tried, and the goal of making this technology a more effective treatment modality through greater safety and control is coming within reach. The first clinical trial of iPS cells has begun, and cell mediated gene therapy products have reached phase Ⅲ in some countries. The potential for tumorigenicity and immunogenicity are still concerns with these products, so physicians should understand the biological aspects of engineered cells in the clinic. In this review article, we attempted to provide a summary update of the current state of knowledge regarding this technology: that is, we reviewed products that have finished clinical trials, are still in clinical trials and/or are at the research stage. We also focused on the challenges, future directions, and strategies for making this technology available in the clinic. In addition, the available measures for making gene therapy products safer are within the scope of this article. It is also important to understand the manufacturing process for gene therapy products, because cell characteristics can change during the cell expansion process. When physicians use gene therapy products in the clinic, they should be aware of the viability, temperature sensitivity and stability of these cells because biologic products are different from chemical products. Although we may not be able to answer all possible questions and concerns, we believe that this is the right time for physicians to increase their interest in and understanding of this evolving technology.

Key words: Cell mediated gene therapy; Review; Physicians; Clinical and research stage; In the clinic

Core tip: In this review article, the authors attempted to provide an up to date summary of the current knowledge regarding cell mediated gene therapy that is, we reviewed products that have finished clinical trial, are in clinical trial and at the research stage. The authors also tried to cover the challenges, future directions, and strategies to make this technology available in the

clinic. This is the right time for the physicians to have knowledge of this evolving technology that already reached the bedside.

Noh MJ, Copeland O, O'Mara M, Lee KH. Cell mediated gene therapy: A guide for doctors in the clinic. *World J Med Genet* 2015; 5(1): 1-13 Available from: URL: http://www.wjgnet.com/2220-3184/full/v5/i1/1.htm DOI: http://dx.doi.org/10.5496/wjmg.v5.i1.1

INTRODUCTION

When the first gene therapy trial of hematopoietic cells and lymphocytes was reported[1], it seemed that gene therapy might be the answer to treating most orphan diseases without major complications. However, in early 2000, after the death of a patient during a gene therapy clinical trial[2,3], many researchers became very cautious about this new technology. The subsequent development of cancer as a result of *ex vivo* gene therapy treatment in 2003[4,5] made the regulatory authorities even more conservative. In 2009, after years of progress, the return of gene therapy was declared[6], and gene therapy was honored as "twenty-first century medicine"[7]. Three products hit the market: p53-expressing Gendicine™ and Advexin™, conditionally replicating adenovirus Oncorine™ and thymidine kinase + ganciclovir therapy, Cerepro™[8]. In 2012, it was reported that over 1800 gene therapy clinical trials had been completed in 31 countries[9]. In addition, some countries' governments have declared their intention to assist in the development of gene-related therapies at the federal government level.

More positive news for the field of gene therapy came with the award of the Nobel Prize for research on iPS cells in *ex vivo* gene therapy[10]. This technology has produced a seismic shift in stem cell research[11] and resulted in an increase in research for disease modeling of iPS cells[12]. The ultimate goal of these studies is the treatment of diseases that have not been treatable by conventional methods. Safety issues, manufacturing issues and product quality issues need to be addressed before these products become available in the clinic. Genetic engineering can make the cells more vulnerable to cancer development and therefore alternative engineering methods are needed[13]. To address these safety concerns, many papers have been published and several clinical trials have been performed to develop gene therapy technology for diseases in which there is no current available treatment.

Recently, the first clinical trial of iPS cells started in Japan[14], and *ex vivo* gene therapy for degenerative arthritis has reached phase Ⅲ clinical trials[15]. In this review, we will discuss the status of cell mediated (*ex vivo*) gene therapy, including clinical trials, safety issues[16,17], and manufacturing issues for clinical applications[18,19]. In the clinic, physicians should understand the characteristics of the treatments that they are using. Cell-related products are new to most doctors, and their use presents a challenge because these treatments were not available during most doctors' medical school education and resident training programs. In the area of regenerative medicine, these treatments cannot be confined to their own subspecialty. Understanding the mechanism of action of each product at the molecular level is necessary for doctors. We acknowledge that doctors can resist change, but we believe that understanding molecular medicine is similar to understanding new electronic technologies in everyday life.

ENGINEERED CELLS FOR CLINICAL TRIAL AND RESEARCH

The idea of multiple treatment modalities is familiar to physicians. For mesenchymal stem cell (MSC) differentiation, different combinations and effects of growth factors have been reported[20]. For gene therapy, the integration of multiple genes into cells (transfection) has been studied in many laboratories. The observation that cells into which multiple genes have been integrated show the characteristics of embryonic stem cells (ESCs) was first reported by the Yamanaka group in Japan[21]. This group inserted four factors, Oct3/4, Sox2, KLF4 and c-Myc, into fibroblasts, making them pluripotent. They showed that the engineered cells were similar to ESCs in terms of morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes and telomerase activity. Furthermore, these cells could differentiate into all of the cell types of the three germ layers *in vitro*. Other researchers showed the regenerative potential of iPS cells, with their ability to differentiate into various cell types[22-24]. To improve the safety of treatment with these cells, other researchers induced the differentiation of these cells without viral vectors[25,26] and even with recombinant proteins[27]. These strategies were reported to effectively eliminate any risk of modifying the target cell genome with exogenous genetic sequences. Consequently, the authors demonstrated the possibility of generating safer iPS cells. However, the stability of these cells has not been reported.

For clinical applications, the manufacturing process should produce batches of cells with the same characteristics. If the cells' characteristics change in the presence of different concentrations of a protein, the manufacturing process is not validated for consistency. Safety analysis should also

be performed prior to clinical trials. iPS cell-based therapies need to be thoroughly evaluated in pre-clinical animal models before they can be applied to human subjects[28,29]. Since 1998, the Food and Drug Administration (FDA) has been regulating cell therapies, beginning with "Guidance for human somatic cell therapy and gene therapy". In 2008, the FDA released the guidelines "Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs)". In 2011, they released the guidelines "Potency tests for cellular and gene therapy products". If cells are engineered to generate a product, the harvesting method (Good Tissue Practice), engineering method, and potency testing protocols should be clearly defined. From the beginning of the development process, the investigators should clearly understand the mechanism of action of the product to develop a manufacturing process with a method for harvesting cells and for testing them, to consistently produce well characterized, high-quality cell-based products. For example, Crook *et al*[30] (2007) reported on the activities and requirements for producing cGMP hESC lines including the derivation, banking, and characterization of these cells.

In 2014, Nature Medicine, in collaboration with the Volkswagen Foundation, organized a meeting with a panel of experts in regenerative medicine to identify the most pressing challenges, as well as to formulate the crucial strategies and stem cell concepts that could best help advance the field of translational regenerative medicine[31]. The panel identified four major issues: first, harnessing the potential of endogenous stem cells; second, deciphering therapeutic reprogramming; third, meeting the challenges of cell integration and function; and fourth, removing roadblocks to the translation of stem cell therapies. We believe that these opinions summarize the present and future of cell mediated gene therapy. Researchers have begun to address these challenges. In particular, one patient-specific pluripotent stem cell therapy has been reported to be safe with respect to immunogenicity and is nearing clinical trials[32]. Neurodegenerative disorders are very interesting clinical targets for this technology[33].

An enormous number of cell therapies have been tested worldwide for the treatment of rare diseases[34]. Regenerative medicine cannot be defined without MSCs and ESCs; however, gene therapy is also an important area in regenerative medicine. Many regenerative medicine companies have started up, and various umbrella organizations such as the Alliance for Regenerative Medicine have been formed. In 2009 and 2013, three very interesting studies were reported[35,36]. These studies integrated a lentiviral vector into patient hematopoietic stem cells and showed promising clinical results for Wiskott-Aldrich syndrome and X-linked adrenoleukodystrophy. In 2013, another group reported promising clinical results using lentiviral *ex vivo* gene therapy for metachromatic leukodystrophy[37]. These serial successes for rare diseases are a hallmark of this technology in the clinic. It is clear that this technology is the flag bearer for the future of treating rare diseases. It was a triumphant success to overcome the pessimistic environment that existed after leukemia developed in X-linked severe combined immunodeficiency (X-SCID) patients treated with retroviral gene therapy[38]. In contrast to retroviral gene therapy, they extracted hematopoietic stem cells from a patient and transduced these cells with a lentiviral vector carrying specific genes (Wiskott-Aldrich gene, ABCD1 and ARSA), producing cell clones that did not carry integrations near oncogenes. Consistent with this, the authors did not observe evidence of clonal expansions in the patients for up to 20 to 32 mo after gene therapy treatment[35].

In 2007, induced pluripotent stem cell lines derived from human somatic cells were developed[39]. In 2008, it was reported that to better understand amyotrophic lateral sclerosis (ALS) and to develop a treatment, induced pluripotent stem cells were generated from a patient with the disease[23]. The authors generated iPS cells from an 82-year-old woman diagnosed with a familial form of ALS. These patient-specific iPS cells possessed properties of embryonic stem cells and were successfully directed to differentiate into motor neurons, the cell types destroyed in ALS. In 2009, an animal study of treating hemophilia A with iPS cells was reported[40]. The authors inserted the genes encoding 3 transcription factors, Oct4, Sox2 and Klf4, into somatic cells. The plasma FVⅢ levels in these mice increased to 8% to 12% of wild type, the hemophilia A phenotype was corrected. This study shows the possibility for future expansion of iPS cells into the clinic. Furthermore, Zhang *et al*[24] differentiated iPS cells into mature pancreatic insulin-producing cells. This work not only provides a new model with which to study the mechanism of human pancreatic specialization and maturation *in vitro* but also enables the possibility of utilizing patient-specific iPS cells for the treatment of diabetes[24].

Since the iPS cell researchers won the Nobel Prize, Japan has pioneered the clinical application of these cells in rare diseases. Previous success at treating degenerative disorders with gene therapy led researchers to pursue gene therapy for degenerative eye disorders[41,42]. In a proof-of-concept study, they showed that iPS cells developed a structured outer nuclear layer with complete inner and outer segments

Table 1 Current cell mediated gene therapy protocols in clinical and R and D stages

Target disease	Stage	Gene modification	Target cell	Ref.
Hemophilia A	Preclinical	Oct4, Sox2, Klf4	Fibroblast	Xu *et al*[40]
ALS	Preclinical	Oct4, Sox2, Klf4, c-Myc	Fibroblast	Dimos *et al*[55]
Alzheimer	Preclinical	Oct4, Sox2, Klf4, c-Myc	Fibroblast	Israel *et al*[46]
ALS	Preclinical	Oct4, Sox2, Klf4	Fibroblast	Chen *et al*[54]
Cardiac failure	Preclinical	Gata4, Mef2c, Tbx4	Fibroblast	Ieda *et al*[22]
Diabetes	Preclinical	Oct4, Sox2, Klf4	Fibroblast	Zhang *et al*[24]
Schizophrenia	Preclinical	Oct4, Sox2, Klf4, c-Myc	Fibroblast with 15q11.2 del	Yoon *et al*[12]
Barth syndrome	Preclinical	Oct4, Sox2, Klf4, c-Myc	Fibroblast	Wang *et al*[52]
Parkinson's disease	Preclinical	Oct4, Sox2, Klf4, c-Myc	Fibroblast	Chung *et al*[50]
Macular degeneration	Phase Ⅰ	Oct4, Sox2, Klf4, c-Myc	Fibroblast	Kamao *et al*[14]
Degenerative arthritis	Phase Ⅱ/Ⅲ	TGF-β1	Chondrocyte	Ha *et al*[15]
X-linked adrenoleukodystrophy	In the clinic	ABCD1	$CD34^+$ Bone marrow cell	Cartier *et al*[36]
Wiskott-aldrich syndrome	In the clinic	WASP	$CD34^+$ Bone marrow cell	Aiuti *et al*[35]
Metachromatic leukodystrophy	In the clinic	ARSA	$CD34^+$ Bone marrow cell	Biffi *et al*[37]

ALS: Amyotrophic lateral sclerosis; TGF-β: Transforming growth factor-beta.

in 3D sheets. The authors also observed host-graft synaptic connections by immunohistochemistry. Eventually, they characterized and developed a human pluripotent stem cell-derived retinal pigment epithelium cell sheet for use in clinical trials. A clinical trial on macular degeneration is ongoing in Japan[43,44]. In their research on retinitis pigmentosa, the authors generated patient-derived iPS cells that recapitulated the disease phenotype and expressed markers of cellular stress. This research created the opportunity for understanding the disease by creating iPS cell models of degenerative disorders[45].

Recent trends in iPS cell research have allowed a shift to using iPS cells to model disease[46]. Degenerative disorders have been the main focus of this research. iPS cells use the same transcriptional network as ESCs to generate neuro-epithelia. iPS cells can be differentiated into functionally appropriate neuronal cell types over the same developmental time course as human ESCs in response to the same set of morphogens[47]. Therefore, researchers have suggested the possibility of employing human iPS cells in pathological studies, therapeutic screening and autologous cell transplantation[48]. Glial progenitor cells have also been suggested to be useful for modeling neurological diseases[49]. Chung *et al*[50] exploited the mutational correction of iPS cells and conserved the proteotoxic mechanisms to reverse the phenotypic response to α-synuclein, a key protein involved in Parkinson's disease[50,51]. In 2014, iPS cell-based *in vitro* modeling of cardiomyopathy was also reported[52]. The authors combined patient-derived and genetically engineered iPS cells to elucidate the pathophysiology underlying the cardiomyopathy of Barth syndrome, a mitochondrial disorder caused by mutation of the gene encoding taffazzin (TAZ). Using Barth syndrome iPS-cell-derived cardiomyocytes, they defined metabolic, structural and functional abnormalities associated with *TAZ* mutation. Another interesting study reported modeling the genetic risk of schizophrenia by using iPS cells[12]. In addition, pathways that are disrupted in motor neurons in human ALS were also identified through the genetic correction of mutant *SOD1*[53]. The authors combined the reprogramming and differentiation of iPS cells with genome engineering and RNA sequencing to define the transcriptional and functional changes that are induced in human motor neurons by mutant *SOD1*[54,55].

Human pluripotent stem cells hold great potential for regenerative medicine, but the available cell types have limitations[56]. Recently, an international cell bank for iPS cells was created. In addition, Ma *et al*[57]'s approach of genome-wide analysis may be the future of iPS cell characterization. These authors examined the DNA methylation and transcriptome profiles of ESCs and somatic nuclear-transferred cells. They observed that the DNA methylation and transcriptome profiles of iPS cells retained residual DNA methylation patterns typical of parental somatic cells. Therefore, they concluded that human somatic cells can be accurately reprogrammed to pluripotency by somatic cell nuclear transfer and are therefore ideal for cell replacement therapy. This approach has opened an interesting field for the characterization of iPS cells in the future. A summary of selected clinical and preclinical research programs involving cell-mediated gene therapy is presented in Table 1.

SAFETY AND EFFICACY ISSUES

The fact that chondrocytes can be induced to differentiate into fibroblasts during cell culture spurs questions about potential differentiation issues. The continuation of the cell division process relies on the interaction of the cells with a microenvironment that consists of other cells and the extracellular

matrix[58]. Cells respond to a variety of growth factors. The autocrine and paracrine modes of cell stimulation are key elements in this process. The differentiation of murine C3H10T1/2 mesenchymal cells into chondrocytes in response to bone morphogenic protein has been observed[59]. Similar papers have reported on the differentiation of specific cells into cells with different characteristics[60]. Dedifferentiation-associated changes in morphology and gene expression have also been reported in primary human chondrocytes in culture[61]. These observations make the transforming growth factor-beta (TGF-β) superfamily proteins possible target modifiers of cartilage and bone formation. However, there is also a concern with regard to safety and efficacy because changes in other characteristics can change the properties of a cell therapy and therefore make the cells unacceptable as a treatment. With respect to efficacy, these findings brought forth the need to identify clear biomarkers to identify and characterize cells for potential transplantation.

Some researchers previously thought that MSCs do not induce immunogenicity. A report showing that human stem cells modulate the allogeneic immune response made people change their view about this concept[62]. In 2005, several papers were published relating to the immunogenicity of mesenchymal stem cells. At this time, there was some confusion regarding the immunogenicity of mesenchymal stem cells. The T cell response to allogeneic human MSCs was evaluated for immunogenicity, tolerance and suppression[63]. The authors concluded that MSCs can initiate the activation of T cells but do not elicit a T cell proliferative response because of an active suppression mechanism. These data were in support of Aggarwat and Pittenger's work that was previously cited. However, somewhat contradictory data were also reported[64,65], namely that MSC immunogenicity was increased upon differentiation after transplantation into human and murine ischemic myocardium. This paper indicated that differentiation of cells after injection into organs can cause problems, and the authors recommended a requirement for immunosuppressive therapy. They also indicated that allogeneic MSCs are not intrinsically immune privileged and that allogeneic MSCs can induce a T-cell response under appropriate conditions. In a pig study, intracardiac allogeneic porcine MSCs elicited an immune response despite their low immunogenic profile *in vitro*[66]. This suggests that the *in vivo* characteristics of allogeneic MSCs might differ and emphasizes the importance of pursuing research both *in vitro* and *in vivo*. More specifically, allogeneic MSCs have been shown to induce immunogenicity, which limited their long term benefits for myocardial repair[22]. The authors of this study concluded that the long-term ability of allogeneic MSCs to preserve function in the infarcted heart is limited by a biphasic immune response whereby these cells transit from an immune privileged state to an immunogenic state after differentiation. We believe that not only the cell type but also the injection site is an important factor for immunogenicity.

To determine the potential for the biodistribution of gene-modified cells, the FDA recommends intravenous administration in an animal study to assess systemic distribution and persistence of the cells. Transduced genes can be the target for determining the distribution in cell-mediated gene therapy. After a single intravenous administration, the major organs should be checked to review the clearance of the cells for at least 30 d and for up to three months after injection. At this stage of preclinical development, the maximum tolerated dose should be calculable[67]. For safety and efficacy tests in animals, it is the standard practice to perform testing in two animal species. Clinical and histological analyses are included among the safety and efficacy tests, and treatment-related and dose-related toxicities can be assessed.

Single and multiple dose toxicity testing with up to a 1-year follow-up period may be required depending on the nature of the disease and the mode of treatment. The no-observed-adverse-effect level can be evaluated with these studies[68]. This can be defined as the highest experimental point that is without adverse effects, although it does not address risk based on toxicologically relevant effects nor does it consider the progression of effects with respect to the duration or dose. In characterizing cell lines, the FDA may request tumorigenicity testing in accordance with the "Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals" guidelines. Further studies of gene-modified cells may be recommended to assess the potential for these cells to adversely differentiate for 6 wk or up to 6 mo. Additional studies can be requested depending on the characteristics of the treatment modality. For allogeneic cells, studies to determine immunogenicity are required and may include assessing the HLA antigen expression by the cells, anti-HLA antibodies, the T cell response and multiple cytokines in animals and/or humans.

TUMORIGENICITY

Karyotype analysis by using chromosome banding is the standard method for identifying numerical and structural chromosomal aberrations. A novel karyotyping technique, termed spectral karyotyping, was developed to increase the sensitivity of karyotyping[69]. After reports of problems with viral vectors in gene therapy[70,71], this karyotyping issue becomes important in the cell-mediated gene therapy field. The FDA had concerns regarding injecting cells

with chromosomal abnormalities in human clinical trials[72,73]. This concern arose as a result of the leukemia observed in the clinical trial of X-SCID patients. Oncogenesis or tumorigenicity has been considered a clinical hurdle for pluripotent stem cell therapies by certain authors[74,75]. These authors identified the seven risks of iPS cell therapy: integration of the gene into the host cell, chromosomal damage, clonal selection, incomplete programming, failure to silence pluripotent networks, DNA damage during cell culture and aberrant regulation of the imprinting process[76]. Adeno-associated virus was considered a relatively safe vector, but several authors still consider that this vector can induce chromosomal abnormalities[77,78]. For cells in which there is a karyotyping change after transfection[79], the FDA recommends rendering the cells replication incompetent (*e.g.*, *via* irradiation) prior to their being used in a clinical setting.

In 2003, one of the most successful, problematic and influential cases of gene therapy was reported: LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1[4,80]. The authors previously showed the correction of X-linked severe combined immunodeficiency in 9 out of 10 patients by using retrovirus-mediated gene transfer to autologous $CD34^+$ bone marrow cells. However, 3 years after this gene therapy, uncontrolled, exponential clonal proliferation of mature T cells occurred in two patients. This incident was reported as an occurrence of leukemia following gene therapy[81]. Although these patients overcame the problem, the impact of this incident was sufficient to change the arena in terms of regulation, investment and research effort. Baum *et al*[38] (2003) reviewed the side effects of this technology that are related to target cell manipulation, vector production, transgene insertion and expression, selection procedures for transgenic cells and immune surveillance. This unfortunate leukemic side effect of gene therapy can be used as a learning tool for developing safer, more effective gene therapies in the future.

To overcome these potential side effects of gene therapy, many possible solutions have been devised[17,82]. Chromosomal insulators, co-transfection of suicide genes under control of an inducible promoter, conditional expression of the transgene only in appropriate target cells, targeted transduction, cell type specific expression, targeted local administration, splitting of the viral genome, and site-specific insertion of the retroviral vector have all been proposed. A global iPS cell library has been proposed to preselect the donor genotype for immunological matching. This approach was proposed for immune compatibility[83], and similar approaches to reducing the potential for tumorigenicity can be devised. Individual cell lines that have undergone insertion site[84] and gene expression analyses and have otherwise been evaluated for tumorigenicity and determined to be non-tumorigenic can be identified and used for other clinical trials. To reach this goal, more clinical and *in vitro* and *in vivo* non-clinical data should be generated.

MECHANISM OF ACTION AND BIOMARKER

Some mesenchymal stem cell populations are relatively easy to harvest: for example, bone marrow MSCs. However, clinical application of these cells without preclinical evidence of efficacy and safety is unacceptable and will delay the development of clinically useful therapies[16]. In particular, we need to better understand the mechanisms of action of stem cells after transplantation and learn how to control stem cell proliferation, survival, migration, and differentiation in pathological environments[33]. Stem cell-based approaches have received much hype as potential treatments for neurodegenerative disorders. Indeed, they showed that transplantation of stem cells in an animal model of neurodegenerative disease can improve function by replacing lost neurons and glial cells and by mediating remyelination, trophic actions and modulation of inflammation. However, a clear understanding of the mechanism of action and a description of a biomarker for demonstrating efficacy should be devised for use in clinical trials.

Proposals submitted to the FDA for MSC-based products are undergoing a rapid expansion and are characterized by increased variability in donor and tissue sources, manufacturing processes, proposed functional mechanisms and characterization methods[85]. Mendicino *et al*[85] attempted to elucidate the FDA's current perspective on the characterization of MSC-based products for clinical trials. The FDA proposed to characterize cell surface markers for IND applications of MSC-based products. Additionally, they found significant heterogeneity in the description of MSC bioactivity characterization in situations in which a candidate marker for a given assay has been defined. It remains unclear which particular set of markers will be sufficient to describe this complex and heterogeneous product class. Markers that can predict potential therapeutic benefit may allow the correlation of MSC characterization data with clinical data as they become available. For iPS cells, it can be more complicated.

iPS cells have been enthusiastically presented as a tool for aiding drug discovery by drug discoverers and commercial reagent and service providers alike[86]. The future trend for research in this area relates to the maintenance of pluripotency and cellular reprogramming[87]. To bring a product to the clinic, licensing, intellectual property and legal issues are also important. At this time, there may not be enough

information for financial and business development professionals to evaluate the marketability of these products. Issues with developing cellular products and related assay development are only some of the challenges that are faced when integrating iPS cells into drug discovery. However, the potential for these cells to markedly improve the symptoms of patients motivates those involved in drug discovery to invest time and money to advance this technology.

MANUFACTURING OF CELL PRODUCTS

One of the hurdles limiting the development of cellular therapies is the difficulty of cell expansion and the mass production required for a commercial product. The manufacturing technology for these products has developed in parallel with research on these products. Since the initial reports of the use of disposable bioreactors for cell culture that use wave-induced agitation[88,89], many innovations in single-use bioreactors have been reported[90]. The technology ranges from 175 cm^2 tissue culture flasks to methods for scalable expansion of the cells[91]. Routine commercial and clinical applications of human cells and their progeny require increasing cell quantities that cannot be provided by conventional adherent culture techniques. Straightforward protocols for the expansion of undifferentiated ESC and iPS cells in suspension culture have been developed and reported. For ESCs, different methods for expansion to improve the culture conditions have been evaluated[92]. A scalable GMP-compliant suspension system for human ESCs was reported by Chen *et al*[18,93]. This suspension culture system provides a powerful approach for scaling-up the expansion of hESCs under defined, serum-free conditions for clinical and research applications[94].

Disposable bioreactors are already widely accepted and in use for protein manufacturing[92,95]. Disposable components and systems are increasingly favored, both for improved process reliability and for the economic advantage they offer. For this reason, many biotech producers of protein molecules are moving to disposable bioreactor modules that are pre-sterilized and meet the applicable regulatory requirements. In 2010, Eibl *et al*[96] published a paper regarding disposable bioreactors, including the current state-of-the-art and recommended applications in biotechnology. In their paper, they provided a summary overview of the disposable bioreactors that were commercially available and described the domination of wave-mixed, orbitally shaken and stirred disposable bench top systems. They concluded that these novel systems are a viable alternative to traditional cell culture bioreactors at the bench top scale.

In 1997, Genzyme gained approval for autologous chondrocyte transplantation. Although this technology was not a commercial success, it fueled the development of mass culture methods for chondrocytes. Not only have chondrocytes been used for the regeneration of cartilage in the knee of patients[97], but they have also been used for intervertebral disc regeneration[98]. To expand these cells for commercial usage, chondrocytes have been cultured and expanded in a microcarrier system[60,99,100]. They investigated human chondrocyte expansion in four macroporous gelatin microcarriers using two manufacturing processes that differed with respect to the amount of emulsifier used during the initial preparation and the gelatin cross-linking medium. The authors observed a strong chondrocyte donor effect during the initial expansion phase. The final cell yield differed significantly between the microcarriers, and the result indicated that manufacturing differences affected chondrocyte densities.

For iPS cells, large-scale culture relies on the combined use of multiple growth components, including media containing various growth factors, extracellular matrices, 3D environmental cues and modes of multicellular association[18]. Chen *et al*[18] describe the criteria, considerations and suggestions for achieving optimal iPS cell growth.

RELEASE TEST AND QUALITY CONTROL

As for chemical drugs, biological drugs should be consistent, active, pure, toxin-free and stable. Growth, harvesting and distribution into vials of cells should be conducted in a controlled GMP environment (Table 2). Throughout the manufacturing process, in-process and release testing should be performed to ensure that each batch of product is safe and consistently meets the criteria for identity, purity and potency prior to administration to humans. The characteristics, viability and potency of the cells should be evaluated. The identity of the cells can be defined by morphologic examination and by markers of specific functions. For example, Type Ⅱ collagen and GAG production are relevant criteria for cartilage cells. Techniques such as immunohistochemical staining and RT-PCR are also good tools for this purpose. Cell potency can be measured by a quantitative analysis such as ELISA for specific therapeutic protein production, or by other product specific assays depending on the therapeutic mechanism. Cell viability and proliferation can be determined by specific staining (*e.g.*, tryptophan blue), MTT assay and/or by automated methods using fluoroscopy. For gene-modified cells generated with a viral vector, replication-competent retrovirus detection may be required as a safety evaluation (Table 3).

The cells should also be tested for contamination by checking sterility, detecting mycoplasma and measuring endotoxin levels. The long-term stability of cells should also be determined under the con-

Table 2 Different types of production methods based on cells needed per batch

Cells/lot (billions)	Cell culture	Harvest	Filling	Freezing
1	T175 flask or hyperflask, 10 layer cell factory	Bucket centrifugation	Hand/manual fill	Bench top control rate freezer
10	10 layer cell factory, hyperstack-12, Xpansion systems	Bucket centrifugation	Semi automated fill machine or hand/manual fill	Bench top control rate freezer
50	Hyperstack or Xpansion, cell cube	Tangential flow filtration, continuous centrifugation	Automated fill machine	Large scale control rate freezer
100	Factory automation of Xpansion or Hyperstack technologies	Tangential flow filtration of continuous centrifugation	Automated fill machine	Large scale control rate freezer
500	Bioreactors using microcarriers	Continuous centrifugation	Automated fill machine	Scale out large scale control rate freezer

Table 3 Suggested release tests advised by Food and Drug Administration for cell product

Category	Test	Key measurement
Sterility	Sterility	Microbiological testing
	Mycoplasma	After pooling of cultures but before washing
Identity	Identity	Cell identity and assess heterogeneity
Purity	Impurity	Residuals contaminants
	Endotoxin	Contaminants during the process
Potency	Specific to product	Relevant function of the cells
Other testing	General safety	Cellular therapy products are exempt
	Viability	Generally > 70%
	Cell number	Minimum and maximum cell number
Title 21 of the code of federal regulations applied to cell therapy products		
The tissue rules: Part 1271		
The biologics requirements: Part 600 and 610		
The investigational new drug requirements: Part 312		
The drug manufacturing requirements: Parts 211 and 212		

ditions of storage, use and shipping. This may require testing under different conditions such as room temperature, refrigeration, and frozen (at temperature ranges corresponding to dry ice and the vapor phase of liquid nitrogen storage).

IN THE CLINIC

One of the first routes of delivery of cellular therapy was intra-articular because of some inherent advantages[101,102]. Some of the benefits of local delivery over systemic delivery include increased bioavailability, fewer adverse events, and lower total drug costs. Additionally, the intra-articular injection of cell therapies has advantages over intravenous administration in that systemic exposure is reduced and exposure to the antigen surveillance system is limited[103]. For these reasons, we have developed a cell mediated gene therapy for degenerative joint disease[104-106] and are currently conducting clinical trials with normal and engineered chondrocytes (called TG-C) injected into the knee joint for the treatment of osteoarthritis (Figure 1).

We are currently completing a Phase Ⅱ study entitled 'A Phase Ⅱ Study to Determine the Efficacy and Safety of Allogeneic Human Chondrocytes Expressing TGF-β1 in Patients with Grade 3 Chronic Degenerative Joint Disease of the Knee' in the United States. Phase Ⅲ testing of this product is currently ongoing in South Korea. To develop this product for clinical use, it was necessary to establish the stability of the product under frozen storage conditions and at room temperature for injection. The temperature of the product as it was transferred from manufacturing to storage to the clinic and within the clinic was carefully controlled and monitored. To date, more than 220 patients have been injected with TG-C. A schematic diagram of the manufacturing and injection processes is presented in Figure 1. The gene-modified cells in TG-C are irradiated to render them replication incompetent. These cells therefore produce the active TGF-β protein for only two weeks. These irradiated cells also produce Type-Ⅱ collagen and glycosaminoglycan (GAG) by an autocrine mode of action and induce normal chondrocytes to produce Type II collagen and GAG by a paracrine mode of action in response to the TGF-β protein produced. The releasing tests during the manufacturing of TG-C are described in Table 4.

As TG-C is an allogeneic product; all patients in this study are being monitored for an immune response. To date, no immune response or serious adverse events attributable to TG-C have been observed. The decreased HLA-type antigenicity of the cells and the relatively immune privileged nature of the intra-articular injection site are thought to be contributing factors to the lack of immune response.

CONCLUSION

Our understanding of the nature of cell-based products and the diseases that they are intended to treat is being increased empirically: that is, through the scientific process of trial and error. Human clinical trials with cutting edge technology and novel products are part of that process and accordingly have their failures and successes, which are necessary if we are to learn and improve our understanding. Industry's perspective of cell-based therapy has changed in response to scientific developments and discoveries[41]. Although there is a long way to go, a tremendous amount of clinical data have already

Table 4 TG-C release test

Test	Method	Specification
Release tests and specifications for hChonJ cell		
Identification		
Cell growth and morphology	Visual (test code 30117)	Fibroblast-like: spindle shaped/bipolar or multipolar cells
Viability	Trypan blue dye exclusion (test code 30458)	> 70%
Mycoplasma	1993 Points to consider (test code 30055)	Negative
RAP test	*In vivo* and ELISA (test code 30163)	Negative
Sterility (direct inoculation method)	21 CFR 610.12 (test code 30744)	Negative
Endotoxin	LAL (test code 37653)	< 5 EU/mL
Release tests and specifications for hChonJb#7 Cell		
Identity		Epithelial-like: fried egg or polygonal-shaped appearance
Cell growth and morphology	Visual (test code 30117)	
Type II collagen assay	RT-PCR (test code 30412)	Type II collagen present
TGF-β1 presence	PCR (test code 30959)	TGF-β1 present
Potency (TGF-β1 assay)	ELISA (test code 30444)	1-50 ng/10^5 cells/24 h
Viability	Trypan blue dye exclusion (test code 30458)	> 70%
RCR	Co-culture of end of production cells (test codes 30628)	Negative
	Supernatant amplification (test code 30633)	Negative
Mycoplasma	1993 points to consider (test code 30055)	Negative
Rat antibody production	*In vivo* and ELISA (test code 30163)	Negative
Endotoxin	LAL (test code 37653)	< 5 EU/mL
Sterility	21 CFR 610.12 (test code 30744)	Negative

RCR: Replication competent retrovirus; LAL: Limulous amoebocyte lysate; RT-PCR: Reverse transcription polymerase chain reaction; CFR: Code of federal regulation; RAP: Rat antibody production.

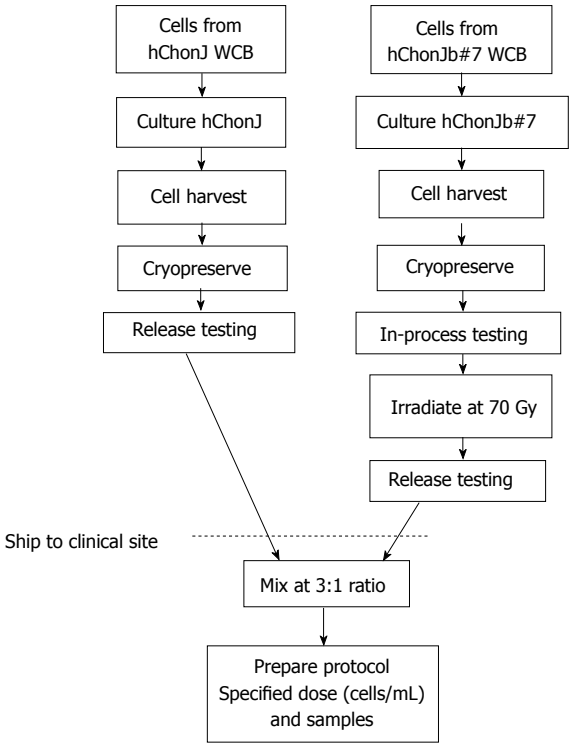

Figure 1 Schematic figure of TG-C manufacturing. WCB: Working cell bank.

been generated. In this review, we summarized the ongoing scientific effort to move cell mediated gene therapy into the clinic. While some may still believe that such therapy is far from the clinic, it is our belief that now is the time for physicians to understand and embrace this new technology.

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REVIEW

Clinical applications of high-throughput genetic diagnosis in inherited retinal dystrophies: Present challenges and future directions

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Abstract

The advent of next generation sequencing (NGS) techniques has greatly simplified the molecular diagnosis and gene identification in very rare and highly heterogeneous Mendelian disorders. Over the last two years, these approaches, especially whole exome sequencing (WES), alone or combined with homozygosity mapping and linkage analysis, have proved to be successful in the identification of more than 25 new causative retinal dystrophy genes. NGS-approaches have also identified a wealth of new mutations in previously reported genes and have provided more comprehensive information concerning the landscape of genotype-phenotype correlations and the genetic complexity/diversity of human control populations. Although whole genome sequencing is far more informative than WES, the functional meaning of the genetic variants identified by the latter can be more easily interpreted, and final diagnosis of inherited retinal dystrophies is extremely successful, reaching 80%, particularly for recessive cases. Even considering the present limitations of WES, the reductions in costs and time, the continual technical improvements, the implementation of refined bioinformatic tools and the unbiased comprehensive genetic information it provides, make WES a very promising diagnostic tool for routine clinical and genetic diagnosis in the future.

Key words: Next generation sequencing; Identification of novel causative genes; Inherited retinal dystrophies; Genetic diagnosis; Whole exome sequencing

Core tip: This review focuses on the application of next generation sequencing (NGS)-based methods [whole genome sequencing, whole exome sequencing (WES), targeted exome sequencing] for genetic diagnosis and novel gene identification in hereditary retinal dystrophies. Advances over the last two years concerning NGS accuracy, reliability, development of bioinformatics tools, together with the drop in costs and time required for the

analysis have allowed thirty novel genes to be identified, plus a large number of new mutations in previously reported genes. NGS techniques (particularly WES) are revolutionizing genetic diagnosis and have clear applications in clinical practice, helping to pave the way for personalized medicine. Present challenges and future directions are also discussed.

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INTRODUCTION

Inherited retinal dystrophies (IRDs) consist of a group of highly heterogeneous disorders at the genetic and clinical level. Until recently, the ever increasing number of causative genes (more than 200 so far) and mutations (more than 5000) (https://sph.uth.edu/retnet/) posed an enormous challenge for molecular diagnosis and limited the effectiveness of conventional mutational screening. However, the advent of next generation sequencing (NGS) technologies has completely revolutionized genetic diagnosis[1,2]. Since the first application of exome sequencing using NGS to identify the causative gene in a very rare autosomal recessive disorder[3], more than 150 new Mendelian disease genes have been reported using similar approaches[4]. Focusing on *IRD* genes, NGS approaches [whole exome sequencing (WES), or whole genome sequencing (WGS)] have rapidly identified new causative genes, increasing the success rate of molecular diagnosis from 40% to almost 80%, depending on the number of cases analysed and the informativity of the family[5-7]. It is foreseeable that NGS-based methods will be the technique of choice for future routine DNA diagnosis in IRDs and similar heterogeneous Mendelian disorders, since accuracy and efficiency increase while costs and time requirements drop continually[8,9].

NGS-BASED DIAGNOSIS

The challenge posed by the molecular diagnosis of heterogeneous disorders prompted researchers to devise novel conceptual and technical approaches to help clinicians classify diseases, inform patients and families, and offer genetic counselling and prenatal diagnosis. The approaches they devised also provide the basis for a more efficient molecular-based therapy. Since the draft of the human genome was published, several high-throughput techniques have been devised. In the field of IRDs, commercially available microarrays for direct mutational screening (http://www.asperbio.com/asper-ophthalmics), customized resequencing microarrays (restricted to several large diagnostic centres/units)[10] and whole genome or targeted gene *SNP* genotyping arrays for linkage analysis (cosegregation and homozygosity studies) have paved the way either for mutation screening in reported known genes, or for the highlighting of new loci for candidate causal genes[11]. Diagnostic efficiency ranged from 15%-44% in direct mutation screening microarrays-depending on the pathogenic allelic frequencies in the population, to 30%-70% for resequencing microarrays-depending on the number of genes included and the sequence quality[12]. Indeed, direct analysis of known mutations and genes requires constant updating, and even so, many mutations remain undetected because they are private[13]. Moreover, SNP genotyping for homozygosity mapping and cosegregation analysis has become a very informative genetic tool in many cases[14,15].

WES EFFICIENCY IN THE DIAGNOSIS OF MENDELIAN DISORDERS

A survey of the IRD (syndromic and non-syndromic) genes identified in the last two years (up to 29) showed that all the approaches used to identify them involved NGS. The success of NGS as a diagnostic tool is due to: (1) the power of an unbiased genome scale analysis; (2) the increasing number of databases containing information on SNP allelic frequencies in different populations, which allows rare presumptive mutations to be discriminated from frequent genetic variants; (3) the relative simplicity of the currently standardized protocols; (4) powerful bioinformatics analysis; and (5) the fact that the data gathered is useful on its own.

Nonetheless, additional genetic information is still instrumental to increase the yield of molecular diagnosis since, despite the power of WES, gene identification in recessive IRDs (24) is far more successful than it is in dominant cases (5) (Table 1). The difference in this outcome is to be expected, since finding the relevant causative mutation in heterozygosis amidst the great number of genetic variants identified by WES (more than 20.000 on average) is not a straight-forward endeavour[16]. In contrast, the requirement of a double heterozygous mutation (or even homozygosis) in the same gene for recessive cases, greatly diminishes the noise associated with such massive collection of data, and thus the number of putative causative genes, after the data has been filtered. While WES alone has pinpointed the causative gene in around 30% of the recessive IRD cases (years 2013-2014), adRD causative genes have proved to be more difficult to identify and require a combination of genetic approaches, such as linkage analysis, deletion mapping and targeted capture of candidates, to eventually single out the pathogenic mutation in a novel gene (10%)[17]. The informativity of these genetic approaches has also greatly favoured gene identification in recessive cases (60%)[9] (Table 1).

Table 1 List of retinal dystrophy causative and candidate genes identified in 2013-2014 and the strategy of identification

Gene	Retinal phenotype	Methodological approach
ABCD5	Recessive CRD, spastic parapesis, white matter disease	Homozygosity mapping combined with WES[25]
ADAMTS18	arRD early onset	Homozygosity mapping combined with WES[43]
ARLBP2	arRP	Homozygosity mapping combined with WES[44]
BBIP1	arBBS	WES[45]
C12orf65	Recessive optic atrophy, spastic paraplegia and neuropathy	Linkage mapping WES[46,47]
C21orf2	Recessive CRD	Homozygosity mapping combined with WES[25]
CSPP1	Recessive JS	WES[48-50]
DHX38	arRP (early onset with macular coloboma)	Homozygosity mapping combined with candidate gene approach[51]
DTHD1	Recessive LCA, myopathy	Homozygosity mapping combined with WES[25]
EMC1	arRP	Homozygosity mapping combined with WES[25]
GDF6	arRD	Candidate gene sequencing[52]
GPR125	arRP	Homozygosity mapping combined with WES[25]
HK1	adRP, nonspherocytic hemolytic anemia, and neuropathy	Linkage mapping and WES[53]
IFT27	arBBS	Homozygosity mapping combined with candidate gene approach[42]
IMPG1	Dominant MD	Linkage mapping
	Recessive MD	WES and candidate gene sequencing[54-56]
ITM2B	Dominant RD, dementia	WES combined with linkage mapping[57]
KIAA1549	arRP	Homozygosity mapping combined with WES[25]
KIZ	arRP, arCRD	WES[58]
LRIT3	arCSNB	WES[59]
MVK	arRP, recessive mevalonic aciduria	WES[60]
NEK2	arRP	WGS[6]
NR2F1	Dominant optic atrophy, intellectual disability	Deletion mapping
		WES and deletion mapping[61,62]
PCYT1A	arCRD with skeletal disease	WES and targeted candidate gene sequencing[63,64]
POC1B	Recessive CRD	WES[65]
PRPF4	adRP	Targeted capture NGS[41]
RAB28	arCRD	Homozygosity mapping combined with WES[66]
RDH11	arRP	WES[67]
SLC7A14		WES[68]
TUB	arRD with obesity	Homozygosity mapping combined with WES[69,70]
TTLL5	Recessive cone and CRD	WES[71]

ar: Autosomal recessive; ad: Autosomal dominant; CRD: Cone-rod dystrophy; RD: Retinal dystrophy; RP: Retinitis pigmentosa; BBS: Bardet-Biedl syndrome; JS: Joubert syndrome; LCA: Leber congenital amaurosis; MD: Macular dystrophy; CSNB: Congenital stationary night blindness; WES: Whole exome sequencing; WGS: Whole genome sequencing; NGS: Next generation sequencing.

WGS *VS* WES *VS* TARGETED EXOME SEQUENCING IN ROUTINE DIAGNOSIS

At present, many groups rely on NGS-based techniques for genetic diagnosis of IRDs (and other Mendelian disorders)[18]; WES is the most common of these approaches (Table 1). Nonetheless, a few attempts using WGS or targeted exome sequencing have also been reported. In the latter, long polymerase chain reaction amplimers spanning the exons of reported *RP* genes[17] or lately, customized exome capture of the coding exons of a selected set of genes, have been developed with a wide range of diagnosis success (40%-80%)[19-22]. Customized approaches allow different degrees of refinement and are dependent on the optimization of the techniques and the prioritization of the type of mutations to be identified. For instance, if copy number variants (CNVs) are suspected, the coverage and high quality of the reads constitute one focus of improvements[23]. Nonetheless, the cost of customized capture arrays for a list of causative disease genes is still much higher than that of conventional capture arrays for WES, and the genetic information provided is limited to the candidates analysed. Mutations in non-selected or previously unreported genes will remain undetected. WES is becoming the most popular choice, particularly since the reliability of the technique and the quality of the analysis software have greatly increased (though there is still room for improvement), and microRNAs and transcript untranslated regions are also included in some exon capture array versions[24]. Overall, the reported success rate for IRD diagnosis in randomly selected familiar and simplex cases account for 74%-80% of the mutation pool in some studies[25].

WGS for the molecular diagnosis of retinal dystrophy (RD) has been attempted with moderate success (56% of molecular diagnosis and the identification of a new causative gene)[6]. The main reason behind this massive genome sequencing approach was to analyse coding and noncoding regions in order to detect structural and copy number variants and to evaluate highly polymorphic SNPs. Although the WGS reported in this work facilitated the detection of two structural pathogenic variants (which would probably have escaped detection with WES), the fact that no pathogenic mutation in the large noncoding fraction of the genome was identified, and that 7 out of 16 patients remained undiagnosed after the considerable effort required to screen the whole genome, pose some questions about the suitability of WGS in routine RD genetic diagnosis.

Table 2 Possible genetic cause in undiagnosed patients after whole exome sequencing

Genetic variants	Technical restrains	Alternative approaches
MicroRNAs and lncRNAs	Not sequenced	Inclusion in the capture
Deep intronic	Not sequenced	RNASeq
		WGS
		Targeted re-sequencing
Variants in regulatory regions	Not sequenced	WGS
		Targeted re-sequencing
Large deletions	Mostly undetected	Detectable in homozygosis
		In heterozygosis can be detected in comparison with controls (if high coverage)
		WGS
		Targeted re-seq
CNVs	Mostly undetected	High coverage
		WGS
		Targeted re-seq
		CGH
Pathogenic trinucleotide repeats	Short reads not covering the whole expansion	Triple repeat based PCR
Structural chromosomal variants	Undetectable	FISH
		WGS
		Targeted Long PCR coupled to NGS
Aneuploidies	Undetectable	Conventional cytogenetics FISH
		WGS

lncRNAs: Long non-coding RNAs; CNVs: Copy number variants; RNASeq: RNA sequencing; WGS: Whole genome sequencing; CGH: Comparative genome hybrdization; PCR: Polymerase chain reaction; FISH: Fluorescent *in situ* hybridization; NGS: Next generation sequencing.

PRESENT LIMITATIONS OF WES IN GENETIC DIAGNOSIS

Although NGS-based methodologies allow comprehensive genomic analysis on an unprecedented scale, none of them is free from technical constrains. The conventional WES diagnostic strategy is based on exon capture by nucleic acid hybridization. Even though continuous improvements to the method have continually been implemented (capture optimization, and higher coverage and sequencing accuracy), not all the pathogenic mutations can be detected[26]. One main issue that needs to be addressed without delay is the implementation of unified bionformatics tools for accurate mapping and reliable variant-calling software, particularly for small indels (insertions/deletions) and CNVs[8,27]. Other pending issues include the detection of mutations in genomic regions that escape the capture methods currently available, such as small exons, regulatory regions, deep intronic variants and chromosomal structural variations that do not affect exons (inversions and deletions) (Table 2).

When the main focus is basic research and the analysis is restricted to a small genomic region highlighted by linkage or homozygosity, custom targeted genome re-sequencing is a viable alternative to WES[22,28]. However, for daily routine diagnosis, standard WES offers an appealing compromise between cost, time, comprehensiveness of data processing and efficiency.

UNEXPECTEDLY HIGH NUMBER OF IRD RECESSIVE PATHOGENIC VARIANTS IN THE CONTROL POPULATION

Knowledge of the underlying genetic structure of human populations provides very valuable clues to help successfully identify pathogenic genes[25,29], particularly in highly consanguineous cohorts where homozygosity by descent is suspected. Current data indicates that this assumption should be extended even in the absence of a positive family history, where both parents may be heterozygous for the same pathogenic allele. Not only may the unsuspected homozygosity of pathogenic alleles reveal a founder effect-which is informative in itself- but it is also one of the most useful genetic assumptions that can lead to the identification of novel causative alleles after WES[25,30].

Notably, the wealth of genome information gathered by WES suggests that control individuals carry 10-20 pathogenic recessive mutations causative of Mendelian disorders[3]. RD stands out as one of the most highly genetically heterogeneous monogenic disorders, and when we focus on the IRD causative genes-even when only null alleles are considered-22% of the control population (1 in 4-5 individuals) is heterozygous for at least one pathogenic mutation[31]. This high prevalence is still an underestimate because missense and splicing mutations have not been included, and neither have all the *IRD* genes been identified, which overall would probably account for 1 in 2 control individuals carrying a pathogenic recessive *RD* mutation. Such a high frequency of unaffected carriers has an important impact on genetic diagnosis since: (1) consanguinity would increase the risk of blindness in the offspring; (2) the comparison of a newly identified genetic variant with control individuals in databases to assess pathogenicity could be misleading; and (3) many patients would by chance bear an additional pathogenic allele besides the causative mutations, which would hamper the molecular diagnosis. This last point would lead to

Table 3 List of prioritized candidates according to the clinical phenotype or X-linked pattern of inheritance

Main candidate gene	Disease
CNGB3, CNGA3	Achromatopsia
RHO	adRP
VMD2	Best disease
CYP4V2	Bietti crystalline dystrophy
RDS/PRPH2	Central areolar choroidal dystrophy
CHM	Choroideremia
LRPO5, FZD4, TSAPN12	Familiar exudative vitreoretinopathy
RDH5, RLBP1	Fundus albipunctatus
NR2E3	Goldman-Favre-Enhanced S-cone syndrome
CEP290	LCA
MFRP	Nanophthalmia
NDP	Norrie disease
SAG	Oguchi disease
RS1	Retinoschisis
RECQL4	Rothmund-Thompson syndrome
ABCA4, RDS/PRPH2	Stargardt disease
USH2A	Usher syndrome
VCN	Wagner syndrome
RPGR	XLCD, XLCRD
RPGR, RP2	XLRP, RP simplex

adRP: Autosomal dominant retinitis pigmentosa; LCA: Leber congenital amaurosis; XLCD: X-linked cone dystrophy; XLCRD: X-linked cone-rod dystrophy; XLRP: X-linked retinitis pigmentosa.

false assumptions of dominant effects of recessive alleles, and explain compound heterozygosis in some consanguineous pedigrees, and open the can of worms of digenic inheritance[31]. In addition, reports of the synergic addition of pathogenic alleles in families with several phenotypes are now emerging, which would seem to call for a new conceptual molecular framework for genotype/phenotype correlations.

Another issue revealed by WES when trios (two parental samples in addition to the patient sample) are analysed is the unexpectedly high frequency of *de novo* mutations, which strengthens the case for reconsidering dominance along side recessivity in simplex cases[22].

PENETRANCE AND EXPRESSIVITY REVISITED: MODIFIER GENES AND WES

Incomplete penetrance and variable expressivity are two genetic phenomena frequently associated with human disease, mainly due to additional genetic factors influencing the final phenotype. From the molecular point of view, genes and proteins interacting and/or regulating the function of the causative gene exert a modifying effect, which could enhance or diminish the pathological outcome in patients bearing the same causative mutation. Identifying the modifier genes has been, and still is, one of the most important challenges in clinical and genetic diagnosis. WES is instrumental in unveiling modifier alleles by direct comparison of the DNA sequences of affected members of the same family, frequently displaying different phenotype severity[6,28,32].

As there is a continual increase in WES-generated data on genetic variants, the pool of modifier genes likewise grows and diagnostic inferences will become more accurate, thus providing the grounds for a more precise prognosis.

EMPOWERING GENETIC DIAGNOSIS OF IRDS BY WES: CANDIDATE PRIORITIZATION CRITERIA, GENETIC INFORMATION AND INTERACTION NETWORKS

So far, NGS-based approaches have mostly been considered for the identification of causative genes in very rare Mendelian disorders when the gene is unknown or mutation screening involves a large number of genes and exons, as is the case of highly heterogeneous diseases. However, after progressive and substantial methodological refining, WES and other NGS-based techniques have leapt from bench to bedside, and are now feasible and attractive alternatives for routine diagnosis. They allow for comprehensive genomic screening, are increasingly affordable and robust, and last but not least, the bioinformatics analysis is becoming more accurate and user-friendly (even though a common standard framework for downstream variant mapping and calling analysis is still lacking)[8,33].

Monogenic disorders caused by mutations in a major gene also will benefit from WES (NGS)-based diagnosis. The costs of Sanger sequencing of a large gene (*e.g.*, *ABCA4*, *CEP290*, *etc.*) are no less than those of full exome sequencing (WES), but the benefits from the comprehensive information gleaned *via* the latter technique are far superior. To mention just a few: minor causative genes are included in the analysis, additional disease causing alleles in modifier genes will be also detected-and so their impact in the population genetic reservoir can be assessed; the molecular basis of rare clinical entities with ambiguous diagnosis can be identified; genotype-phenotype correlations will be more precisely defined; and genetic data on the patient drug response (pharmacogenetics) will be included. Indeed, the analysis of NGS-based data should be prioritized for the genes and variants that are most prevalent for a particular IRD and pattern of inheritance (for instance, in X-linked disorders) (Table 3). If no pathogenic variants are identified, the list of candidates should be expanded following prioritization criteria that include less frequent causative candidates for the same (or similar) phenotype, and finally, all the variants detected by WES under all possible assumptions of Mendelian inheritance should be considered[22,34]. This is particularly relevant in simplex cases and pedigrees with a small number of patients, where dominant *de novo*, X-linked or very rare recessive mutations should be carefully considered. In this context, exhaustive human gene mutation repositories will be extremely informative tools to perform a rapid screening of reported mutations

and thus, simplify the genomic analysis[35].

Indeed, intersection with previous or parallel genetic analysis has been and still is instrumental in pinpointing pathogenic alleles. For instance, SNP genotyping microarrays (6K Illumina) for linkage or homozygosity studies (Table 1 and references therein), or SNP-based cosegregation chips[12] highlight the genetic loci where the gene/mutation identification efforts should be focused. This greatly simplifies matters and provides statistical support for the final molecular diagnosis. In fact, only one third of the novel *IRD* genes identified by NGS over the last two years (Table 1) were discovered without resorting to candidate prioritization using genetic data.

TAKING ON THE FUTURE: PARTS LIST, MAP, DIAGNOSIS, THERAPY

How many novel *IRD* causative genes remain to be identified? Based on the latest NGS results where all new genes explain either rare syndromic disorders with an accompanying IRD phenotype or cases with private mutations affecting very few patients, it seems very unlikely that any novel gene will account for a substantial fraction of unassigned cases[6]. As most technical approaches do not cover the whole panoply of causative mutations, a percentage of mutations in already reported genes might have been overlooked. In fact, transcriptome analysis of healthy human retinas revealed more than one hundred previously unannotated genes, almost 30000 unreported exons (around a 3% increase) and over 20000 3′ and 5′ alternative splicing sites[36]. This unprecedented transcript diversity is a serious challenge for mutation identification, as these regions are not yet included in commercial exome enrichment kits and RNASeq of patient neural tissues is not feasible. Thus, optimization of molecular diagnosis in IRD demands, on the one hand, technical improvements for easy implementation and accuracy, and on the other, the widening of the genomic regions to include novel genes, exons and other regions of interest.

The great wealth of data gathered by conventional as well as high-throughput approaches demands a framework based on systems biology[37]. To this end, unveiling the genetic networks underlying IRDs, although still fragmentary, is a valid approach. Ongoing efforts to integrate interactomes of photoreceptors[38-40] are beginning to show the first promising candidates[41,42]. Further work will allow the translation of this genetic information to the cellular and tissular contexts. Only a comprehensive view of the retinal pathways in health and disease can pave the way for effective therapies.

Finally, although not the main aim of this review, we should not overlook that any genetic laboratory working on WES and WGS data should abide to strict ethical guidelines that concern incidental findings relevant to the patient's health status but unrelated to the focus of the genetic testing.

CONCLUSION

To sum up, the generalized implementation of NGS-based analysis will foster more reliable genotype/phenotype correlations and provide a more holistic view of the genetic factors that cause and modify the severity of the phenotype. Even though 100% diagnosis will not be reached soon and there are new challenges and questions to address, the comprehensive genetic data gathered by NGS will definitely help the clinicians and patients in securing diagnosis, improving prognosis and recommending therapy. It is foreseeable that in the near future, clinical management of the patient will become more personalized and thus more effective.

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Comparison of next generation sequencing-based and methylated DNA immunoprecipitation-based approaches for fetal aneuploidy non-invasive prenatal testing

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Conflict-of-interest: Elisavet A Papageorgiou is currently employed by and owns shares of NIPD Genetics. Philippos C Patsalis also owns shares of NIPD Genetics. Elisavet A Papageorgiou and Philippos C Patsalis have filed a PCT patent application for the MeDIP real time qPCR based NIPD approach (PCT Patent Application No.PCT/1B2011/O00217). Voula Velissariou and Georgia Christopoulou declare that they have no conflict of interest.

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Abstract

Over the past few years, many researchers have attempted to develop non-invasive prenatal testing methods in order to investigate the genetic status of the fetus. The aim is to avoid invasive procedures such as chorionic villus and amniotic fluid sampling, which result in a significant risk for pregnancy loss. The discovery of cell free fetal DNA circulating in the maternal blood has great potential for the development of non-invasive prenatal testing (NIPT) methodologies. Such strategies have been successfully applied for the determination of the fetal rhesus status and inherited monogenic disease but the field of fetal aneuploidy investigation seems to be more challenging. The main reason for this is that the maternal cell free DNA in the mother's plasma is far more abundant, and because it is identical to half of the corresponding fetal DNA. Approaches developed are mainly based on next generation sequencing (NGS) technologies and epigenetic genetic modifications, such as fetal-maternal DNA differential methylation. At present, genetic services for non-invasive fetal aneuploidy detection are offered using NGS-based approaches but, for reasons that are presented herein, they still serve as screening tests which are not readily accessed by the majority of couples. Here we discuss the limitations of both strategies for NIPT and the future potential of the methods developed.

Key words: Next generation sequencing; Differential methylation; Epigenetics; Fetal aneuploidy; Methylation dependent immunoprecipitation; Non-invasive prenatal testing

Core tip: Non-invasive prenatal screening and diagnosis of fetal aneuploidies has been a challenging field

for many researchers. Different methodologies have been developed, mainly based on next-generation sequencing and epigenetic modifications. At present, non-invasive prenatal testing services are offered using next generation sequencing-based technologies which have great potential, but currently they present with certain limitations. Epigenetic approaches may overcome some of these limitations and seem to have promising potential for wider applications.

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INTRODUCTION

Invasive procedures such as chorionic villus sampling (CVS) and amniocentesis are a prerequisite for the prenatal diagnosis of fetal chromosomal abnormalities, either by conventional and/or molecular fetal karyotyping, or other molecular cytogenetic methods. Although these approaches yield accurate results, the rate of pregnancy loss attributed to CVS or amniocentesis is estimated to be 1.0% to 2.0%[1]. This considerable procedure-related risk of pregnancy loss has motivated researchers to try to develop non-invasive approaches in order to provide safer healthcare service.

Since the discovery that fetal cells circulate in the maternal blood during pregnancy[2], numerous researchers worldwide have put great effort towards exploring the possibility of non-invasive prenatal investigation of the fetal genetic constitution. Initially, the focus of investigation was on circulating fetal nucleated cells, where detection of fetal gender and aneuploidies was made possible, mainly by applying FISH subsequent to cell sorting[3-5]. Even though preliminary results were promising, the development of a commercially available application has failed to date, mainly due to certain inherent limitations of the method. Firstly, the rarity of fetal cells in the maternal circulation made it very difficult to isolate a satisfactory number for investigation[4-6]. Secondly, the poor quality of the isolated fetal cells made the application of FISH on the nuclei problematic, resulting in less reliable results. Most importantly, the observation that fetal cells may remain in the maternal circulation for several years after their release, presents a serious problem for non-invasive prenatal investigation of subsequent pregnancies[7,8]. Nevertheless, researchers have not given up this approach entirely and attempts are still being made to overcome limitations[9-13].

The discovery of cell free fetal DNA (cffDNA) in maternal plasma during pregnancy by Lo *et al*[14] in 1997, gave rise to a whole new opportunity in the field of non-invasive prenatal testing (NIPT). Its origin is proven to be either trophoblastic or from embryonic cells in the maternal blood which have undergone apoptosis[15]. It has also been demonstrated that cffDNA is cleared from maternal plasma within a few hours after delivery[16], making its study specific to the current pregnancy. Although cffDNA is detectable from the early stages of pregnancy[17] and increases during its progression[18,19], it is demonstrated to account only for 3.0% to 6.0%[14,20] of total free DNA in maternal plasma. A more recent study utilizing microfluidics, re-estimated the cffDNA fraction to a median of 9.7% in the first trimester[21,22]. The relatively small amount of fetal DNA in maternal plasma presents one of the most serious technical challenges for whichever technology is implemented for investigation. Furthermore, the fact that fetal DNA is 50% identical with that of the mother makes the attempts for fetal aneuploidy testing even more challenging.

CURRENTLY APPLIED METHODS

During recent years independent teams from all over the world have focused on developing methods for NIPT using cffDNA, mainly testing for fetal aneuploidy[23]. Despite applying different strategies including SNP and allelic ratio analyses, none have managed to produce a widely available test, mainly because they depend on informative genotypes or fetal gender[24-26]. On the other hand, next-generation sequencing (NGS) technologies have made great progress in the field, resulting in commercially available NIPT services. In recent years, the use of commercially available tests for NIPT for trisomy 13, 18, 21 and sex chromosome aneuploidies has been introduced into routine antenatal care. Massively parallel direct sequencing reads from a tested chromosome are compared to others with the aid of sophisticated bioinformatics software, resulting in a relative chromosome dose. NGS-based methods are polymorphism independent and have the ability to detect aneuploidies. In a recently published meta-analysis of clinical validation and implementation studies the pooled weighted detection rate for trisomy 21 is reported to be > 99% and the false positive rate to be < 0.01%[27]. Commercially available tests based on NGS technologies have been validated on large numbers of cases and have a very high sensitivity and specificity[28-34] as well (Table 1).

Another promising prospective in NIPT is provided by methylated DNA immunoprecipitation-based (MeDIP-based) approaches. The discovery of fetal-maternal differentially methylated regions (DMRs)[35] has facilitated the development of NIPT strategies by combining MeDIP with other downstream applications. Using the "epigenetic approach", a NIPT method based on MeDIP combined with quantitative polymerase chain reaction which proved to be of high precision in a proof of principle (100% sensitivity, 100% specificity)[36] and larger validation study[37] (100% sensitivity, 99.2%

Table 1 Validation and verification comparison of the most widely used commercially available non-invasive prenatal testing for trisomy 21

Company	Sequenome	Verinata (Illumina)	Ariosa	Natera
Test	"Materni T21 PLUS"	"Verify"	"Harmony"	"Panorama"
Sensitivity	99.6%-99% (209/212)	> 99% (90/90)	100% (81/81)	> 99% (25/25)
Specificity	99.8% (1468/1471)	99.8% (409/410)	99.97% (2887/2888)	> 99% (242/242)
False positive	0.2% (3/1471)	0.2% (1/410)	0.03% (1/2888)	0
No result rate	3.4%	5.8%	4.7%-5.7%	5.4%

specificity) was developed.

ADVANTAGES AND DISADVANTAGES

Approaches based on NGS are extremely powerful. Besides detecting whole chromosome aneuploidy, they have the potential to detect smaller chromosomal imbalances allowing for microdeletion/microduplication syndrome NIPT[38]. However, although NIPT is already commercially available for the detection of a certain number of microdeletion/microduplication syndromes, further validation studies are needed[39]. Taking into account the vast amount of data that NGS is capable of producing, it could be potentially be combined with other methodologies to generate non-invasive fetal whole genome sequencing[40]. As impressive as this may seem at present, it is quite possible that this will materialize in the near future.

The impressive developments of NGS technologies are accompanied however by certain drawbacks. One important limitation is the low level of fetal DNA which is available for testing. This is overcome in MeDIP-based technologies which are based on fetal DNA enrichment, which then increases sensitivity substantially. However, MeDIP by which cffDNA hypermethylated regions are selectively enriched is a stage wherein bias may be introduced, influencing the test results. Therefore, it is very important to carefully select DMRs, optimize this stage and evaluate the overall performance allowing for this. Another drawback of NGS-based approaches is that the equipment/technology required is still not available in all clinical settings, making the service feasible only in large centers, such as those in the United States and China. Furthermore, the requirements for significant infrastructure, complex laboratory procedures, highly trained personnel and challenging bioinformatics analyses make NGS-based technologies costly and complex. In contrast, the "epigenetic approach", uses equipment that is available in most genetic diagnostic laboratories offering established genetic services, it is considerably cheaper and simpler and therefore it may be applied potentially worldwide and offered to a broader population. However, current MeDIP-based approaches focus mainly on fetal trisomy 21 and at present have not yet demonstrated their ability to detect other fetal aneuploidies and submicroscopic aberrations that NGS-based technologies have proven to be capable of detecting. Moreover, large validation studies and future clinical application feedback data are awaited in order to re-evaluate the advantages and disadvantages of MeDIP-based NIPT tests.

THE FUTURE OF FETAL ANEUPLOIDY NIPT

Both NGS and MeDIP-based approaches yield risk classification results at present. This means that a probability is given for each condition investigated, and depending on whether the pregnancy is assessed as being high risk or not, the couples are counseled to proceed with confirmatory invasive diagnostic testing, usually fetal karyotyping after CVS or amniocentesis. False positive results lead to unneeded invasive procedures posing an undesirable risk of pregnancy loss, while false negative results may lead to the birth of an abnormal child. There is an argument that false negative NIPT results for trisomy 18 or 13 are unlikely to result in the birth of an abnormal child because both syndromes are most likely to present with serious ultrasound findings during pregnancy. Conversely, cases with trisomy 21 (Down syndrome) may not have any indications throughout the pregnancy and consequently, NIPT false negative trisomy 21 fetuses are more likely to be born[41]. Therefore, NIPT for trisomy 13, 18 and 21 should be considered as a screening test rather than a diagnostic test, which should be robust, rapid and cost efficient. We believe that MeDIP-based tests meet these requirements for the reasons already presented, and moreover have certain advantages compared to NGS-based methods and therefore show great potential for large scale public service access. At present, if treated as a replacement for current biochemical screening tests, the resulting risk could be combined with that derived from ultrasound markers such as nuchal translucency measurement and others. The combined NIPT-U/S risk for fetal aneuploidy may provide a safer screening strategy compared to that offered to most couples today[42]. The future aim is to eventually avoid invasive procedures and develop NIPT (testing) into NIPD (diagnosis).

For any NIPT used caution is needed when it comes to

genetic counseling, in order to avoid misunderstandings concerning diagnosis. There is an ongoing debate on ethical and policy issues related to NIPT and the European Society of Human Genetics/American Society of Human Genetics invite the scientific community to contribute to setting future guidelines for NIPT[43].

CONCLUSION

During recent years there have been enormous advances in the field of fetal aneuploidy NIPT. Relevant genetic services are offered by academic centers and commercial companies worldwide, but not all future parents have access to this service. Our team is working towards developing a commercially available MeDIP-based test, that will be relatively inexpensive and easy to apply and from which more people can benefit. Looking ahead, we predict that epigenetic based approaches in combination with genetic-based approaches and advanced technologies (digital PCR, NGS) will contribute to the development of NIPT for more subtle fetal genetic abnormalities[44], such as point mutations, microdeletion/microduplication syndromes, *etc*.

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EDITORIAL

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

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.

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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 commmonly used AAV serotypes and variants are summariezed 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 actuary 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 amourosis, 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, Dentritic 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, oveary, skin, fibroblast, lung	-	-	[81]

AAV: Adeno-associated virus.

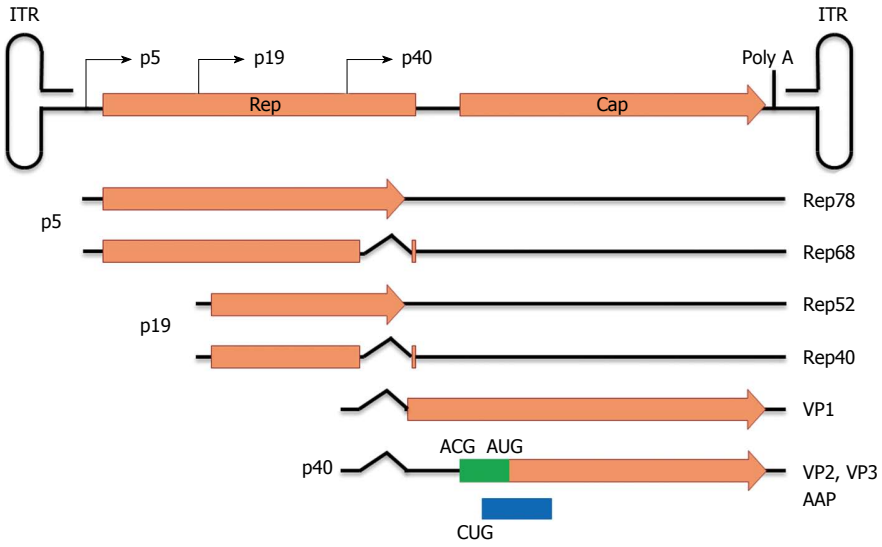

Figure 1 Diagram of adeno-associated virus genome depicting the inverted terminal repeats, promoters, polyadenylation sequence, and mRNAs coding for rep, cap, and assembly-activating protein proteins. The rep open reading frames codes for four rep proteins (Rep78, Rep68, Rep52, and Rep40) that are synthesized 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.

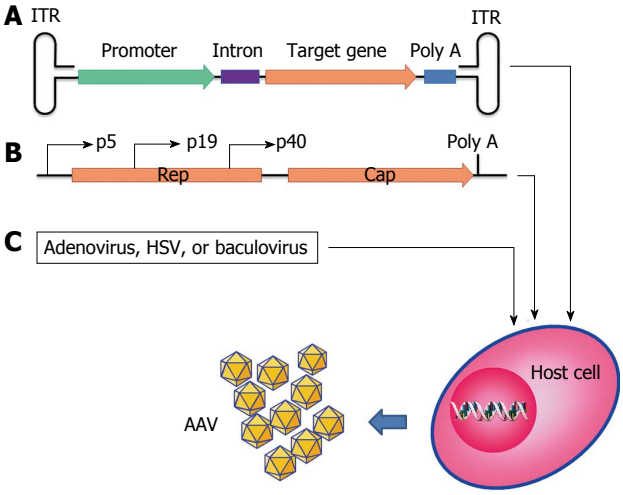

Figure 2 Diagram of adeno-associated virus vector production. A: The expression cassette containing promoter, intron, target gene, and polya-denylation sequence is flanked with adeno-associated virus (AAV) inverted terminal repeats (ITRs); B: AAV rep and cap sequences without ITRs are provided in trans; 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 de-signated 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 proapoptic 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[41].

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 galactocerebrosidase 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 AAV's 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 Ca^{2+} 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-vitreal 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-vitreal 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 trasport 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-vitreal 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 trance*, 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 posts 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, $\geqslant 10^{16}$ 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,

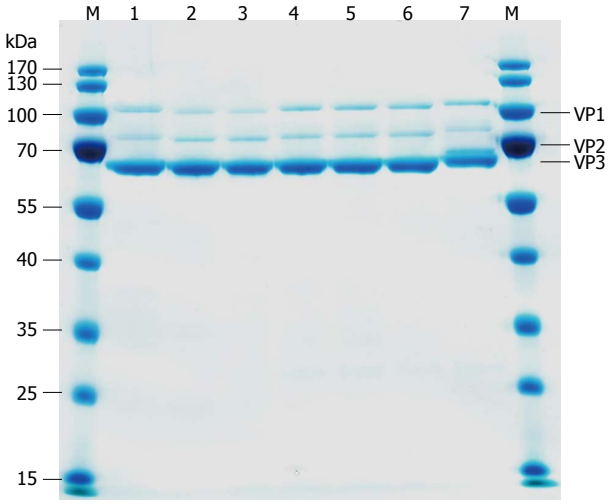

Figure 3 SimplyBlue SafeStaining of purified adeno-associated virus vectors on SDS-PAGE gel. Seven purified lots of adeno-associated virus (AAV) vectors at the amount of 1e + 11 vg per lane were loaded on a 10% Tris-glycine gel. The gel was stained with SimplyBlue SafeStain Kit (Invitrogen). Lanes 1, 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 VO_2 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 VO_2 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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EDITORIAL

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

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]
	GETAREA	curie.utmb.edu/getarea.html	[31]
Stability prediction	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	decrypthon.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	[46]
Aggregation tendency, amyloid formation and chaperone binding	TANGO	tango.crg.es/	[47]
	WALTZ	www.switchlab.org/bioinformatics/waltz	[48]
	LIMBO	www.switchlab.org/bioinformatics/limbo	[49]
Sequence conservation	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	11βHSD2	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]

11βHSD2: 11β-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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EDITORIAL

Oncofertility in adolescent and young adult hereditary cancer: Considerations for genetics professionals

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Abstract

Adolescents and young adults (AYA) with a cancer diagnosis or those at risk for cancer due to hereditary cancer syndromes may benefit from genetic counseling and testing not only to manage personal risk but also to address reproductive concerns, especially fertility. The opportunity for genetic counselors to provide important risk information is relevant to both the newly diagnosed as well as to unaffected carriers and survivors. However, genetic counselors may need additional training in reproductive options related to AYA cancer to provide this valuable counsel. This commentary uses hereditary breast and ovarian cancer syndrome as a model to highlight important considerations when discussing preimplanatation genetic diagnosis and prenatal diagnosis, particularly in the context of expanded testing for hereditary cancer risk including multigene panels or whole exome or whole genome sequencing. Other hereditary cancers are also addressed; however, less is known about the psychosocial and fertility concerns in these AYA populations. Additionally, we provide an overview of the concept of "oncofertility" - the linkage between cancer care and reproductive medicine that aims to expand the reproductive opportunities of cancer patients - and offer support for the expansion of guidelines to include genetic counselors in AYA cancer patients' treatment planning related to reproductive health and fertility.

Key words: Fertility; Oncology; Genetic counselors; Decision-making; Oncofertility; Adolescent young adults; Training; Health professionals

Core tip: Genetic counseling and testing holds great promise for adolescents and young adults (AYA) with cancer or potentially at risk for cancer. Oncofertility, the connection between reproductive medicine and oncology, provides expanded prospects for AYA to achieve childbearing and parenting goals. Genetic counselors and experts may benefit from expanded oncofertility training to provide counsel to AYA and aid in improving quality of life. Newer genomic technologies available for testing such as multi-gene testing and whole exome sequencing combined with advances in assisted reproductive technology offer novel opportunities for AYA to achieve reproductive goals.

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INTRODUCTION

Every year over 70000 adolescents and young adults (AYAs) are diagnosed with cancer in the United States, accounting for approximately 6% of all cases of newly diagnosed invasive cancers. The incidence of specific cancers in the AYA population varies considerably across the age continuum typically defined as between 15 and 39 years[1]. Hodgkin and non-Hodgkin lymphomas, melanoma, testicular cancer, female genital tract cancers, thyroid cancer, bone and soft tissue sarcomas, leukemia, brain and spinal cord tumors, breast cancer, and non-gonadal germ cell tumors account for 95% of all cancers in this age group[2]. Importantly, many AYAs with these diagnoses, particularly if they have a family history of cancer, are candidates for genetic counseling and possibly testing for hereditary cancer risk[3]. Genetic counseling typically entails a comprehensive discussion with a trained genetics professional (*i.e.,* medical geneticist or genetic counselor) to: (1) obtain a risk assessment based on personal and family cancer history; (2) educate about hereditary cancer risks and management; and (3) discuss potential benefits and limitations of genetic testing[4-8]. Goals of this initial session are to determine the appropriateness of genetic testing based on the patient's history and risk assessment[9], increase knowledge about hereditary cancer risks and implications, assess and address psychosocial concerns, and facilitate patient decision-making about genetic testing and risk management[10-12].

One key and highly relevant issue for AYAs is the impact of a cancer diagnosis and associated treatment on future fertility[13-17]. Numerous organizations including the American Society of Clinical Oncology (ASCO), the Royal College of Physicians in the United Kingdom, and the Clinical Oncology Society of Australia have developed clinical practice guidelines for fertility preservation for patients of reproductive age[18-21]. The ASCO guidelines, in particular, recommend that in addition to medical oncologists, the responsibility for discussion of and referral for fertility preservation also extends to other physician specialties and allied health care professionals in the oncology care setting[18]. However, for the subset of individuals at increased risk for hereditary cancer, there may be the additional concerns about genetic risk for future offspring. The possibility of transmitting a mutation to a child is often a concern among individuals affected with hereditary cancer, perhaps to the extent that some carriers may avoid childbearing[22-28]. To address this important concern, the National Comprehensive Cancer Network (NCCN) - an affiliation of some of the world's most prominent cancer centers that establishes frequently-updated, expert-reviewed, evidence-based guidelines regarding cancer care and treatment - recommends that patients of reproductive age should be counseled about the options of prenatal diagnosis (PND) and pre-implantation genetic diagnosis (PGD) for several hereditary cancer syndromes[4,29,30]. Indeed, genetics professionals often see patients at a critical juncture, in which AYA patients are not only acclimating to their diagnosis and treatment, but are also learning about fertility preservation options while considering potential risk to offspring that could impact their future parenting decisions.

This commentary uses hereditary breast and ovarian cancer syndrome as a model to highlight important considerations when discussing PGD and PND, particularly in the context of expanded testing for hereditary cancer risk including multigene panels or whole exome or whole genome sequencing. Other hereditary cancers are also addressed; however, less is known about the psychosocial and fertility concerns in these AYA populations. Additionally, we provide an overview of the concept of "oncofertility" - the linkage between cancer care and reproductive medicine that aims to expand the reproductive opportunities of cancer patients - and offer support for the expansion of guidelines to include genetic counselors in AYA cancer patients' treatment planning related to reproductive health and fertility[31].

DISCUSSION

Hereditary cancers

Hereditary cancers are those in which increased susceptibility is generally passed down within a family. They result from germline gene mutations and comprise 5% to 10% of all cancers[32]. Most cancers that affect the AYA age group, particularly those diagnosed under age 30, appear to be "sporadic" - or not arising from any recognized inherited susceptibility or environmental risk factors[1]. However, AYAs with cancer, and especially those with family histories of cancer suggestive of a

Table 1 Prevalent adolescents and young adults hereditary cancer syndromes

Syndrome	Description	Genetic Testing recommendations
HBOC	Breast cancer or breast and ovarian cancers among multiple family members	Testing for *BRCA1* and *BRCA2*
LFS	Increases risk for many cancers including sarcoma, breast, brain, lymphoma, lung, and others	Testing for p53
Retinoblastoma	Intraocular tumors (not always hereditary); nonocular tumors common in hereditary retinoblastoma	Testing for *RB1*
MEN and FMTC	Increases risk of endocrine tumors; FMTC is a common type of MEN	Testing for *MEN1*, *RET*, and *CDKN1B*
Lynch syndrome	Increases risk for colorectal cancer	Testing for *MLH1*, *MSH2*, *MSH6*, *PMS2*, or *EPCAM*
FAP	Increases risk for colorectal cancer; existence of multiple adenomas is passed down within family members	Testing for *APC* and M*UTYH*
Cowden Syndrome	Increased risk for breast, thyroid, endometrial (uterine lining), and other cancers	Testing for *PTEN*
Von Hippel - Lindau Syndrome	Increased risk for kidney cancer and multiple noncancerous tumors, including pheochromocytoma	Testing for *VHL*
Familial Melanoma	Increased risk for malignant melanoma and pancreatic cancers	*CDK2NA* and *CDK4*

HBOC: Hereditary breast and ovarian cancer; LFS: Li-fraumeni syndrome; MEN: Multiple endocrine neoplasia; FMTC: Familial medullary thyroid carcinoma; FAP: Familial adenomatous polyposis.

hereditary cancer syndrome, are good candidates for genetic counseling and testing[3]. Characteristics of hereditary cancer are dependent upon cancer type and include: Premature onset of cancer, multiple primary cancers within an individual, bilateral cancer in paired organs, rare tumors and uncommon tumor histology, and unusual cancer such as male breast cancer[4,33-35]. Additional characteristics related to family history include: Clustering of matching cancers in immediate family members, cancers spanning across generations of a family, rare cancers correlated with birth defects, and certain ethnic or geographic populations that are at particular high risk of hereditary cancers[33,36-38]. Table 1 highlights the most prevalent AYA hereditary cancer syndromes[4,39-45].

Hereditary breast and ovarian cancer

Hereditary Breast and Ovarian Cancer syndrome (HBOC), primarily caused by mutations in the *BRCA1* and *BRCA2* (*BRCA*) genes, is associated with very elevated risks for breast, ovarian and other cancers, affecting about 5% of women with breast cancer and 10% of women with ovarian cancer having HBOC[46,47]. While approximately 12% of all women will develop breast cancer during their lifetimes, the inheritance of a harmful *BRCA1* mutation increases this risk to up to 65% and a *BRCA2* mutation increases it to roughly 45%; these mutations increase the likelihood of developing ovarian cancer from 1.3% of all women to 39% for *BRCA1* and up to 17% for *BRCA2*[45]. Because effective management strategies exist for breast cancer screening and mortality reduction for ovarian and breast cancer vis-à-vis bilateral salpingo oophorectomy, many professional associations recommend *BRCA* counseling and testing for women at high risk of HBOC Syndrome[4,40,41,48]. For example, the National Society of Genetic Counselors (NSGC) identified critical components of the testing and counseling process that include: The ascertainment of medical and family histories, determination and communication of cancer risk, assessment of risk perception, education regarding the genetics of HBOC, discussion of molecular testing for HBOC if appropriate (including benefits, risks and limitations) and any necessary follow-up[9,40]. Additionally, the United States Preventive Services Task Force recommends genetic counseling for women with high risk family histories[41].

Management of HBOC risk in women may include aggressive and early breast cancer screening with breast magnetic resonance imaging beginning at age 25, mammography starting at age 30, or consideration of bilateral risk reducing mastectomies[4]. Bilateral salpingo-oophorectomy is recommended by age 35-40 and when childbearing is completed[4]. These surgeries and therapies have implications for future fertility and parenting considerations. For example, women facing decisions about oophorectomy may wish to know that oocytes can be preserved and through the use of assisted reproductive technology (ART) such as *in vitro* fertilization (IVF), they can still carry a pregnancy[49-51]. For women recommended to use tamoxifen or undergoing chemotherapy or other adjuvant therapy to manage risk, it is imperative for them to be aware that pregnancy is contraindicated during this time[52] and oocyte freezing may be a consideration for delayed childbearing[53-55]. An emerging ovarian cancer risk reduction option includes a two-step surgical strategy that includes bilateral salpingectomy prior to menopause followed by postmemopausal oophorectomy. Ovarian preservation could lead to an opportunity to maintain some fertility preservation options for a more extended period of time, reduce cardiovascular disease and bone loss and improve quality of life. However, this relatively new approach for ovarian cancer risk reduction must be considered in light of limited data regarding optimal timing of the two surgeries and whether timing should differ based on the specific cancer predisposing mutation. Additionally, the short and long term impact of this option on cancer risk reduction, quality of life,

physical and psychosocial functioning, as well as other cancer prevention behavior remains largely unknown[56,57].

Aside from fertility concerns, a woman who has had bilateral mastectomies will not be able to breastfeed her child. This is an important consideration for women who perceive breastfeeding as an essential parenting role. These women would benefit from counseling about additional ways to establish bonding with their infants and to take this information into consideration when making decisions about risk management[58]. Each of these examples highlights the importance of women being aware of how hereditary risk may affect fertility and parenting concerns. The genetic counselor is thus important in both providing a woman with personal risk reduction information, and addressing family planning goals with options and strategies.

Fertility and cancer

AYAs with any type of cancer, heritable or not, face several challenges including unique psychosocial consequences. AYA cancer patients and survivors often experience disruption in education, employment, relationships, and personal growth[59]. One key quality of life issue among this childbearing-aged population is the threat to reproductive health, including risks like loss of fertility, compromised fertility, and concerns about transmission of cancer susceptibility gene mutations to future offspring[60]. ASCO, NCCN, the Royal College of Physicians, and the European Society for Medical Oncology, as well as other prominent organizations, have all created guidelines suggesting the most effective way to deal with these challenges is to discuss options and preservation methods prior to cancer treatment and document this discussion in the medical record[18-20,61,62]. However, recent research evidences low rates of documentation, which may equate to low rates of actual discussion[63].

Established options for fertility preservation include sperm, oocyte, and embryo cryopreservation. Experimental options include testicular and ovarian tissue freezing. Still other options, often referred to as alternative family building, include the use of donor sperm, oocytes or embryos or the use of a gestational carrier. While these options are available for AYA cancer patients and survivors numerous studies have documented poor communication about potential infertility risks and preservation or family building options between patients and health care providers. Additionally, health care providers report discomfort and lack of knowledge regarding some assisted reproductive technologies, like PGD, which may be an important resource for cancer patients concerned about passing on cancer-specific gene mutations to their future offspring[64-66]. Consequently, many patients do not receive timely and accurate information about the impact of their diagnosis on future reproductive health[67-70]. Even when these risks are communicated, however, patients may not be provided with additional resources for related issues beyond immediate treatment impact, including referrals to specialists like reproductive endocrinologists and genetic counselors, who can answer important questions and provide individualized guidance for AYA cancer patients. While oncologists and oncology nurses are necessary primary sources of information on cancer diagnosis and treatment impact on future fertility, sessions with genetics professionals can expand upon this initial information by discussing how hereditary cancer risks may affect the patient's childbearing concerns and goals.

Genetic testing for hereditary cancer syndromes in future offspring

Prior reproductive considerations were largely limited to hereditary cancer syndromes following an autosomal dominant inheritance pattern *(e.g., BRCA, PTEN*). Thus, counseling was focused on reproductive implications based solely on the proband's test results. However, with expanded gene panel testing, reproductive counseling must also consider genetic disorders that follow an autosomal recessive inheritance patterns. For example, the addition of the Fanconi anemia (FA) genes (*FANCD1/BRCA2, FANCJ/BACH1/BRPI1, FANCN/PALB2, FACNCO0/RAD51C*, and *FANCA*) to cancer testing panels raises the possibility of identifying risk for FA. Thus, reproductive implications for offspring are also informed by the carrier status of the proband's current (or future) partners. If both are heterozygotes, there is a 25% risk that an offspring will be a homozygote and have FA. Similar considerations would arise for probands carrying mutations in *ATM* and *MYH* genes.

For most individuals with autosomal dominant hereditary cancer syndromes *(e.g.,* associated with *BRCA1/2, PTEN*, or *TP53* mutations), reproductive options exist for prenatal and PGD to detect heterozygous offspring. However, with the advent of panel testing, more individuals are being identified with heterozygous mutations in a broad array of genes that had been previously identified primarily in homozygotes. These homozygous individuals are biallelic mutation carriers, having inherited a mutation from each parent through autosomal recessive inheritance. For example, female *ATM* heterozygotes are at increased risk for breast cancer, but biallelic carriers have a neurologic condition known as ataxia telangiectasia. Similarly, *BRCA2* homozygotes and others with biallelic mutations in genes in the FA pathway *(e.g., BRIP1, PALB2, RAD51C*) develop FA. Recently, the rare finding of biallelic *BRCA1* carriers appears to manifest with a similar FA phenotype. Individuals with two mutations in some genes associated with Lynch syndrome may develop a severe condition known as constitutional mismatch repair deficiency. Thus, an individual tests positive for one mutation in genes such as these, counseling about reproductive implications needs to address. Not only the risks associated with autosomal dominant

Table 2 Hereditary breast and ovarian cancer syndrome pre and post treatment options with reproductive implications

Genetic counseling needs for AYAs with a new cancer diagnosis	Genetic counseling needs for AYA cancer survivors
Surgical treatment (*e.g.,* contralateral prophylactic mastectomy at the time of initial diagnosis for *BRCA* carriers)	Risk reduction surgeries post treatment (*e.g.,* salpingectomy *vs* bilateral salpingo oophorectomy for *BRCA* carriers)
Chemotherapy (*e.g.,* clinical trials focused on poly ADP ribose polymerase inhibitors for *BRCA* carriers)	Use of Tamoxifen for management of disease recurrence among *ER* + *BRCA* carriers.

AYA: Adolescents and young adults.

inheritance but also the potential risk to have a child with two deleterious biallelic mutations that could result in a severe condition. Therefore, assessing the tested individual's partner (*i.e.,* his or her personal and family history, as well as ethnicity) is important. In the unlikely event that both parents are heterozygous for specific mutations, there is a 25% risk that a child will be homozygous and could have a severe phenotype. Thus, the couple should be made aware of reproductive options such as PGD.

Preimplantation genetic diagnosis

AYAs with hereditary cancers may have concerns about future offspring and transmission of the hereditary cancer[22-28,66,71,72]. Technologies exist for individuals with cancer susceptibility gene mutations to avoid the birth of a child with such mutations. PGD is a type of ART allowing couples to choose which fertilized embryos, created through IVF, are implanted into a woman's uterus for further gestation[73]. These embryos are tested for genetic disorders with the intent that the selected embryo will result in a child who does not carry the genetic mutation[73,74]. To date, over 20000 cases of PGD use have been reported in the United States and over 200 genetic disorders or conditions can be identified using PGD[75].

PGD is not without its ethical concerns. Studies of the general public and families with hereditary cancers suggest concerns that PGD is akin to "playing God" and a slippery slope for the creation of "designer babies"[76,77]. Although oncology healthcare providers may be willing to discuss PGD with patients, many studies show physicians and nurses lack sufficient knowledge and confidence to initiate PGD discussions[78]. A study of 373 gynecologic oncologists and obstetrics and gynecologists reported that 68% of participants had incorrect or limited knowledge of PGD for hereditary cancer[79]. Another study with 201 oncology nurses showed more than half of respondents (78%) were unfamiliar with PGD and those familiar with PGD had limited knowledge[79]. Studies with individuals at increased risk for hereditary cancer syndromes reported low levels of knowledge about PGD for hereditary cancers, moderate rates of acceptability, and high levels of need for information about PGD[80]. With respect to patients with hereditary cancer, although a few studies indicate some would not consider PGD personally, most individuals agreed that it is important for health care providers to provide information about the option of PGD[64,81-83].

PND

PND can be used to identify hereditary cancer risks in the developing fetus. This process typically includes chorionic during the eleventh through fourteenth weeks of pregnancy or amniocentesis in later weeks[84]. PND has been used to identify gene mutations in *RB1*, which causes retinoblastoma; *NF1* and *NF2*, which cause neurofibromatosis; and a host of others that help determine cancer predisposition[85]. PND is more likely to be used for childhood onset hereditary conditions like *RB1* and less likely to be used for HBOC, of other adult onset cancer syndromes.

Genetic counseling for cancer risk

As defined by the NSGC genetic counseling is "the process of helping people understand and adapt to the medical, psychological, and familial implications of genetic contributions to disease"[86]. Through the cancer risk assessment, genetic counselors can navigate patients through the process and provide education and counseling. This opens the floor for discussions regarding potential test results as well as the genetic test's risk and benefits.

Genetic counseling for AYAs at-risk for or with cancer

Although some AYA programs include access to genetic services as part of their umbrella of AYA cancer care, most institutions do not have a specific AYA program. Further, the number of trained oncology genetic counselors is low and may not be available to meet the needs of this growing population. Thus there is great need for genetic counselors in AYA programs and other settings who are trained not only in the discussion and assessment of risk to the individual but who also can discuss fertility, general preservation options for those whose fertility is at risk, and the impact the hereditary cancer may have on future offspring and ways to manage that risk. Expanding the training of genetic counselors to include oncofertility knowledge, resources, and decision-making tools, may greatly improve the quality of life and quality of care for AYA with hereditary cancer risk. Using HBOC, Table 2 provides examples of pre and post treatment genetic counseling and testing options for breast cancer survivors that may have reproductive implications for AYAs.

CONCLUSION

Genetic counselors may benefit from training on communicating about reproductive health risks and options for managing risks in AYA populations. This training should include not only information on fertility preservation, PGD, PND, and ART techniques but also strategies to communicate this information to patients in ways that facilitate informed decision-making and which consider the values and preferences of the patient and if applicable his or her family and partner. Genetic counseling education programs should consider didactic courses for learners on these same reproductive health options so that future genetic counselors are trained to address these important issues with their AYA patients. Improved communication on reproductive health issues and options for patients with hereditary cancers will greatly improve their future quality of life and expand the cadre of oncofertility health care providers.

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