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"Gene Therapy in the Eye" – Basics and recent developments in its success

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Abstract:

Ever since the genetic basis of phenotypic variance and disease occurrence was discovered, scientists and clinicians have hoped that "correcting" mutations may be the panacea for many human ailments. Over the past several years, Gene therapy [GT] has evolved from an idea to a reality, with over 1800 clinical trials being piloted worldwide. The target ailments vary from cancer to inherited diseases to complex disorders. A variety of GT modalities have emerged, which typically range in their delivery methods to the target tissues. Both viral and non-viral methods of gene delivery are getting advanced, but viral vectors have gained greater attention due to their superior gene delivery efficiencies. The GT process has been particularly successful in the eye and holds great promise for curing various forms of blinding disorders. This review will discuss the basics of GT process, prospect of ocular GT, and its recent developments.

Introduction: Many human disorders are genetic in origin and caused due to a defective gene function. Gene therapy (GT) is an advanced therapeutic application of recombinant DNA technology wherein a gene is delivered to the target cell or tissue against a defective gene to restore its function and regulation, thereby curing the disease.[1]The process of GT can be *in vivo* where a gene is introduced directly into a living system or *ex vivo* where it is first introduced into the targeted cells in a lab setup, and then gene-corrected cells are infused into the living system for intended therapeutic benefits. Somatic gene therapies involving transfer of the therapeutic genes into diploid living cells are in practice. In contrast, germline gene therapies, where germ cells (sperm or egg) could be genetically manipulated, are not permissible.[2] The main motive for GT development is to find treatment alternatives for those genetic diseases for which other forms of management are unsuccessful. Although the concept of GT was first realized in the 1960s, it took more than 20 years to get the FDA (Food and Drug Administration) approval for the first-ever GT clinical trial in the 1990s to treat adenosine deaminase-based severe combined immunodeficiency (ADA-SCID). After this, many GT studies started for various genetic diseases, and more than 1,800 clinical trials have been registered worldwide.[3] In the context of ophthalmic GT, its recent success and advancements in efficacy and safety have precluded it as a desirable method to cure complex and untreatable visual disorders. This review will address the gene therapy modalities depicting ocular gene therapy, its present status, clinical development, and future prospective.

Modalities of Gene Therapy: A successful GT involves the transfer of an appropriate doses of a therapeutic gene designed to get expressed to the target cells or tissue of the host organism without substantial toxicity. In a gene therapy process, a particular gene needs to be cloned with many other DNA elements to get expressed in the target tissue post-delivery. The delivery of a gene can be mediated through viral methods or non-viral methods [Table-1][4] Although non-viral vectors are considered to be safe, they are often limited by far less delivery efficiency and transient gene expression in contrast to viral methods, which could transfer genes more efficiently with prolonged expression.[5] Therefore, viral vectors have gained more application to be used as a potent gene delivery vehicles.

Viral vectors in GT: Viruses have natural ability to infect the cells by high jacking host cellular machinery of replication, transcription and translation. For gene therapy purposes, these viral vectors are engineered in such a way that they can carry, deliver and express the therapeutic gene of interest. Using recombinant DNA technologies, the viral genes are substituted with the gene of choice. The viral genes are supplied in trans (separately) during their cultivation or production. Other forms of engineering of GT viruses may involve different strategies such as adding tissue-specific promoter and enhancer sequences, inserting transcriptional or translational signals, creating point mutations, introducing homologous recombination sequences, generating gene splicing sites etc. Due to the enclosed capsid structure, there is a certain maximum limit of the size of gene a particular virus can accommodate within it, known as its packaging capacity. In order to expand the viral packaging capacity, strategies like trans-splicing, overlapping vectors**,** and hybrid vector system have been developed.[6] For r-virus production, simplified methodologies such as triple transfection methods have been established wherein three different DNA constructs, one with the gene of choice and other two with the viral genes for its replication and capsid formation are supplied

in a cell culture system, which generates recombinant viruses within cells. The produced viruses then undergo the series of isolation and purification processes. [7]

Suitable GT viral vectors: The GT recombinant viruses include gamma retrovirus, lentivirus, foamy virus, adenovirus, adeno-associated virus and herpes simplex virus. Each viral vector system has its own advantages and limitations for GT purposes, (reviewed in many publications) [8,9]. Among these, the current trends of AAV based viral gene therapy got momentum because of several unique advantages it possess without eliciting a significant immune response, (ii) can transduce a wide variety of dividing and non-dividing cells (iii) are capable of providing long-term expression of transgenes (iv) various serotypes of AAV are available which can be specifically efficient for particular target tissues. These properties make it the best-suited viral vector for GT, especially ocular GT[10]. GT based therapeutic interventions for various types of incurable disorders like cancer, neurodegenerative disorders, arthritis, muscular dystrophy, cystic fibrosis, hemophilia B, Stargardt's disease, Leber's congenital amaurosis, Fuch's corneal dystrophy and many more are being implemented through AAV vectors.[11] However, the limiting factor of rAAV for GT application is its low gene carrying capacity, being limited to less than 4.7 kb. Nevertheless, these limitations can be overcome by adopting various strategies like cis-activation, overlapping vectors, trans-splicing, hybrid vectors, capsid mutant, and self- complimentary design.[12]

Non-viral vectors: Non-viral vectors for gene delivery include free DNA delivery or delivery of gene with certain physical agents such as gene gun or chemical agents like lipofectamine, calcium phosphate or with nanoparticles. They are considered a better choice for gene delivery as they do not induce much immunogenic response and do not possess the risk of oncogenic integration. However, practically they are limited by their poor gene delivery efficiency in vivo conditions.[13]

Approaches in gene therapy: Various approaches in gene therapy have been developed to cure a disease. In a classical gene augmentation-based gene therapy a normal copy of gene is delivered into target cells using viral or non-viral vectors. In a gene silencing approach, a defective gene is targeted for its inhibition using molecules like si RNA, Ribozyme, antisense oligonucleotide, which can be delivered using viral or non-viral vectors.[14,15] In gene-editing approach using tools like TALEN (Transcription activator-like effector nuclease), ZFN (Zinc finger nucleases), and recently evolved CRISPR (clustered regularly interspaced short palindromic repeats) the defecting gene can be corrected or inhibited.[16] The therapeutic benefits using gene therapy can be achieved through different mechanisms involved. Delivering normal copies of gene, it may integrate into the host genome or remain as episomal elements and gets expressed using cellular machinery; providing apoptotic/anti-apoptotic genes or toxin-producing genes may cause the death of harmful or cancerous cells; delivering immunogenic factors can activate the immune system to aid killing of diseased cells; delivering an agent which can convert pro-drug into a useful drug, and providing targeted small molecules which can inhibit undesirable expression of a particular gene.[17]

Process in GT: Like any other drug development, GT goes through extensive research, preclinical studies, and clinical trials. GT experiments pass

through *vitro* and *in vivo* animal model studies as a proof of concept followed by a - series of clinical trials where the safety and efficacy of the treatment would be assessed in humans. Gene transfer protocols require supervision by regulatory agencies like Food and Drug Administration (FDA), Recombinant DNA Advisory Committee [RAC], Institutional Biosafety Committee [IBSC], and Institutional Review Board [IRB] who need to certify it as GRAS (generally recognized as safe) and only GRAS- certified GT protocols would be approved. After approval clinical-grade vectors for GT applications are made by carefully adhering to the guidelines of Good Manufacturing Practices and are subjected to an extensive series of quality control assays. [18],

Gene therapy in eye: Eye being a compartmentalized system, provides a suitable and promising platform for testing Both posterior and anterior parts of the eye have been served for the gene therapeutic benefits using different strategies. Eye provide routes for gene delivery including topical, sub-retinal, intravitreal, and intracameral**.** Few parts of eye are avascular (cornea and fovea centralis) preventing tissue specific immune response. They are also immune privileged lacking lymphatic channels and antigen presenting cells (APC), and have blood ocular barrier. The imaging techniques for ocular tissue have been well developed which helps in easier clinical characterization, and the other untreated eyes always can serve as an experimental control.[19].

Table.1 : Gene Delivery Methods

Viral methods	**Non-viral methods**			
Exploit the natural tendency of virus to infect host cells and interact with their genome	Allow DNA[genes] to enter inside cells under certain chemical or physical influence			
GT viruses	**Injection of Naked DNA**	**Physical Methods**	**Chemical Methods**	**Hybrid method ʊ**
Retroviruses	Vein injection	Electroporation	Synthetic Oligonucleotides	Combine two or more methods. Eg. Virosomes
Lentiviruses	Artery injection	Sonoporation	Lipoplexes	
Adenoviruses	Portal injection	Magnetofection	Polyplexes	
Adeno-associated viruses	Blood occlusion	Gene Gun	Cationic Peptides	
Herpes Simplex Virus	Local injection		Inorganic Nanoparticles	

Ophthalmic GT developments: Many diseases which were believed to be incurable are now being tried for GT-based treatments. These benefits have been reflected remarkably in several ophthalmic retinal - conditions like Leber's congenital amaurosis [LCA], retinitis pigmentosa, Stargardt's disease, red-green colour blindness, age-related macular degeneration [AMD], and primary open angle glaucoma [POAG].

Table 2: List of ocular gene therapies and methods/strategies

Posterior chamber gene therapy	**Methods/strategies**
Leber congenital amaurosis	Successful delivery and expression of RPE65, AIPL1,GUCY2D, RPGRIP1 genes[35]
Autosomal dominant retinitis pigmentosa	Combined knockdown of the toxic gene and co-expression of a human rhodopsin cassette.[36 37]
Glaucoma	Rho and rho kinase inhibition in trabecular meshwork and expressing neurotropic factors like CNTF, BDNF, [38] [39]
Wet Age related macular degeneration	Adeno-associated virus [AAV]-mediated expression of anti VEGF factor sFlt-1, **[40] [41]**
Retinal and Choroidal neovascularisation	Adenovirus-mediated delivery of short hairpin RNAs targeting VEGF. Intraocular injection of an adenoviral vector expressing PEDF.[42]
Achromatopsia	Successful delivery of ion channel genes Gnat3,Cnga3 and CNGB3.[43]
Stargardt macular dystrophy	Successful delivery of ABCA4 gene.[44]
Mitochondrial GT for LHON	AAV mediated intravitreal injection of MT-ND1, MT-ND4 gene administrations[45]
Anterior chamber Gene therapy	**Methods/Strategies**
Wound healing and prevention of fibrosis in diabetic cornea	AAV mediated Decorin overexpression.[46] Inhibition of TGF-β by delivering SMAD-7.[47] TGFR-II[48]
Prevention of corneal graft rejection	Indoleamine dioxygenase therapy. lentivirus-mediated delivery of anti-apoptotic genes Bcl-xl, Bcl-2, Survivin, p35.[49]
Corneal neovascularization	Inhibition of VEGF via [sVEGFR1, sFlt-1, Flk1, angiostatin, PEDF].[50]
Chronic allergic conditions of lacrimal gland and conjunctiva	Inhibition of inflammatory cytokines. Expression of anti-inflammatory cytokines [IL-10, IL-4, sTNFr, anti TNF-a].[51]
Congenital hereditary endothelial dystrophy	AAV mediated SLC4A11 gene augmentation[52]
Fuch's endothelial corneal dystrophy	CRISPR based COL8A2 knock out [53]

Other ocular disorders which have followed suit into GT clinical trials include Leber's hereditary optic neuropathy, diabetic retinopathy, choroideremia, superficial corneal opacity and diabetic macular edema.[20] (Table2). On the other hand, the corneal gene therapies, though potentially stronger for its outcome being immune-privileged, developments are yet to be expanded as none of the corneal GT studies have reached clinical trials. Initial corneal gene delivery studies were limited to reporter gene delivery, its location and expression in vitro and in vivo system. Further few developments were achieved in preclinical studies for the prevention and the treatment of corneal fibrosis, corneal graft rejection neovascularization, haze, and herpetic stromal keratitis.[21] Uveal GT studies have also been started. Cytokines such as, IL1 and IL10, which show anti-inflammatory effects in uveitis, have been subjected for systemic and subconjunctival adeno virus mediated transfer in experimental uveitis, which showed suppression of autoimmune response [22] Many ophthalmic disorders have been linked with mitochondrial genes alterations. Mitochondrial gene therapy approaches has also been developed in these mitochondrial inherited disorders such as Leber Hereditary Optic Neuropathy [LHON].[23] The other ailments such as diabetic retinopathy, age-related macular degeneration, and glaucoma are also the potential targets.[24] Among all ocular disorders, the retinal GT treatments have been extensively expanding. The breakthrough in retinal GT came with the success of LCA gene therapy, which represents the first successful ophthalmic GT for treating blindness. The success story of LCA gene therapy helps us to understand the entire concept, work flow, challenges and outcome of an ocular gene therapy.

About LCA: Leber congenital amaurosis (LCA); [Mendelian Inheritance in Man [MIM] #204,000] is a genetically inherited retinal disorder causing severe visual impairment in infants and children. The reported incidence of LCA is 1 in 30, 000 live birth globally though; incidence seems to be more in consanguineous populations like south India. Many children in blind schools are blind due to LCA.[25] Clinically, LCA is characterized by severe visual loss identifiable in early infancy (1-2 years) of life. The loss of vision is accompanied with some mostly occurring primary features, including sensory nystagmus, sluggish eye pupil responses, high refractive errors [hyperopia or myopia] and light insensitive rod and cone cells, detected by electroretinogram [ERG] which appears flat against normal wavy appearance. The secondary features which may or may not show include oculodigital sign (pressing and poking of eyes), photoaversion (photophobia), nyctalopia, (night blindness) and keratoconus (central corneal thinness) [25,26] LCA arises as a result of mutations in the genes important for the ocular visual system. There are more than 20 such genes, and new genes have periodically been identified. For one type of LCA caused due to RPE65 mutation, gene therapy development led to a successful outcome to cure it.

LCA GT : The proof–of–concept for LCA GT aroused from a Briard Dog experiment where, these naturally blind dogs carrying a defective RPE65 gene were treated subretinally with recombinant AAV vectors carrying a normal copy of the RPE65 gene. After a five-year follow-up, most of these dogs showed improved vision and navigation.[27] After this milestone, the human clinical trials started in many centers, including the Centre for Cellular and Molecular Therapeutics at Children's Hospital of Philadelphia, USA, Moorfields Eye Hospital, UK, University of Pennsylvania and University of Florida,

USA. [28,29]**.** The genetically engineered vectors, AAV2.hRPE65v2 carrying a normal RPE65 cDNA were injected through a very fine needle in the sub-retinal space of one severely affected eye of LCA patients after vitrectomy under general anesthesia. After a month follow-up, the patients showed a modest improvement in retinal function, visual navigation, and other subjective visual acuity tests without any serious immunological response. Since then, many additional clinical trials assessing their safety, efficacy, dosage effect, injection sites, and bilateral injection were performed successfully[30,31] In Phase 3 study with 31 participants with one year follow-up it was found all participants with visual impairment could gain the ability to navigate an obstacle thus showing their gain in vision.[33] Finally, in 2017 the FDA approved the recombinant AAV GT drug in the commercial name LUXTURNA [https://luxturna.com/about-luxturna/] for its commercial use on patients. It became the first cure to reverse inherited blindness. Other LCA genes, including GUCY2D, CEP290, AIPL1 and RPGRIP1 are also in gene therapy developmental process. [39]. With the remarkable success of LCA GT there is a reasonable hope that in the coming decade, GT can be applied to patients as part of standard ophthalmic treatment for many untreatable ocular disorders.

Limitations of GT: Despite various developments, there are many limitations associated with GT. The efficiency of in-vivo gene delivery in the target tissue always remains low due to multiple barriers to the cellular system the body delivers. Immunogenic responses mediated by the host body system are the next challenge. The preexisting antibody against viral vectors in our immune system may neutralize the effect of delivered genes. The surgical process required to provide the gene in internal tissue or organs. There are limited players for GT developments and, therefore, very few success rates. Many countries, including India, have no major involvement in GT development programs. Finally, once any GT drug crosses all these barriers and succeeds in getting the status of approved drug, the market price gets extraordinarily costly, in millions of dollar.[34] Thus, it remains unapproachable to many needy patients. Therefore, there is a need to establish a collective approach by various stack holders in GT development programs all across the world to work in this direction and make GT drug a successful, effective and affordable treatment regime in the future.

Discussions: Although the full potential of GT is yet to be realized, it is continuing to make steady progress towards becoming the future drug for the treatment of several diseases. Ophthalmic GT though new is evolving fast with promising results, thus providing options for incurable ocular disorders. Various viral vector systems owing different properties have been extensively used for GT purposes. New improved viral methods and non-viral methods are in process to enhance the efficiency and safety aspects of GT. Various strategies including gene replacement, gene targeting and gene silencing, have been established as potential way to cure the gene defects. Both posterior and anterior ocular gene therapies are in progress but the former have got more expansion. The corneal and uveal gene therapy is limited to preclinical studies. The success of LCA GT proved to be a model towards the treatment of inherited retinal blindness. GT strategies for glaucoma, retinitis pigmentosa, Stargardt disease, red-green colour blindness, age-related macular degeneration [AMD], diabetic retinopathy, and diabetic macular edema are being targeted for clinical trials. GT for achromatopsia, choroideremia and mitochondrial GT for Leber's hereditary optic

neuropathy [LHON] are the latest developments. Mitochondrial gene therapy to be developed in many ophthalmic diseases could be the next step in ophthalmic GT. The majority of results have been attained from *in vitro* and *in vivo* animal model studies. More human clinical trials are required before it becomes a part of standard ophthalmic medical practice in the future.

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