CHAPTER 2
LITERATURE REVIEW

Eye is the organ responsible for the vision, which is considered one of the most important sense of human being. Retina is the inner most layer at the posterior part of the eyeball which is a clear, thin, and photosensitive tissue. This structure converts light into electrical signals which is enabled by specialized cells, the photoreceptors, in a process termed phototransduction. There are two types of photoreceptors cells: rods and cones, which are located in humans in the peripheral and central retina, respectively. Rod cells are involved in the dim light vision while cone cells are associated with color detailed vision. In addition to the photoreceptors, retinal pigmented epithelium cells also play an important role in maintaining retinal function. (**Figure 1**) Losing either one or both of these photoreceptors as well as retinal pigment epithelium lead to a condition called retinal dystrophies.[10]

There are many types of retinal dystrophies, affecting only one cell type or both and different progression with age. These disorder phenotype range from achromatopsia, Stargardt's disease, cone dystrophy, cone-rod dystrophy, retinitis pigmentosa, to Leber congenital amaurosis. (**Figure 2**) Amongst others, Leber congenital amaurosis is one of the most severe and it accounts for ~20% of children in the special school for the blind.[3]

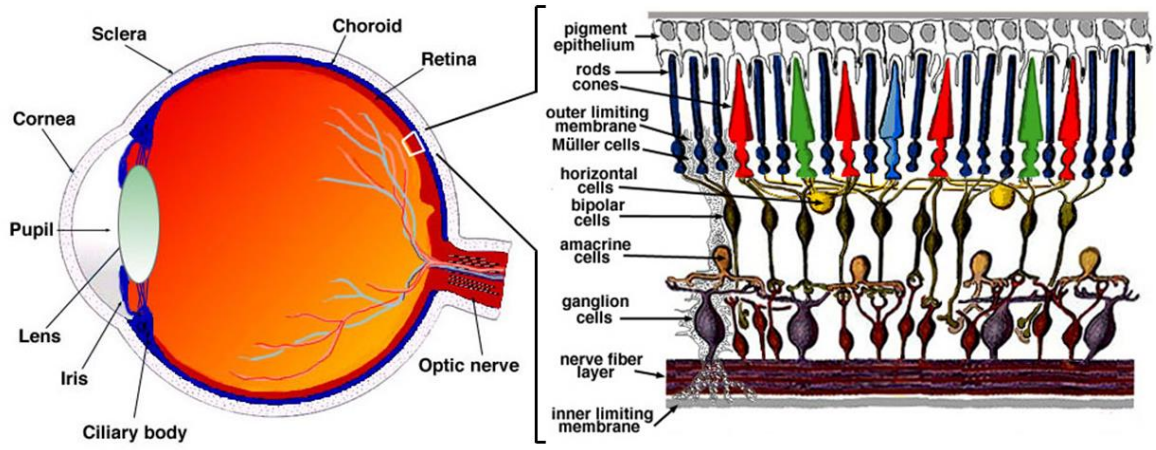

Figure 1. Anatomy of the eye and retinal structure. Structure of the eye and the retina, which consists of many different cell layers. (Adapted from: http://webvision.med.utah.edu/book/part-i-foundations/simple-anatomy-of-the-retina/)

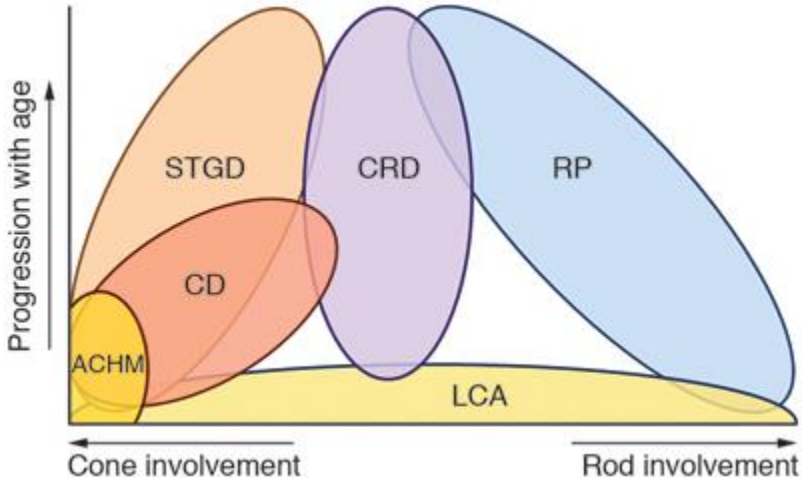

Figure 2. Classification of IRDs according to age progression and cell involvement. There are two kind of photoreceptor cells involvement: rod and cone. Rod is responsible for dim light and peripheral vision. Cone is responsible for color and central vision. STGD: Stargardt disease, CD: cone dystrophy, ACHM: achromatopsia, CRD: cone-rod dystrophy, RP: retinitis pigmentosa, LCA: Leber congenital amaurosis.[10] (Adapted from: den Hollander, *et al*. 2010)

2.1. Leber Congenital Amaurosis

Leber congenital amaurosis (LCA; OMIM 204000) is the most severe retinal dystrophy with the early onset in the first year of life. It was described for the first time by the German doctor, Theodore Leber, in 1869. He found that the disease was characterized by unresponsive pupil, wandering nystagmus, and a fundus that initially appears normal and then turn to be typical for retinitis pigmentosa in early childhood. He introduced this disease as the congenital form of retinitis pigmentosa.[11] Pinckers thought that the disease Leber found actually was more or less the same as what we call now as neuronal ceroid lipofuscinosis.[12] This disease accounts for ~5% of all inherited retinopathies with a prevalence of approximately 1:50.000.[13.14]

2.1.1. Clinical Characteristics

The three main features of LCA are congenital onset of visual loss, amaurotic pupils; no response in light reflex, and nystagmus.[11] Franceschetti and Dieterle mentioned the importance of elecroretinogram in retinal degeneration.[15] Absent electroretinogram became one of the diagnostic criteria other than bilateral congenital blindness.[16] Foxman and colleagues suggested that elevtroretinogram (ERG) should be examined before the age of one year.[17]

Visual acuity in patients with LCA varies extensively. Typically from 20/200 to only light perception (LP) or negative LP.[1] Studies from 90 LCA patients showed that the majority

(75%) of visual acuity of the patients remained stable while visual deterioration and even improvement were observed in 15% and 10% patients, respectively.[18-20]

Table 1. Fundus photography results of LCA patients with known mutations in causative genes.[1]

Genotype	Fundus Photography
AIPL1 (P.Thr124Ile/p.Pro376Ser)	Macular coloboma, retinal arteriolar narrowing, and optic disc pallor
CEP290 (p.Cys998*/p.Cys998*)	Choroidal sclerosis, pale optic discs, non-visible retinal vessels, relative preservation of the posterior pole
CRB1 (p.Cys948Tyr/p.Cys948Tyr)	Preserved para-arteriolar RPE (PPRPE) and nummular pigmented retina
CRX (p.Pro9fs*/+)	Maculopathy, relatively normal appearing vessels and optic disc
GUCY2D (p.Leu954Pro/p.Ser981fs)	Relatively normal retinal appearance
LCA5 (g.19612-18015del1598/g.19612-18015del1598)	Optic disc drusen and mild vessels narrowing
LRAT (p.Met73fs*/p.Met73fs*)	Mild vessel narrowing and very mild salt and pepper appearance with normal optic disc
RDH12 (p.Cys285Tyr/p.Cys285Tyr)	Maculopathy
RPE65 (p.Tyr368His/p.Tyr368His)	RPE translucency
RPGRIP1 (p.Arg89*/ p.Arg89*)	Bone spicules appearance and vessel dragging
TULP1 (c.718+2T>C/c.718+2T>C)	Perivofeal yellow annular ring and mild pigmentary changes

All LCA patients in this table have recessive mutations, except for the patient with *CRX* mutation.

Fundus photography of the retina from LCA patients reveal an extensive disparity, from basically unaffected retina, to retinal arteriolar narrowing, retinal pigmentation (bone spicule, salt and pepper, or nummular), and maculopathy (**Table 1**). A study showed a

correlation between preserved para-arteriolar RPE and *CRB1* LCA-causing mutation. However, it is still difficult to have a convincing genotype-phenotype correlation in IRDs.[1,21]

ERG is essential to assess the visual function of LCA patients. ERG serves to measure the function of cone and rod cells. The electrical signal a-wave comes from the photoreceptor and the b-wave comes from bipolar and Müller cells. ERG test shows nonresponsive signals in LCA patients while a-wave and b-wave are detectable in normal persons. This is especially important to diagnose LCA and also to figure out the genotype-phenotype correlation in LCA. Some studies showed that LCA patients carrying mutations in *AIPL1* have a rod ERG impairment, in *GUCY2D* cone ERG impairment, and in *RPGRIP1* both cone and rod impairment. Carriers with *CRB1* heterozygous mutation may develop regional retinal dysfunction that can be determined using multi-focal ERG.[1]

Clinical characteristics are also important in order to understand, characterize, and predict the prognosis of the disease. While fundus photography provides a wide variety of retinal appearances, full-field ERGs never give positive results after treatment in previous study.[22] Pupillometry and nystagmus assessment were used as an objective measurement in clinical trial of LCA patients. Still, subjective measurements such as best corrected visual acuity, Goldmann visual-field examination, and mobility testing can be very useful.[9]

2.1.2 Genetic Causes and Heritability of Leber Congenital Amaurosis

There are different approaches to identify the genetic causes of LCA such as linkage analysis, identity-by-descent (IBD) mapping, candidate gene analysis and whole exome sequencing (**Table 2**).

Table 2. Mutation identification strategy.[1]

Method	Linkage Analysis	IBD	Candidate Gene Analysis	Next Generation Sequencing
Gene identified	*AIPL1*, *GUCY2D*, *RDH12*	*CEP290* and *LCA5*	*LRAT*, *RPE65*, *RPGRIP1*, *CRB1*, *CRX*, and *IMPDH1*	*BBS4*[23]
Locus identified	*LCA9* on 1p36, *LCA3* on 14q24	-	-	-

Several methods were used as a mutation identification strategy. This allowed scientist to design cost-effective research based on available facilities.

Table 3. Prevalence of LCA causative genes.[6]

Gene	Location	Protein	Prevalence
GUCY2D	17p13.1	Retinal-specific guanylate cyclase	6%-21%
CEP290	12q21.32	Centrosomal protein 290 kDa	~20%
RPE65	1p31.2	Retinal pigment epithelium-specific 65 kD protein	3%-16%
CRB1	1q31.3	Crumbs homolog 1	9-13%
AIPL1	17p13.2	Arylhydrocarbon-interacting receptor protein-like 1	4%-8%
RPGRIP1	14q11.2	RP GTPase regulator-interacting protein 1	~5%
RDH12	14q24.1	Retinol dehydrogenase 12	~4%
CRX	19q13.32	Cone-rod *OTX*-like photoreceptor homeobox transcription factor	~3%
LCA5	6q14.1	Lebercilin	1%-2%
SPATA7	14q31.3	Spermatogenesis associated protein 7	Unknown
NMNAT1	1p36.22	Nicotinamide nucleotide adenylyl transferase 1	Unknown
IMPDH1	7q32.1	Inosine monophosphat dehidrogenase 1	
RD3	1q32.3	RD3 protein	Unknown
LRAT	4q32.1	Lecithin retinol acyltranseferase	Unknown
TULP1	6p21.31	Tubby-like protein 1	Unknown
KCNJ13	2q37	Inwardly-rectifying potassium channel subfamily J member 13	Unknown
IQCB1	3q13.33	IQ motif containing B1 protein	Unknown
OTX2	14q22.3	Orthodenticle homeobox 2 protein	Unknown
CABP4	11q13.1	Calcium binding protein 4	Unknown
DTHD1	4p14	Death domain containing protein 1	Unknown
GDF6	8q22.1	Growth differentiation factor 6	Unknown
MERTK	2q14.1	c-mer protooncogen receptor tyrosine kinase	Unknown

So far, 22 genes have been identified to cause LCA. Around 70% was caused by mutation in *GUCY2D*, *CEP290*, *RPE65*, *CRB1*, and *AIPL1*.

Since most of LCA's causative genes are inherited recessively, non-consanguineous families with at least 6 patients are needed to reach significance in linkage analysis. Only 3-4 patients are required for significant linkage analysis in consanguineous families. Furthermore, IBD mapping can be used in families with small size that are not reaching significance by linkage analysis. Candidate gene analysis can be used because non-syndromic LCA is

restricted to retina, therefore, genes that are expressed specifically in retina or have important function, can be presumed as candidate genes for LCA.[1]

Thus far, there are up to 22 genes involved in this disease (**Table 3**).[6] From those genes, *CEP290*, *GUCY2D*, *CRB1*, *IMPDH1*, and *RPE65* are the most common mutated genes, with a prevalence being 15%, 11.7%, 9.9%, 8.3%, and 6%, respectively in previous studies on Caucasian populations. Since there is no comprehensive data about Asian population, it is important to accomplish the study. Inheritance pattern of LCA is mainly autosomal recessive, apart from *CRX*, *IMPDH1*, *OTX* which can be inherited in an autosomal dominant trait. These genes can be clustered into several group based on the mechanism in which they are involved: phototransduction, retinoid cycle, photoreceptor structure and development, connecting cilium transport system, guanine synthesis, outer segment phagocytosis.[1]

2.1.3 *CEP290*

CEP290 is one of the most mutated genes in LCA (~20%) in Caucasian populations. This gene encodes the centrosomal protein of 290 kDa composed of 2472 amino acids. This protein is localized in the connecting cilium of the retinal photoreceptor cells, which may play a role in the transport system between the inner and outer segment of the photoreceptors. This transport system is very important because the proteins, which required for phototransduction, are synthesized in the inner segment of the photoreceptor cells. These proteins have to be transported to the outer segment, in order to conduct their correct function. In addition, *CEP290* is expressed in almost all of the body cells localizing to the centrosomes or the basal body of the cilium. Cilium is a microtubule-related organelle which acts as the antenna of the cells, transferring sensory information from the extracellular surroundings. The importance of this organelle is represented by numerous disease associated with mutation in the ciliary genes, termed ciliopathies.[24,25]

Mutations in *CEP290* result in a wide range of ciliopathies (**Figure 3**): LCA, Senior-Loken syndrome (SLS), Joubert syndrome and related disorder (JSRD), cerebello-oculo-renal syndrome (CORS), Meckel-Gruber syndrome (MKS), MKS-like, Bardet-Biedl syndrome.[1,26] However, this study will only focus on LCA.

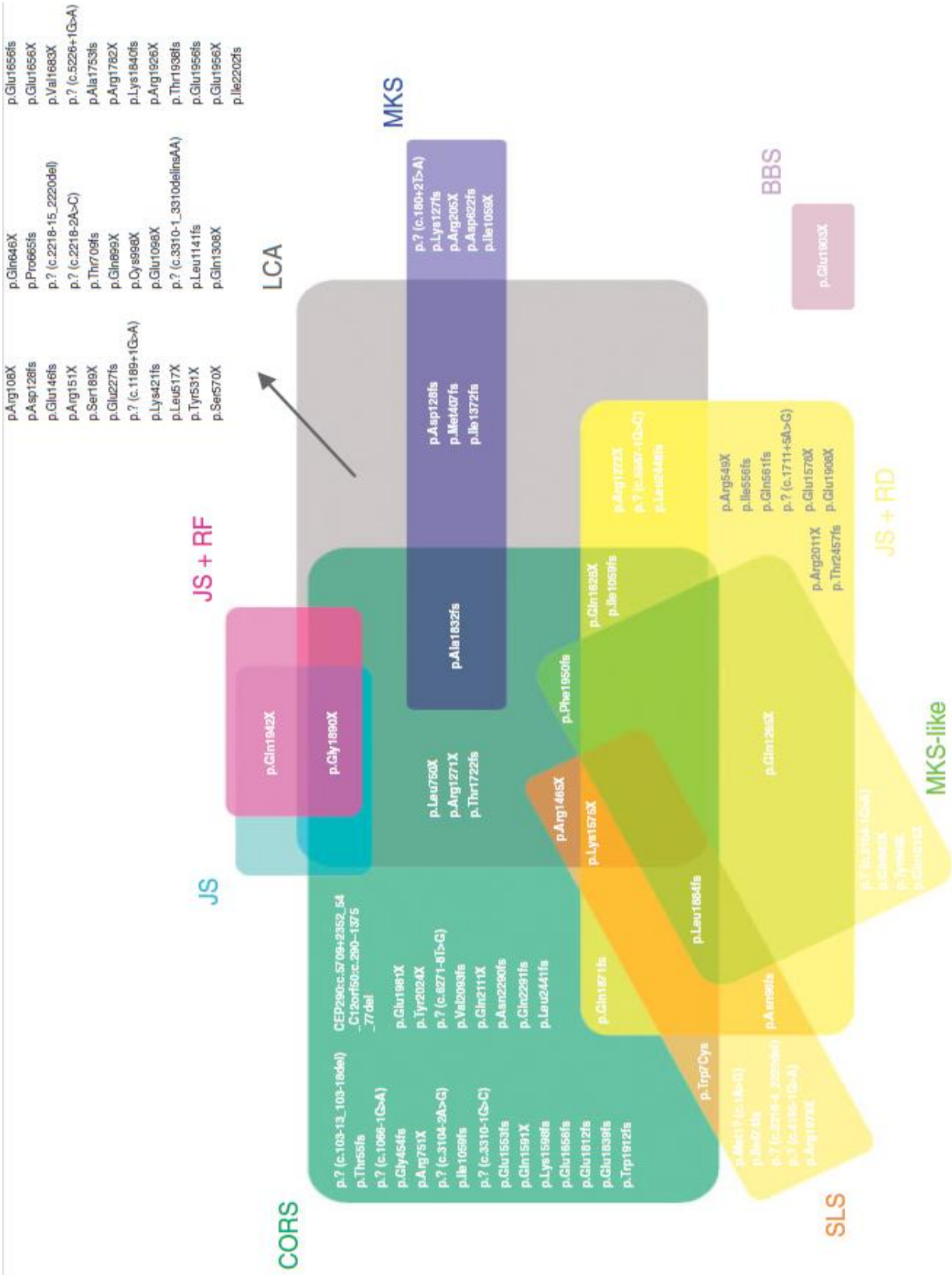

Figure 3. Mutation spectrum in *CEP290.* CORS: Cerebello-oculo-renal syndrome, JS: Joubert syndrome, JS+RF: Joubert syndrome plus renal failure, LCA: Leber congenital amaurosis, MKS: Meckel-Gruber syndrome, BBS: Bardet-Biedl syndrome, MKS-like: Meckel-Gruber like syndrome, SLS: Senior-Loken syndrome. (Adapted from: Coppieters, *et al.* 2010)[26]

2.2. Therapeutic Strategies

Therapeutic strategies for inherited retinal disorders comprise gene augmentation therapy and antisense oligonucleotide-based therapy. Nucleic acid sequence, either DNA or RNA, is used for both of them rather than protein or other molecules. The therapeutic agent is delivered to the target cells and allows restoration of gene function by acting as a replacement (gene augmentation) or in case of splicing mutation, redirecting the correct splicing using antisense oligonucleotide.[27,28]

2.2.1 Gene Augmentation Therapy

Gene augmentation therapy aims to insert a full-length cDNA of a gene, to restore the defect caused by the mutation. As the full-length cDNA is insufficient to enter the cell itself, the presence of a vector is required. There are several vectors available for gene augmentation therapy, including viral and non-viral vectors (**Table 4**).[27,29]

Clinical trials for *RPE65*-associated LCA have already been conducted using adeno-associated virus (AAV) vector containing the full length *RPE65* cDNA. This approach was proved to be safe and effective at least up to 3 years post-therapy.[30]

2.2.1.1 Lentiviral Vector

Lentivirus (LV) is a single-strand RNA retrovirus enable to infect both dividing and non-dividing cells and can be integrated in the chromosome of host cells. This ability gives a benefit of a long-term expression in dividing cells. However, random insertion of LV in the gene and gene spare long interspersed nuclear elements (LINE) can cause insertional mutagenesis which can lead to other genetic diseases such as cancer. In addition, expression levels can be reduced because of moderate immune response. The cargo capacity of this virus is up to 10 kb, large enough for most of retinal dystrophy genes.[31-33]

2.2.1.2 Adeno-associated Viral Vector

Adeno-associated virus (AAV) is a single strand DNA *Dependovirus* which is favorable in IRD gene therapy due to their ability to target various retinal layers with relatively safety and immunogenicity profile. One of the AAV's major strength is the availability of many serotypes and the ability to create hybrid vectors with the same AAV inverted terminal repeats (ITRs) and the capsid from different variant (**Table 5**). This transcapsidation system increases the potential of AAVs for IRD treatment.[34]

Table 4. Vectors used in clinical trial for various retinal diseases. (Adapted from: Rowe-Rendleman, *et al*. 2014)

Indication	Vector/Delivery system	Route of delivery
LCA2	rAAV2.hRPE65 rAAV2/4.hRPE65 rAAV2-CB^{SB}-hRPE65 rAAV2-CB-hRPE65	Intraocular
Choroideremia	rAAV2.REP1	Intraocular
Nonarteritic anerior ischemic optic neuropathy	QPI-1007:siRNA inhibitor targeting caspase-2	Intraocular
AMD	AdGVPEDE11D	Intraocular
Stargardt disease	StarGen equine infectATious anemia virus (EIAV) lentiviral vector expressesing ABCA4	Intraocular
AMD	RetinoStat EIAV lentiviral vector expressing endostatin and angiostatin	Intaocular
RP with Usher syndrome	UshStat EIAV lentiviral vector expressing MYO7A	Intaocular
AMD	AAV2-sFLT01	Intaocular
Retinal disease	rAAV2-VMD2-Hmertk	Intaocular
Metastatic melanoma of the eye	Albumin nanoparticles	Systemic
Diabetic macular edema	Cyclodextrin microparticles	Topical

Abbreviation: rAAV, recombinant adeno-associated virus. hRPE65, human Retinal pigmented Epithelium 65 kDa. CB, Chicken Beta-actin. REP1, Rab Escort Protein 1. siRNA, small-interference RNA. sFLT01, soluble Fms-like tyrosine kinase. MYO7A, Myosin VIIA. VMD2, Vitelliform Macular Dystrophy 2. hMERTK, human c-mer Proto-Oncogen Tyrosine Kinase. AMD, Age-related Macular Degeneration. RP, retinitis pigmentosa.

Despite of the versatility, AAV has a limited cargo capacity (4.7 kb). This constraint limits the use of AAV in IRD caused by mutation in genes whose cDNA exceeds 5 kb, for example: *CEP290* (~8 kb). This can be overcome by splitting the interest gene in two parts

and packing them separately in two different AAV (dual AAV vectors). Dual AAV vectors can be occurred as AAV has the ability to form intermolecular concatemers in the nuclei of targeted cells.[31,36]

Table 5. AAV serotype and target tissue. (Adapted from: Surace , 2008)**.**[35]

AAV Genome Plasmid	AAV Packaging Plasmid	Virion	Target Tissue
Rep 2	Cap 1	AAV2/1	Muscle, Retinal pigment epithelium (RPE), Lung
Rep 2	Cap 2	AAV2/2	Muscle, Liver, Retina
Rep 2	Cap 3	AAV2/3	Inner ear
Rep 2	Cap 4	AAV2/4	SNc, RPE
Rep 2	Cap 5	AAV2/5	Lung, Retina
Rep 2	Cap 7	AAV2/7	Muscle, Retina
Rep 2	Cap 8	AAV2/8	Liver, Retina
Rep 2	Cap 9	AAV2/9	Lung, Heart, SNc

Abbreviations: Rep, Replication. Cap, Capsid. AAV packaging plasmid can be used to target different organs.

2.2.1.3 Nanoparticles

Nanoparticles (NP) have been tested for IRD since it provides relatively large cargo capacity (up to 20 kb) and no insertional mutagenesis. NP are peptides for ocular delivery (POD), which can enter retinal cells *in vivo*. CK30PEG-NP is a POD conjugated with polyethylene glycol.[37] CK30PEG-NP can be used to transfer *ABCA4* in Stargardt's mouse model in which the transgene expression still remained after 2 years and improved the phenotype.[38]

2.2.2 Antisense Oligonucleotide Based Therapy

Antisense oligonucleotide (AON) is a small (13-25 bp) RNA molecule that bind to a specific sequence of the pre-mRNA, manipulating the splicing mechanism.[39,40] Nonetheless, these molecules are easily degraded by ribonucleases, therefore, chemical modifications to increase the intracellular stability are important. The specificity and stability of AONs are important features that determine the efficiency of AON therapeutic effect.[28]

2.2.2.1 AON Sequences

Theoretically, AON sequences are specifically designed to induce a biological effect, such as interfering expression of interest gene via RNase H-dependent mechanism or manipulating aberrant transcript caused by splicing mutation. Basically, two groups of AON are known: RNase H-dependent oligonucleotides, which degrade mRNA, and sterick-blocker oligonucleotides, which interfere with the splicing machinery. Both of them need to be specific, but the action mechanism of AON itself is complex and poorly understood. Several predictive tools have been established, mostly based on the secondary structures of local sequences and thermodynamic properties of AONs. One of them is ESEfinder (http://rulai.cshl.edu/cgibin/tools/ESE3/esefinder.cgi?process=home), which has been used in determining AON sequences as therapeutic approach for *CEP290*-associated LCA.[39,40]

2.2.2.2 Chemical Structure of AON

Phosphorothioate is the most widely used antisense oligonucleotide backbone, which is more soluble than methylphosphonates, the first synthesized oligonucleotides backbones. Modification are also important in the 2'-position of ribose by an O-alkyl group to enhance the stability of AONs inside the cell without decreasing the potency, such as 2'-methoxy (2'-OMe) and 2'-methoxyethoxy (2'-MOE) (**Figure 4**). The stability of these modifications probably due to the structural protection provided by the alkyl group.[40] However, convincing differences between these two chemical modifications are poorly understood yet.[41]

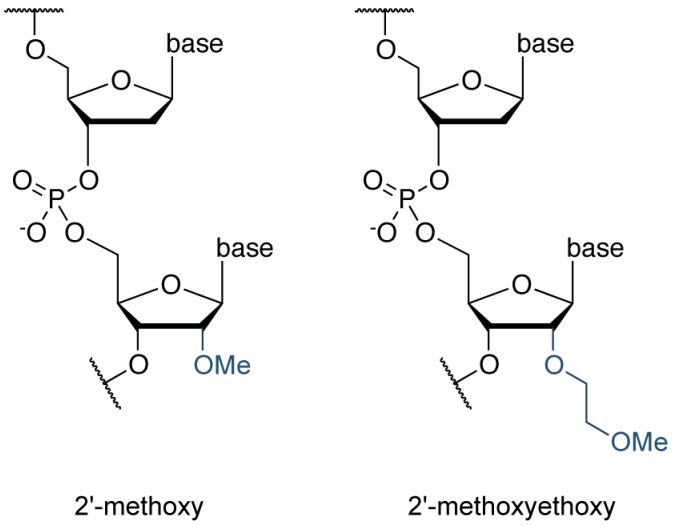

Figure 4. Chemical structure of AON. Adapted from: http://www.atdbio.com/content/13/Oligonucleotides-as-drugs. This study used two kinds of modification in O-alkyl group.