CHAPTER 5
DISCUSSION

5.1 Molecular Genetic Analysis of Indonesian LCA Patients

Up to date, only one study described the molecular genetics of LCA patients in Indonesian population. Study performed by Sitorus and colleagues revealed variants in *RPE65* and *AIPL1* by screening three genes: *GUCY2D*, *RPE65*, and *AIPL1*.[3] In this study, mutation identification strategy based on small number of amplicons containing known/frequent mutations: *CEP290* (c.2991+1655A>G), *CRB1* (exon 7 & 9), *GUCY2D* (exon 12), and *AIPL1* (exon 6) encompassed around 26% of all LCA cases. Reasonably, at least one candidate variant will be found from four patients. This study successfully revealed three candidate variants from two out of four Indonesian LCA patients. This simple and rapid screening can be used in larger cohorts to identify the genetic causes of LCA in Indonesian patients.

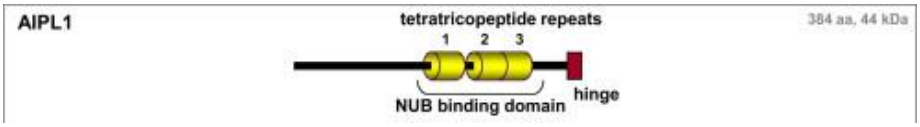

Figure 14. AIPL1 protein domain structures. (Adapted from: den Hollander, et al.) AIPL1 is a 44 kDa protein whixh is interacting with NUB, NEDD8 Ultimate Buster 1 (NUB1) protein.[1]

The variants found in this study were located in *AIPL1* gene, aryl hydrocarbon receptor protein-like 1. Protein from this gene consists of 384 amino acids, forming three tetratricopeptide repeat (TPR) domains (**Figure 14**). These domains act as molecular scaffolds, interacting with NEDD8 Ultimate Buster 1 (NUB1) protein, which is involved in cell-cycle process. AIPL1 also showed reduced cGMP hydrolysis activity, thus causing accumulation of cGMP and photoreceptor cells death. There are three classes of mutation in *AIPL1*: class I missense mutations, located in the N-terminus; class II missense and stop mutations, located in the TPR motifs; and the class III small in-frame deletions located in the C-terminus. Interestingly, missense variant found in this study, p.E318G in exon 6, is located in the C-terminus, which previously reported as a cause of autosomal dominant cone-rod dystrophy and juvenile RP.[45] However, it is also possible that this variant is only a benign rare variant since the difference between physicochemical substitution of this variant is relatively

low (Grantham score: 29). Second hit in *AIPL1* cannot be excluded, since analysis for intronic and untranslated region was not performed in this study.[1,45-47]

Two other variants found in this study, p.R324R in exon 6, and p.G11G in exon 1 were synonymous variants. Remarkably, both of them were predicted to cause an effect on the splicing pattern. It is not uncommon to find a splicing mutation as an LCA cause. Sample of tissue cultured from the patient (i.e. fibroblast) would be a good resource to further study the effect at transcriptional levels using cDNA synthesized directly from the patient's RNA. This approach has been used in the previous study revealing the *CEP290* deep intronic mutation, c.2991+1655A>G, as the most common mutation in the European and North-American LCA population.[48]

Mutational analysis for the unsolved cases can be done using molecular inversion Probes (MIPs), a next generation targeted sequencing approach. There were numerous genes to explore in these patients, such as *RPE65* which has been found to associate with LCA in the previous study of Indonesian population. *NMNAT1* also a good candidate since it accounts for 5% from LCA cases. However, due to time constraints, we were not assessed them yet.

5.2 AON Optimization for *CEP290*-associated LCA Patients

The use of AONs is already being tested in phase I/II clinical trials for Duchenne muscular dystrophy (DMD) with encouraging outcome.[49,50] In DMD, AONs were used to induce exons skipping so the reading-frame of disrupted transcript will be restored. The other therapeutic mechanisms of AON include (i) changing the ratio between different splice isoform, (ii) splicing exons that contain protein-truncating mutation, and (iii) blocking aberrant splice sites, as in *CEP290* intronic mutation.[51]

There are several factors that influence the efficacy of AONs: AON chemistry, AON sequence, and type of target cells.[52] In this study, two phosphorthioate AONs with different chemical modifications were tested: 2'-OMe vs 2'-MOE. Interestingly, AONs with 2'-OMe seems to have a better efficiency than AONs with 2'-MOE in blocking the cryptic splice site present in *CEP290*-associated LCA fibroblasts. Further study needs to be done to explain this phenomenon.

The specificity of sequence is important in regulating AON at cellular levels. As indicated by this study, from 29 AONs tested, only 22 AONs showed to restore the splicing defect at high concentration (1 µM). Most of the inefficient AON sequences at this concentration were targeting against the splice donor site. Remarkably, there are five different AON sequences that still work in sub-optimal condition (0.005 µM): GT2, GT3, GT4, GT20,

and GT22. GT2, GT3, and GT4 were predicted to have an effective redirecting effect from the previous study,[8] while GT20 and GT22 were targeting almost the same region (difference only 1 and 2 basepairs, respectively) with GT4. In this case, the different between chemical modifications might explain the result.

There is a mouse model for *CEP290*-associated LCA, but it does not recapitulate this disease.[53] Therefore, we need an alternative as a model for this disease to further study the efficacy of AON. Fibroblast is a good model because it can develop cilium under the serum starvation condition.[54] However, fibroblast cells do not represent the molecular environment of photoreceptors. Fortunately, fibroblast can be transformed to induced pluripotent stem cells (iPSCs) which can be generated into photoreceptor-like cells.[55] These photoreceptor-like cells have the similar environment and can be used to study ciliary function and target off-function, thus a good candidate to further test the efficacy of AON.

The use of AONs as therapeutic approach for *CEP290*-associated LCA has several advantages. First, *CEP290* c.2991+1655A>G is by far the most frequent mutation in the Caucasian population. Therefore, AON-based therapy for this mutation can benefit at least one thousand patients worldwide, considering LCA prevalence 1:50.000.[26,48] Second, toxic effect caused by overexpression of *CEP290* can be avoided since AONs only work at pre-mRNA levels. Overexpression of *CEP290* has been proved to cause cellular death in a lentiviral-based approach carrying full length *CEP290*.[7] Third, small size of AON fits in AAV, which is currently the most used viral vector in gene therapy field. Furthermore, clinical trials using rAAV targeting retinal cells showed promising safety and efficacy result. Fourth, the small size of AONs also allows them to enter into the cells easily.[9,56]

However, choosing the right AON chemistries and sequences can be problematic. Computer program such as ESE Finder 3.0 can be used to choose effective sequences to block the cryptic splice site. This study proved that the use of 2'-OMe is better than 2'-MOE in fibroblast cells and that AONs are still efficient at 0,005 µM. Nevertheless, efficacy in photoreceptor cells still needs to be assessed.

5.3 Progress of Gene Therapy in Retinal Dystrophies

Retinal dystrophies are excellent candidates to develop gene therapy as retinal structure provide some advantages: (i) it has blood-retinal barrier that makes immune response unlikely to occur, (ii) it can easily accessed through various surgical procedures that ensure the delivery of gene therapy, (iii) visual pathways remain intact, in which photoreceptor preservation hopefully help patient to keep their vision, and (iv) photoreceptor

cells are post-mitotic cells, therefore non-dividing cells, which minimize the risk of dilution effect in gene therapy.[57,58] Gene augmentation therapeutic approaches to restore vision in *RPE65*-associated LCA, choroideremia and other retinal disease are currently in clinical trials.

5.3.1 *RPE65*-associated LCA

Gene augmentation therapy, using rAAV serotype 2 (AAV2-hRPE65v2) as a vector, has been established to deliver full-length *RPE65* cDNA into the retinal pigment epithelium via unilateral sub-retinal injection. In this trial, all patients (from 8-44 years old) displayed improvement which is assessed by subjective and objective measurement, such as: dark adaptometry, pupillometry, elecroretinography, nystagmus, and ambulatory behavior. Patients in this study also have intact and responsive visual brain pathways despite of severe and early onset visual loss. Notably, the best response was showed by children, indicating that early intervention gives better result. Longitudinal study (1.5 and 3 years) showed that gene therapy using AAV2-hRPE65v2 is safe and effective.[5,30,59-61]

5.3.2 Choroideremia

As a part of retinal degeneration, choroideremia (CHM) is an X-linked inherited genetic disease due to loss of function in *CHM*, a gene that encodes Rab Escort Protein (REP1). *CHM* cDNA length ~2 kb, thus can be packaged in the rAAV. This approach has been proved preclinically to restore REP1 enzymatic activity in lymphoblast and iPSCs. Subsequently, clinical trial using rAAV serotype 2 carrying *REP1* (AAV.REP1) through subfoveal injection has been established and showed improvement in photoreceptor function.[56,62]

5.4 Therapeutic possibilities for *CEP290*-associated LCA.

There are several therapeutic approaches for *CEP290*-associated LCA. First, gene augmentation therapy using lentiviral vectors can be used to deliver full-length *CEP290* cDNA, which has been proved to restore ciliogenesis defect in fibroblast cells from the patient. This approach requires careful attention in determining therapeutic dosage, as overexpression of *CEP290* transgene delivered by the vectors results in toxicity.[7] Second, minigene containing N-terminal region of *CEP290* restored vision in zebrafish resembles *CEP290*-associated LCA.[63] This minigene can be inserted to the AAV, in which the full-length *CEP290* cDNA does not fit. Third, since the most common mutation of *CEP290*-associated LCA is the c.2991+1655A>G intronic mutation, the use of AON to redirect normal

splicing also gives benefit in this situation.[8,64] However, each approach aforementioned still needs to be further investigated to optimize its potential.