CHAPTER 4
RESULTS

4.1 Molecular Genetic Analysis of Indonesian LCA Patients

A total of 4 patients from three unrelated families were included in this study (**Figure 7**). Family II was consanguineous family with two affected family members. Patient II:2 (8 years old) in family 3 could not be participated in this study because his parents refused to give their consent for this children.

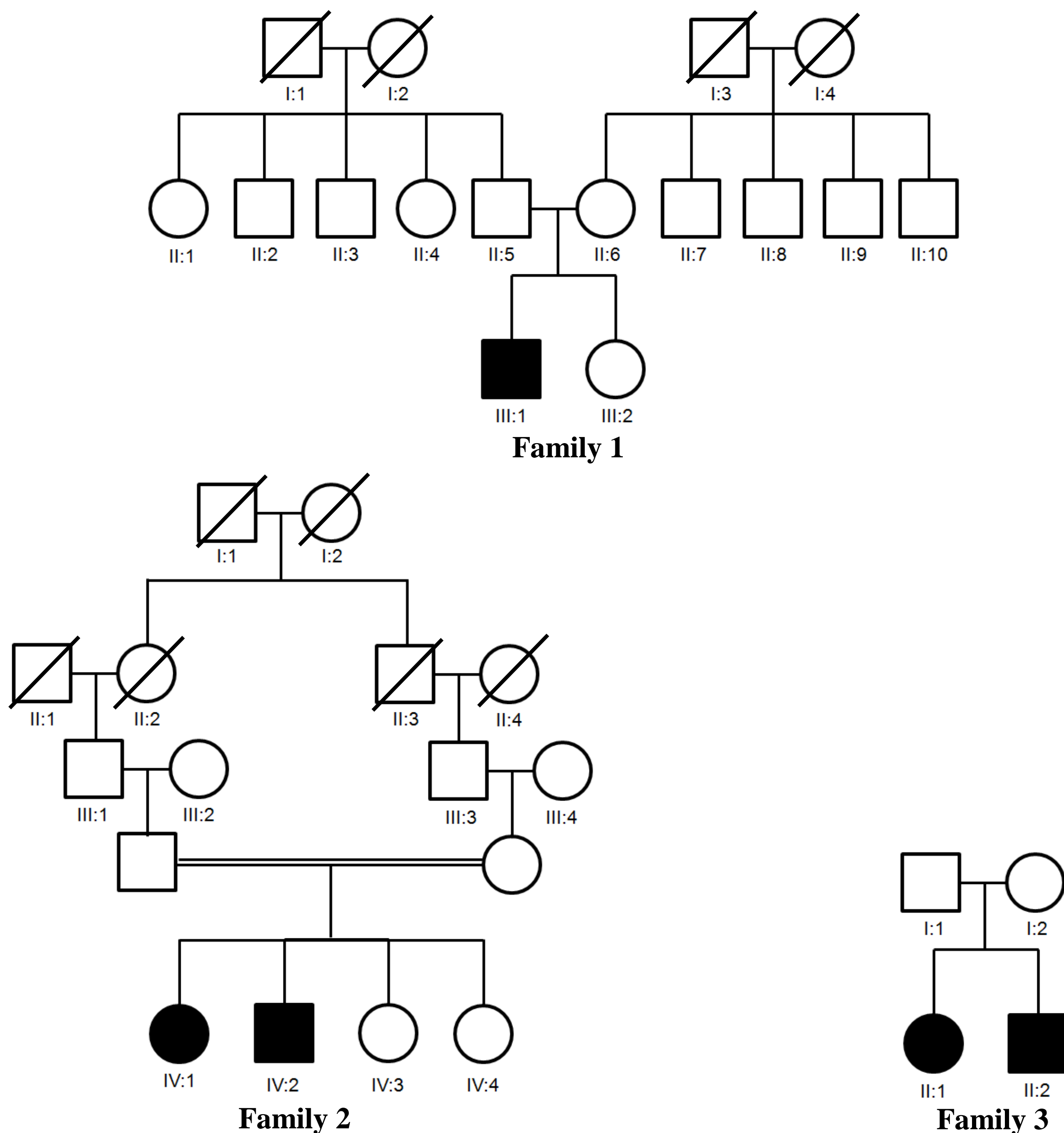

Figure 7. Pedigrees of the families analyzed in this study. Three Indonesian families were participated in this study. Black square (male) and black circle (female) represent affected family member.

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4.1.1 Clinical Findings

All of the patients have a severe visual loss before the first year of life. Funduscopy examination revealed retinal degeneration in all of them. Patients also developed sensory nystagmus and amaurotic pupils. Moreover, Franceschetti oculo-digital sign were found in three out of four patients. Patient number IV:1 and IV:2 from family 2 were also discovered to have a deep-set eyes, probably due to this specific behavior.

4.1.2 Molecular Findings

Table 6. Molecular findings of Indonesian LCA patients.

	Patient	*CEP290* (c.2990+1655A>G)	*CRB1* (p.P1305L)	*GUCY2D* (exon 12)	*CRB1* (exon 7 & 9)	*AIPL1* (exon 6)
Family 1	III:1	+/+	+/+	+/+	+/+	c.952G>C/+ (p.E318G/+)
Family 2	IV:1	+/+	+/+	+/+	+/+	+/+
	IV:2	+/+	+/+	+/+	+/+	+/+
Family 3	II:1	+/+	+/+	+/+	+/+	c.970C>A/+ (p.R324R/+)

	Patient	*AIPL1* (exon 1)	*AIPL1* (exon 2)	*AIPL1* (exon 3)	*AIPL1* (exon 4 & 5)
Family 1	III:1	+/+	+/+	+/+	+/+
Family 3	II:1	c.33G>C (p.G11G/+)	+/+	+/+	+/+

Mutation identification strategy based on, at first, five amplicons revealed variants in *AIPL1* exon 6. Further investigation in *AIPL1* exon 1-5 identified another synonymous variant in *AIPL1* exon 1.

The results for the ARMS PCR for c.2991+1655A>G mutation in *CEP290* and p.P1305L in *CRB1* gene were wild-type. Sanger sequencing of exon 7 & 9 of *CRB1* and exon 12 of *GUCY2D* only revealed wild-type variants. We discovered potential novel variant in *AIPL1*gene, p.E318G and p.R324R in exon 6 of two patients. Subsequently, sequencing performed in the remaining exons of *AIPL1* gene (exon 1-5) uncovered new synonymous variant p.G11G in exon 1 of one patient. The summary of the molecular findings were listed

in **Table 6**. The p.E318G mutation has a phyloP score of 4.64 and Grantham score of 29. The c.970C>A/p.R324R was predicted to diminish SC35 splicing protein and p.G11G mutation was predicted to recruit splicing factors, thus creating a putative new splice site (**Figure 8 & 9**).

Figure 8. Splicing prediction for c.33G>C variant in AIPL1. Using Alamut software, it is predicted that this variant recruits SRp55 and SRp40 proteins.

Figure 9. Splicing prediction for c.970C>A variant in AIPL1. This variant is causing a loss in SC35 protein, which is predicted to change the splicing pattern in this genomic region.

Both c.33G>C and c.970C>A variants were further screened using RFLP in the ~190 Indonesian control sample. Although it seems that variant c.33G>C only appeared in the LCA patients, confirmation study in the cDNA levels is needed. The result from c.970C>A RFLP screening is inconclusive since many of the samples showed unspecific digested products.

4.2 AON Optimization for *CEP290*-associated LCA Treatment

In order to optimize the AON treatment for c.2991+1655A>G mutation in *CEP290* gene, 29 AONs targeting different regions around the mutation were tested (**Figure 10**) using several dilutions. To measure the efficacy, transcriptional analysis was performed by RT-PCR. The best three AONs at RNA levels were subsequently tested at protein and cilium levels.

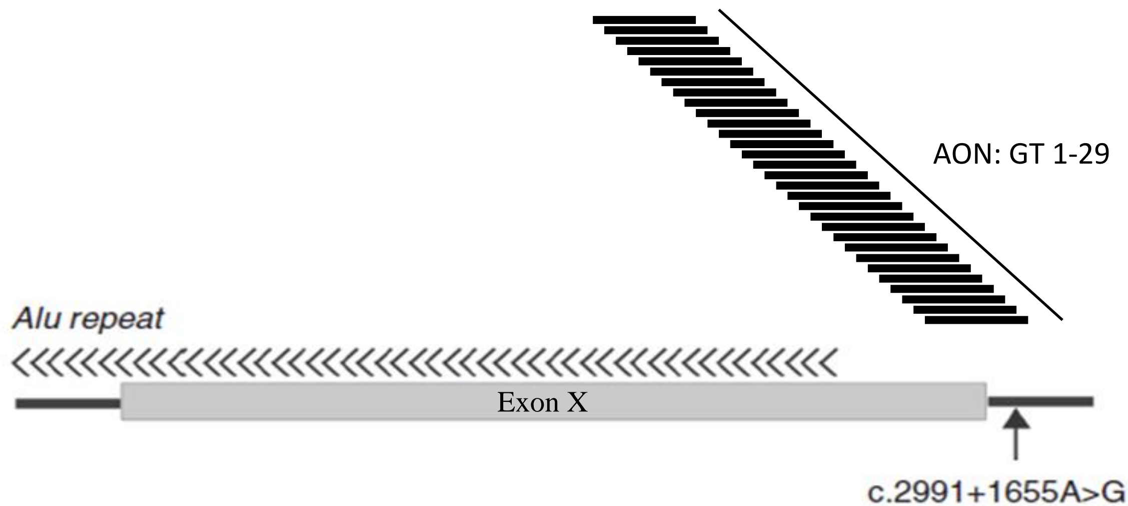

Figure 10. Schematic representation of AONs. The number of AON tested in this study is 29 (GT1-GT29), located in different places around deep intronic mutation (c.2991+1655A>G) in *CEP290* gene. GT1-GT6 used a 2'-OMe while GT7-29 used a 2'-MOE as a chemical modification.

4.2.1 Transcriptional Analysis

Fibroblasts cells derived from *CEP290*-associated patient were used. Transfection of AONs was done in several dilution steps: 1 µM, 0.1 µM, 0.01 µM, 0.005 µM, and 0.001 µM. The AON efficiency was assessed by measuring the ratio between aberrant and total transcript in each dilution. From the 1 µM concentration, we identified that some of the AONs were already not effective in restoring the aberrant transcript. Only those AONs that showed some degree of rescue were further diluted.

Table 7. Efficacy of AONs in serial dilutions.

AON	Chemical Modification	Efficacy				
		1 µM	0.1 µM	0.01 µM	0.005 µM	0.001 µM
GT1	2'-OMe	+	+	-		
GT2	2'-OMe	+	+	+	+	-
GT3	2'-OMe	+	+	+	+	-
GT4	2'-OMe	+	+	+	+	-
GT5	2'-OMe	+	+			
GT6	2'-OMe	-	-			
GT7	2'-MOE	+	+			
GT8	2'-MOE	+	+			
GT9	2'-MOE	+	+			
GT10	2'-MOE	+	+			
GT11	2'-MOE	+	+	-		
GT12	2'-MOE	+	+			
GT13	2'-MOE	+	+			
GT14	2'-MOE	+	+			
GT15	2'-MOE	+	+			
GT16	2'-MOE	+	+			
GT17	2'-MOE	+	+			
GT18	2'-MOE	+	+			
GT19	2'-MOE	+	+			
GT20	2'-MOE	+	+	+	+	-
GT21	2'-MOE	+	+			
GT22	2'-MOE	+	+	+	+	-
GT23	2'-MOE	+	+			
GT24	2'-MOE	-	-			
GT25	2'-MOE	-	-			
GT26	2'-MOE	-	-			
GT27	2'-MOE	-	-			
GT28	2'-MOE	-	-			
GT29	2'-MOE	+	+	-		

2'-OMe: 2'-O-Methyl. 2'-MOE: 2'-Methoxyethoxy

When we continued with lower dilution, the number of AON that work efficiently in restoring the aberrant transcript was also reduced. We found that the number of AONs that worked at 0.1 µM concentration was the same as those at the concentration of 1 µM. From now onwards, the 0.1 µM dilution will be called optimal dilution. Additionally, at 0.001 µM, no AON worked (**Table 7**).

The lowest effective concentration identified in small number of AONs was 0.005 µM, and we called it sub-optimal concentration. The three best AON in the sub-optimal condition were selected to evaluate the effect at the protein and cilium levels. These AONs were: GT2, GT4, and GT22. GT3 at 0.1 µM was used as a control since the characteristic was already known before (**Figure 11**).

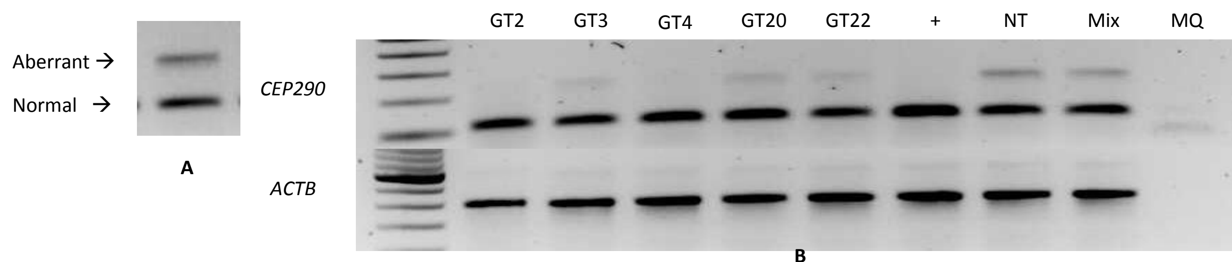

Figure 11. Transcriptional Analysis. A. *CEP290*-associated LCA cells showed aberrant and normal transcript that can be assessed by PCR encompassing exon 26-27 at cDNA levels. **B.** Transcriptional analysis of the best 5 AON at 0.005 µM concentration.

4.2.2 Western Blot and Protein Quantification

Western blot was performed to assess the effect of AON transfection at protein levels. To determine the efficiency of AONs, the quantity of protein were compared between non-treated LCA cells and AON-treated LCA cells *vs* wild-type (WT) cells. In order to do that, we used antibody targeting C-terminal part of CEP290, which is truncated in the mutant allele. Quantification of CEP290 protein was done by measuring the CEP290 band using ImageJ. Tubulin was used to normalize the result. Quantification was done in five series, to see if the result is consistent between each serial.

Non-treated LCA fibroblast cells were producing only 31.75% of CEP290 protein compared to the amount of CEP290 protein produced by the healthy cells (WT). CEP290 protein levels were increased after treatment to 54,91%, which is significantly higher than non-treated cells (**Figure 12**).

This study also proved that longer duration of AON treatment increases the protein levels. Fibroblast cells treated with GT3 for 6 days showed significantly higher CEP290 protein levels than cells treated with same AON for only 3 days (51,93% vs 62,55%).

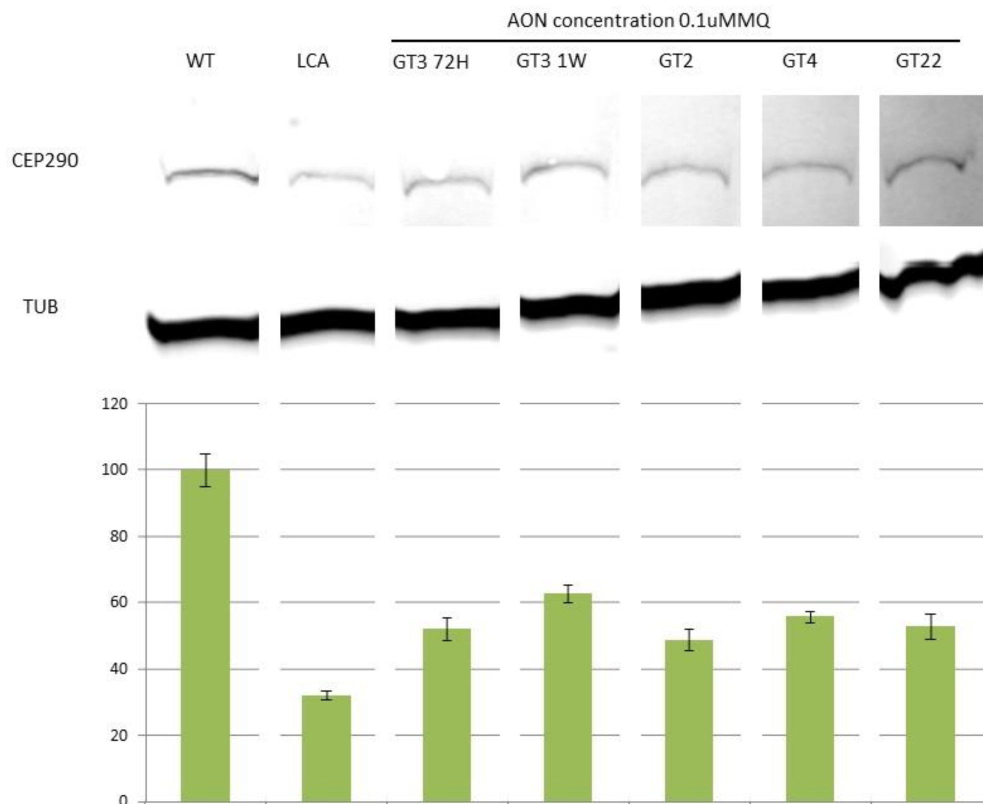

Figure 12. Immunodetection and protein quantification of GT2, GT4, and GT22. Western blot of CEP290 protein from wild-type (WT) fibroblast cells, non-treated fibroblast cells from LCA patient (LCA) and LCA fibroblast cells treated with GT3, GT2, GT4, and GT22. Bar chart showed the quantification of Western blot using ImageJ software. The measurement was performed five times, with the error bars showing standard deviation. Tubulin was used to normalize the result.

4.2.3 Immunocytochemistry

Wild-type fibroblast cells from seven independent experiments were used together with *CEP290*-associated LCA fibroblast cells. The three best AONs selected from previous transcriptional analyses (GT2, GT4, GT22) were used along with GT3 (positive control). Antibody against CEP290 protein used in this experiment is the same as the one used for the Western blot analyses. Cilium length measurements were performed using an antibody targeting acetylated tubulin, which represent the axonemes of primary cilia (**Figure 13A**). The axonemes were measured from the basal body to the tip using ImageJ software. Unpaired

Student's t-test was used for statistical analysis. The mean of non-treated cells cilium length was significantly reduced compare to wild-type cells (3,116 µm *vs* 5,076 µm). Fibroblast cells treated with GT2, GT3, GT4, and GT22 showed increased cilium lengths compare to untreated LCA cells both in optimal and sub-optimal condition (**Figure 13B**).

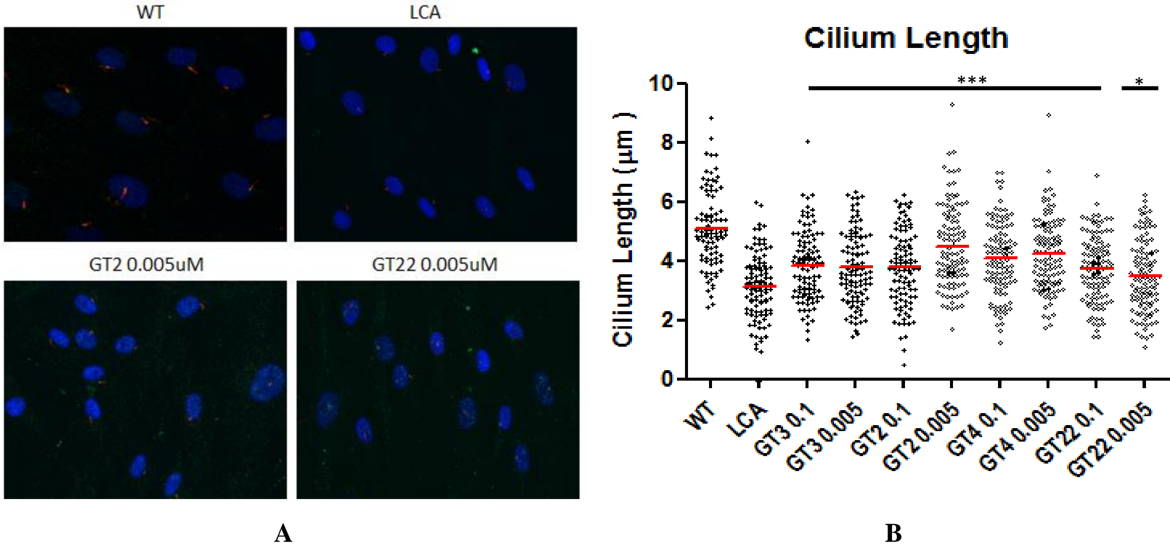

Figure 13. A. Immunocytochemistry using 4', 6-diamidino-2-phenylindole (DAPI, blue stain), rabbit polyclonal anti-CEP290 (green stain), and mouse monoclonal anti-acetylated Tubulin (red stain). **B.** Statistical analysis of cilium length analysis showed that all of the AON-treated LCA cells have a statistically significant longer cilia than the non-treated LCA cells (*p-value <0.05, ***p-value <0.001).