CHAPTER 3
RESEARCH METHODS

3.1 Research Field

This research project is in the field of human genetics predominantly molecular genetics integrated with ophthalmology.

3.2 Research Location

Sample collection and DNA isolation was performed in the Center for Biomedical Research (CEBIOR), Medical Faculty of Diponegoro University, Semarang, Indonesia. Mutational analysis and functional studies were conducted at the Department of Human Genetics of the Radboud University Medical Center in Nijmegen (The Netherlands).

3.3 Research Period

This research was accomplished from June 2013 to November 2014. Samples collection and DNA extraction were done in the 1st semester (June to November 2014), while diagnostic and functional study were done during the 2nd and 3rd semester (November 2014-2015).

3.4 Research Design

This study consists of diagnostic study and functional study. The diagnostic study is a descriptive cross-sectional study whereas the functional study is *in-vitro* study.

3.5 Population and Sample

3.5.1 Population

Individuals with congenital blindness from Low Vision Center, Yogyakarta and Wiyata Guna special school for blindness (Bandung) were included in the diagnostic study. Interview with patients or available family members and pedigree drawing were carried out to acquire information regarding ethnical background and consanguinity.

3.5.2 Sample

Samples were chosen from the congenital blindness patients who clinically were diagnosed with Leber Congenital Amaurosis. The age of patients was between 8 and 27 years

old. Their parents and related family were also included in the study in order to attain the family history and blood samples for carrier testing and segregation analysis. Informed consent were collected from the patients and family members involved in this study following the Declaration of Helsinki.

3.5.2.1 Inclusion Criteria

Patients with congenital blindness, sensory nystagmus, abnormal retinal appearance, and no other causes of congenital blindness such as infection, congenital cataract, glaucoma, and trauma, were included in this study. The diagnosis of LCA was given by two ophthalmologists from the history taking and eye examination. Electroretinogram was not performed due to limited facility.

3.5.2.2 Excluded patient

One patient has been excluded because he refused to do the venous sampling.

3.6 Research Variable

3.6.1 Diagnostic Studies

Variable: Mutation

Scale: Nominal

3.6.2 Functional Studies

Variable: Ratio between aberrant and normal transcript, cilium length, and protein level.

Scale: Ratio.

3.7 Diagnostic Studies: Mutational Analysis of Indonesian LCA Patients

3.7.1 DNA Isolation

The method used to extract the DNA from venous sampling was salting out method as mentioned in the Cebior's manual laboratory.[42] The DNA concentration was measured using NanoDrops.

3.7.2 Primer Design

The primers were designed using Primer3Plus software, http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/ . The optimum primer size is 20, and the optimum annealing temperature is 59^{o}C. GC content was kept around 40-60%.

3.7.3 Polymerase Chain Reaction (PCR)

Two types of PCR were conducted, amplification refractory mutation system (ARMS) and regular PCR to amplify the interest gene for further investigation using Sanger sequencing. There were two regular PCR methods used in this study, standard PCR and touch down PCR.

This study used standard PCR with universal PCR mix consist of: 2,5 µl PCR buffer 10x (Roche), 0,5 µl dNTPs (10 mM), 0,5 µl forward and reverse primer (10 pmol), 0,5 µl polymerase (5 U/µl), and 2,5 µl DNA sample. The amount of H_2O and $MgCl_2$ were adjusted for each primer condition. Universal PCR condition used in this study was 3 minutes at 94^{o}C, 30 seconds for denaturation at 94^{o}C, 30 seconds for annealing which depends on each PCR condition, and 30 seconds of elongation at 72^{o}C. Three last steps were repeated in 35 times of cycle and ended at 72^{o}C for 5 minutes. PCR conditions and primers sequence can be found in **appendix 1-5**.

Touch down PCR was used to decrease unspecific product by decreasing the annealing temperature every second cycle from the upper limit, which is determined by the melting temperature of the primer.[43]

3.7.3.1 ARMS-PCR

ARMS-PCR was done to investigate the presence of mutation in *CEP290* (c.2991+1655A>G;p.C998*) and *CRB1* (c.3914C>T;p.P1305L) gene. ARMS-PCR for wild type *CEP290* was performed under standard PCR while touch down PCR was performed for c.2991+1655A>G mutation.

3.7.3.2 Regular PCR

CRB1, *GUCY2D*, and *AIPL1* genes were amplified by standard and touch down PCR. Two primer sets were used overlapped each other to encompass exon 7 of *CRB1*. We use thee primer sets to encompass exon 9 of *CRB1*. Touch down PCR was used only for *GUCY2D*.

3.7.4 Sanger Sequencing

PCR products were sent to sequence facility using ABI PRISM Big Dye Terminator Cycle Sequencing V2.0 Ready Reaction kit and the ABI PRISM 3730 DNA analyzer from Applied Biosystem. Purification of PCR product was performed before sending to the sequence facility, either using Exo1 and FastAP enzyme or NucleoSpin Gel and PCR Clean-Up (Macherey-Nagel).

3.7.5 Restriction Fragment Length Polymorphism (RFLP)

Mutations found were analyzed with restriction enzyme analysis (RFLP) using HpaI and BsaHI enzymes (New England Biolabs, Ipswich, MA) designed using NEBcutter software http://tools.neb.com/NEBcutter2/ in Indonesian normal population. HpaI enzyme cuts DNA with specific sequence CCGG, resulting in three bands (262 bp, 156 bp, and 82 bp) in RFLP gel result with wild-type variant of *AIPL1*exon 6 while BsaHI enzyme cuts GRCGYC sequence, resulting in 327 bp and 109 bp bands in RFLP gel result containing c.33G>C variant of *AIPL1*exon 1. RFLP was performed in approximately 190 Indonesian control samples.

3.8 Functional Studies: Optimization of Antisense Oligonucleotide (AON) Therapeutic Approach for LCA

3.8.1 Cell Culture

Fibroblast cells from *CEP290*-associated LCA patient were cultured in DMEM medium (Sigma, St Louis, MO) containing 20% (vol/vol) fetal calf serum (Sigma), 10 U/µl penicillin and 10 µg/µl streptomycin (Sigma), and 1% sodium pyruvate. The cells were kept at 37^{o}C incubator with 5% CO_2. Twice a week, the cells were passed in 1:3 dilutions and grow in fresh medium.

3.8.2 Transfection

Transfection mixtures consisted of 100 µl of Opti-MEM, AON in a desired concentration, and 3 µl FuGENE HD transfection reagent (Promega, Madison, WI). The mixtures were incubated for 20 minutes at room temperature and were added to the cells. For RNA and immunocytochemistry studies, the number of the cells was 150.000-300.000 cells, while for the protein study, the number of the cells transfected was ~1.800.000 cells.

3.8.3 RNA Isolation and Reverse Transcription-PCR

Fibroblast cells were harvested and rinsed with 1x-phosphate-buffered saline (1x PBS) before RNA isolation. Total RNA was isolated using NucleoSpin RNA II isolation kit (Machery Nagel, Duren, Germany), following the manufacturer's protocol (**appendix 7**). cDNA synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad, Veenendaal, the Netherlands), and the cDNA was amplified by standard PCR condition enhanced with Q-solution (Qiagen, Hilden, Germany). Primers used in this PCR are listed in the **appendix 6**. Primers for *CEP290* encompass exon 26 and exon 27. Subsequently, PCR products were resolved in the 1.6% agarose gel. In addition, PCR of *ACTB* was done to normalize samples. Pictures were taken by ImaGo^{TM} and bands were quantified using ImageJ software.

3.8.4 Western Blot

Fibroblast cells were collected from 10cm^2 plates and washed with 1xPBS. The cell pellets were homogenized in RIPA buffer (50 mM TRIS pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.5% Na-Deoxycholate, 1% NP40 plus protease inhibitors) sonicated, and centrifuged. The soluble fraction was transferred into a new tube, and protein concentration was measured using BCA protein assay (Thermo Scientific, Rockford, IL). A mixture of 100 µg of total protein was prepared, 80 µg of protein were loaded onto a NuPAGE 3-8% Tris-Acetate gel (Life Technologies, Carlsbad, CA) for CEP290 immunodetection and 20 µg onto a NuPAGE 4-12% Bis-Tris Gel (Life Technologies) for Tubulin detection. Proteins were transferred to PVDF membrane, blocked in 5% non-fat milk in 1xPBS solution and incubated with mouse anti-tubulin (1:2000) and rabbit anti-CEP290 (1:1000) in 0.5% blocking solution for 4 days at 4 ^{o}C. Incubation of secondary antibodies using goat anti-rabbit or –mouse antibodies was done at 1:20.000 for 1 hour. Membrane was washed three times for 10 minutes between each antibody incubation with PBS-Tween 0.1%. Blot was developed using Odyssey infrared imaging system (Li-Cor, Lincoln, NE). Image analysis was done using ImageJ (National Institutes of Health, Bethesda, MA).

3.8.5 Immunocytochemistry

Fibroblast cells were grown on coverslips, transfected as described previously and incubated in serum-free medium to induce cilium formation. Cells were washed in 1x-PBS and fixed in 2% paraformaldehyde. Cells were permeabilized in 1% TritonX100 in 1x-PBS. Samples were blocked in 2% BSA in 1x-PBS for 20 minutes to prevent unspecific binding of antibodies. Subsequently, cells were stained using antibodies targeting CEP290 (rabbit

polyclonal antibody, 1:300) and acetylated tubulin (mouse monoclonal antibody, 1:1000). Goat anti-rabbit and –mouse were used as secondary antibodies at 1:500 in 2% BSA. Coverslips were washed between incubations 3 times for 5 minutes in 1x-PBS, and rinsed in H_2O before mounting in Vectashield compound containing DAPI. Images were taken using Axio Imager (Zeiss, Oberkochen, Germany), processed with ZEN software (Zeiss) and analyzed using imageJ software (National Institutes of Health).

3.8.6 Statistical Analysis

Blind analysis of the immunocytochemistry was performed by counting 100 cells per condition at minimum. Unpaired Student's t-test was used for statistical analysis using GraphPad Prism 6 software.

3.9 Research Flow

Two workflows were used, one for diagnostic analyses and the other for functional studies. Diagnostic analyses were done by amplifying several amplicons, which together explain 26% of LCA cases in Caucasian population.

3.9.1 Diagnostic Study Workflow

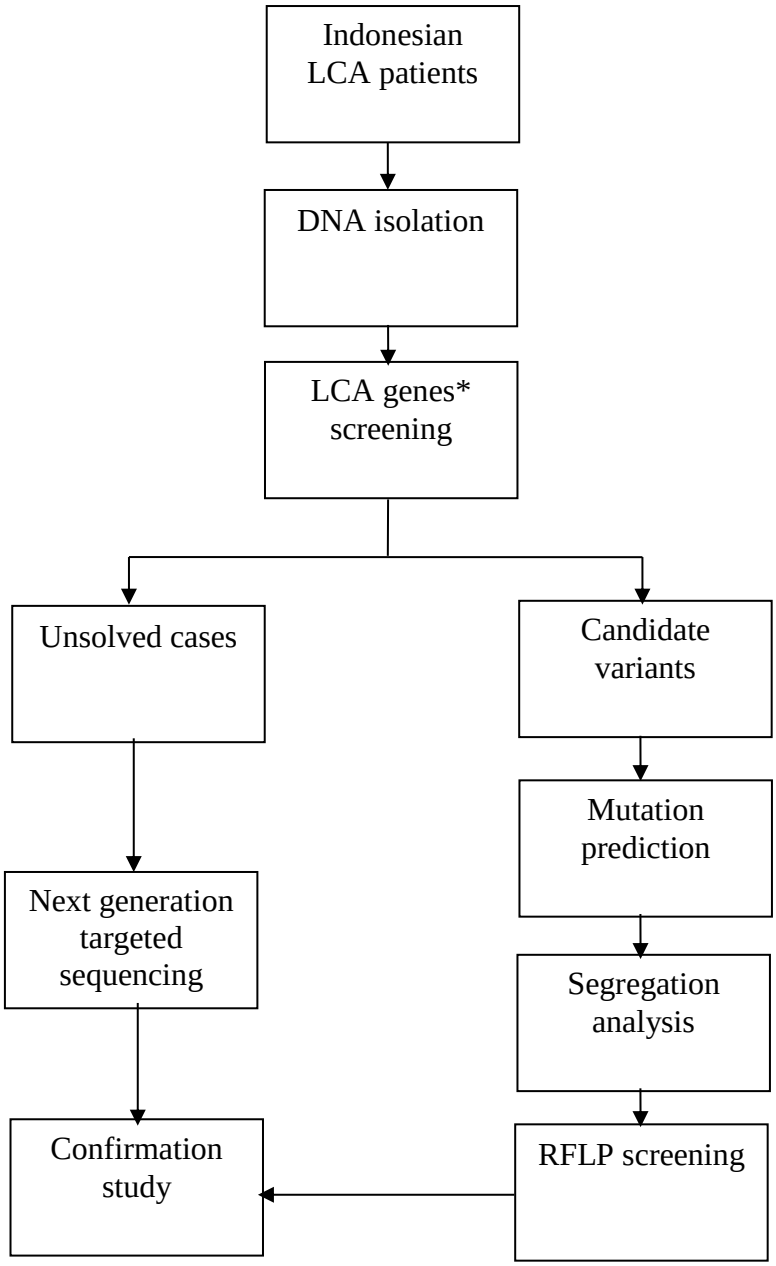

Figure 5. Schematic representation of diagnostic study workflow. *LCA genes screened in this study are *CEP290* (c.2991+1655A>G), *CRB1* (exon 7 & 9), *GUCY2D* (exon12), and *AIPL1* (exon 6). *CRB1* (p.P1305L) also included as a frequent mutation in Indonesian retinal degeneration patients from a previous study.[44]

3.9.2 Functional Study Workflow

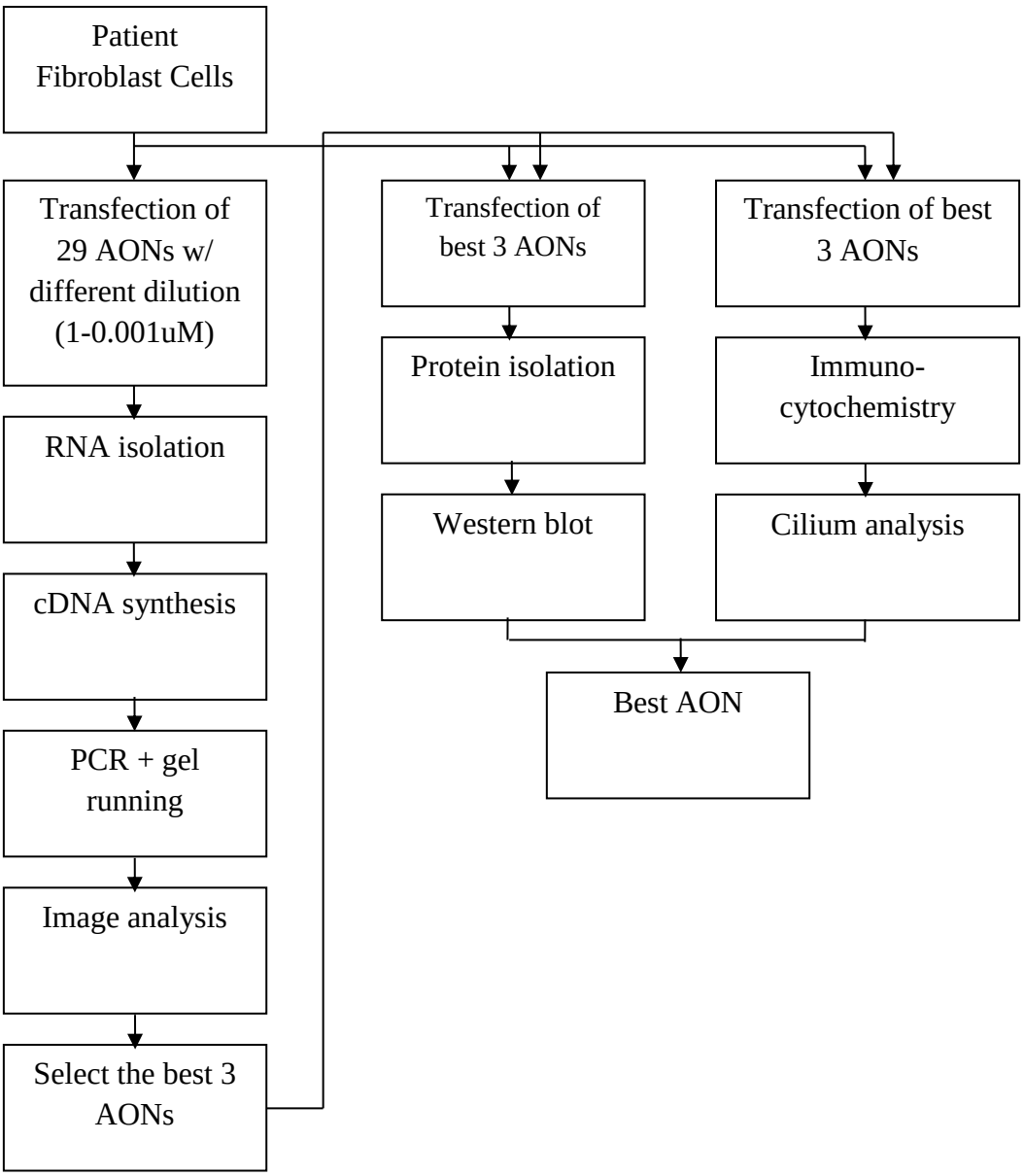

Figure 6. Workflow for functional studies to optimize the potential of AON-based approach. *CEP290*-associated patient fibroblast cells were used to test 29 AON with different sequences. In addition, WT cells from healthy persons were used as a normal control in the Western blot and cilium analysis. Selection of 3 best AONs was done at RNA levels by treating the cells with different dilutions (1 µM, 0.1 µM, 0.01 µM, 0.005 µM, and 0.001 µM).

3.10 Data Analysis

Data analysis for diagnostic study is descriptive and for functional studies is experimental.

3.11 Research Ethics

Informed consents were signed by affected individuals or the caregiver. Ethical clearance was approved by Institutional Review Board of Faculty of Medicine and Kariadi Hospital, Diponegoro University.