ORIGINAL ARTICLE

Clinical Evaluation and Mutation Analysis in Indonesian Autosomal Dominant Polycystic Kidney Disease

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ABSTRACT

Background: Autosomal Dominant Polycystic Kidney Disease (ADPKD) is the most common form of hereditary Polycystic Kidney Disease (PKD) in adults. ADPKD is characterized by massive renal cyst expansion leading to renal failure. Mutation of PKD1 is the most prevalent cause of ADPKD (85% cases). This is the first study which provided genotype and phenotype data of Indonesian patients suspected of ADPKD.
Aim: To provide data on exon 13-15 PKD1 as the highest prevalence region of PKD1 mutations and its impact on the phenotype.
Methods: Fifteen unrelated patients from Kariadi General Hospital, who fulfilled Ravine renal ultrasound criteria for ADPKD and control matched by gender. Nested PCR from long range (LR) PCR was performed to avoid pseudo gene amplification.
Results: History of renal disease in the family was found in 60% subjects. All of the patients were hypertension, and almost half of the cases have end stage renal failure. Two novel variants in PKD1, c.3148G>T and c.3852C>T in two different families were found. Variant c.3148G>T leads to a premature stop: [p.Glu1050*] and was found in the patient and his brother. Variant c.3852C>T is a silent mutation, but software analysis revealed a chance of exon skipping.
Conclusions: Hypertension and renal failure is frequent symptom in ADPKD**.** Pathogenic novel nonsense variant c.3148G>T and a synonymous variant c.3852C>T was found in two different families. Further analysis of synonymous variants for possible effect on splicing should be done on the mRNA level.
Keywords: PCR, ADPKD, genetic testing

INTRODUCTION

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is the most common form of Polycystic Kidney Disease (PKD) in adults[1,2,3,4]. ADPKD is characterized by massive renal cyst expansion leading to renal failure[5]. This hereditary renal disease frequently causes Chronic Kidney Disease (CKD)[6]. End-stage renal disease (ESRD) occurs in the late phase of the disease. In this stage, the majority of patients (50%) have to start renal replacement therapy (dialysis and/or kidney transplantation)[5]. The prevalence of ADPKD worldwide is high, probably due to the very large size of the causative gene, which is a large target for mutations. In the USA it is estimated between 1 in 400 and 1 in 1000, whereas in Japan the prevalence is 1 in 2500. Prevalence for Indonesia is unknown. Based on prevalence estimates in other countries, we expect between 100,000 and 500,000 ADPKD cases in Indonesia.

Mutations of *PKD1* are the most prevalent cause of ADPKD (85% of cases). The remaining cases (15%) are caused by mutations of *PKD2*[7,8]. *PKD1* is located on chromosome 16p13.3. Patients with *PKD1* mutations have more severe clinical manifestations than those with *PKD2* mutations. The kidney volume is larger and onset of ESRD is earlier in *PKD1* compared to *PKD2* patients[9,10].

Currently, no DNA testing for ADPKD is available in Indonesia. DNA testing is important for genetic counselling of families, with option of prenatal testing. It is also important for early diagnosis and start of treatment.

PKD1 is a very large gene that contains 46 exons with more than 52 kb of genomic DNA. *PKD1* encode a protein that is known as polycystin-1 (PC1). PC1 is comprised of 4302 amino acids[11]. PC1 participates in the cells' signal-transduction pathway as a mechanic sensor of fluid flow. PC2 is the protein product of *PKD2* which functions as an ion channel mediating calcium influx into the renal epithelial cells. Mutations in both genes cause disruption of renal cilia function leading to the formation of cysts[3,4,12].

The gene size, presence of hot-spot mutations and GC rich content are obstacles in amplification and mutation analysis of *PKD1*[4]. The most challenging problem in mutation analysis is the presence of duplication regions of exon 1-33 of *PKD1* which are known as pseudo genes *(PKD1P1-P6)* that have very high similarity (97.7%) to *PKD1*[13]. The pseudo genes have mRNA with suboptimal start codons that are not translated or expressed[14]. Mutations in the 5' end of the *PKD1* gene are associated with earlier onset and higher severity compare to mutations in the 3' end region. Mutation studies in the *PKD1* gene reported that pathogenic mutations were more common in exon 15[13].

An efficient PCR design is needed to avoid *PKD1* pseudo genes amplification. Nested PCR from long range (LR) PCR is a good strategy to avoid pseudo gene amplification[15]. PCR High Resolution Melting (HRM) is a fast and sensitive mutation detection technique, based on melting curve analysis after real time PCR[16]. A combination of LR PCR-Nested PCR and HRM is a promising technique to help screen for mutations in *PKD1* cost-

efficiently, which is important for testing in Indonesia, where genetic testing is just getting started and is not covered by health insurance.

A number of mutations have been reported in Asian populations like Korean, Thai[17] and Chinese[18] but not yet in the Indonesian population. This is the first study that provides genotype and phenotype data of Indonesian patients suspected of ADPKD focused on exon 13-15 PKD1 as the highest prevalence region of *PKD1* mutations.

METHODS

Population and samples. Fifteen unrelated Patients were collected from renal clinics Dr. Kariadi Hospital, Semarang. Patients were diagnosed with polycystic kidney disease by physical examination, laboratory examination including ultrasound imaging that fulfil the Ravine criteria for ADPKD. All patients have never previously been tested using molecular diagnostic techniques for ADPKD. Fifteen independent healthy individuals matched by gender to the patients with normal blood pressure and kidney structure as shown by ultrasound were included as control DNA samples. The gender distribution was 6 (40%) females and 9 (60%) males. The youngest age was 25 years and the oldest was 80 years with a mean age of 48 years. Approval for the study was obtained from the Ethical Committee for Medical Research, Faculty of Medicine, Diponegoro University - Dr.Kariadi Hospital.

DNA samples: DNA were extracted from 15 samples (S) and 15 controls (C) who DNA samples were extracted from whole EDTA blood using a salting out method as described elsewhere. DNA quality and quantity were measured by Nanovue Plus GE Healthcare Bio Sciences AB.

LR PCR: Primer sequences, according to Tan YC et al 2012,[15] for fragment LR4 are F 5'- TGGAGGGAGGGACGCCAATC-3' and R 5'- GTCAACGTGGGCCTCCAAGT-3'. The PCR reaction consisted of a total of 25µl: 12,5 µL of Taq Extra Hot Start Ready mix (KAPA); 1 µL with 10 picomole forward and reverse primer; 1 µL with 60-100ng DNA and dHPLC H2O. PCR was undertaken on an ABI 9700 to amplify the region of interest with the following settings: initial denaturation at 95ºC for 3:15; followed with 35 cycles of denaturation at 95ºC for 00:20, annealing at 68ºC for 5:00 and final extension at 72ºC for 7:00 and hold at 4ºC for 10:00. The 0.8% agarose gel was run using electrophoreses at 90V for 60 minutes with FluoroSafe dye. Marker GeneRuler 1 kb was used to determine product size.

Nested PCR HRM: Nested PCR HRM was performed to amplify exon 13-15G (exon 15 was divided in 9 part i.e., 15 A to 15 I) using primer designs previously published by Bataille et al (2011).[20] Each PCR HRM reaction consisted of 5µL Type-It HRM Master Mix, 1µL with 10 picomole forward and reverse primer, 1 µL with 10ng DNA from the post purification of LR PCR products using the *GeneAid Purification* Kit and HPLC H_2O up to a total volume of 10 µL. The RotorGeneQ5Plex HRM 72-well rotor (Qiagen, California, USA) was used to amplify targets with settings: initial denaturation at 95ºC for 10:00 followed by 30 cycles of denaturation at 95-98ºC for 00:15, annealing at 64-70ºC for 00:20 and extension at 72ºC for 00:20. HRM melting was done at 72-95ºC with 0.1ºC temperature increment per second.

Sequencing: Sanger sequencing was undertaken for 3 samples of each exon, which showed baseline graph (WT), highest aberrant graph with positive deflection (VAR) and lowest aberrant graph with negative deflection (VAR). The mutation predictions using LR PCR-Nested PCR HRM were compared to sequencing results.

Mutation analyses: The sequence analysis was compared to the reference sequence from NCBI RefSeqPKD1 (NM_001009944.2). Known mutations were accessed in The Polycystic Kidney Disease Mutation database website (http://pkdb.mayo.edu), NCBI (http://ncbi.gov.nih) and Ensembl (http://ensembl.org). New variants detected were analyzed through Alamut version 2.3.6e software. Pathogenic mutations were analysed through pedigree segregation analysis.

Data analyses: Data are provided in tables and graphs.

RESULTS

Overviews of clinical characteristics of the patients are list in table 1. All of the patient's blood pressures were under maintenance with oral anti-hypertension drugs. Based on the JNC VII criteria, 9 (60%) samples were pre-hypertension and 6 (40%) were hypertension stage II. Mean systolic blood pressure was 144 mmHg and diastolic blood pressure was 87 mmHg. Family history related to renal disease was documented by drawing the pedigree. History of renal disease in the family was found in 9 (60%) samples: 5 with renal cysts, 3 with unspecified renal disease and 1 with chronic hypertension.

Renal and extra renal cysts were assessed by ultrasound examination. Extra renal cysts were found in 7(47%) samples: 6 samples with liver cysts and 1 sample with adnexa cyst. Bilateral with numerous numbers of cysts (>10 cysts) in each kidney was found in 9(60%) samples. Renal enlargement was documented in 7(78%) of those 9 samples. Haematuria was found in 8 (53%) samples. End stage renal disease with routine dialysis was regularly performed in 7(47%) cases.

Variation in gDNA sequence was observed in 4 fragments (Table 2). There were 1 nonsense mutation and 3 synonymous mutations found in the samples. Pedigree and sequencing analysis in family members was undertaken for c.3148G>GT to see the segregation of this variant. Alamut predicted c.3852C>CT to have a chance of exon skipping.

Analysis of LR PCR – Nested PCR HRM showed in Fig 1A. What is striking about the figures is S35 (sample) showed c.3852C>CT mutation which in HRM predicted S35 as VAR (Fig 1D). A pedigree of S35, ultrasounds picture and sanger sequencing of sample 35 and 45 presented in figure 2 (A- G).

Table1: Clinical characteristics of study participants

No	Samples (Initial)	Sex	DOB	Systolic Pressure	Diastolic Pressure	Pedigree collection (Y/N)	Renal Ultrasound Data (Y/N)	Hemodialysis (at the time of blood collection)
1	EG (S1)	F	12/31/1979	170	100	Y	Y	-
2	SKH (S3)	F	20/05/1958	180	90	Y	Y	+
3	AM (S8)	M	16/06/1932	130	80	Y	Y	-
4	SR (S14)	M	08/03/1983	130	80	Y	Y	+
5	DE (S15)	F	23/02/1972	130	80	Y	Y	+

6	YK (S18)	F	04/08/1988	160	80	Y	Y	+
7	RL (S21)	M	24/12/1968	110	80	Y	Y	+
8	KS (S24)	M	05/07/1960	120	80	Y	Y	-
9	KSI (S26)	F	15/05/1967	130	80	Y	Y	+
10	SRJ (S34)	M	17/07/1951	130	80	Y	Y	-
11	DJS (S35)	M	30/06/1969	180	120	Y	Y	+
12	MS (S36)	M	07/05/1965	170	110	Y	Y	-
13	AK (S38)	F	11/05/1952	120	80	Y	Y	-
14	NG (S39)	M	12/01/1957	170	90	Y	Y	+
15	SH (S42)	M	10/02/5194	130	80	Y	Y	-

Table 2: List of variation in c.DNA sequence

Fragments	c.DNA changes	Protein Changes	Effects	Samples	Previously described
13	c.3148G>GT	p.Glu1050*	Nonsense Mutation	S35	Novel
15B	c.3852C>CT	p.Ala1284Ala	Synonymous Mutation	S3	Novel
15D	c.4674G>GA	p.Thr1158Thr	Synonymous Mutation	S1, S3, S39, C2, C11	rs79884128
15G	c.5847C>CT	p.Ser1949Ser	Synonymous Mutation	S24, S42	rs80111665

*STOP codon, S = sample; C = healthy control

Fig 1: Analysis of LR PCR – Nested PCR HRM in exon 15B PKD1 compared to sequencing
A. Analysis of LR PCR – Nested PCR HRM. Yellow is graph for WT group whereas blue (highest deflection) and red (lowest deflection) were graphs for VAR group.
B. Forward Sequence for C1 (healthy control). HRM predicted C1 as WT agreed with sequencing result.
C. Forward Sequence for C13 (healthy control). HRM predicted C13 as VAR whereas sequencing showed as WT.
D. Forward Sequence for S35 (sample) showed c.3852C>CT mutation. HRM predicted S3 as VAR agreed with sequencing result.

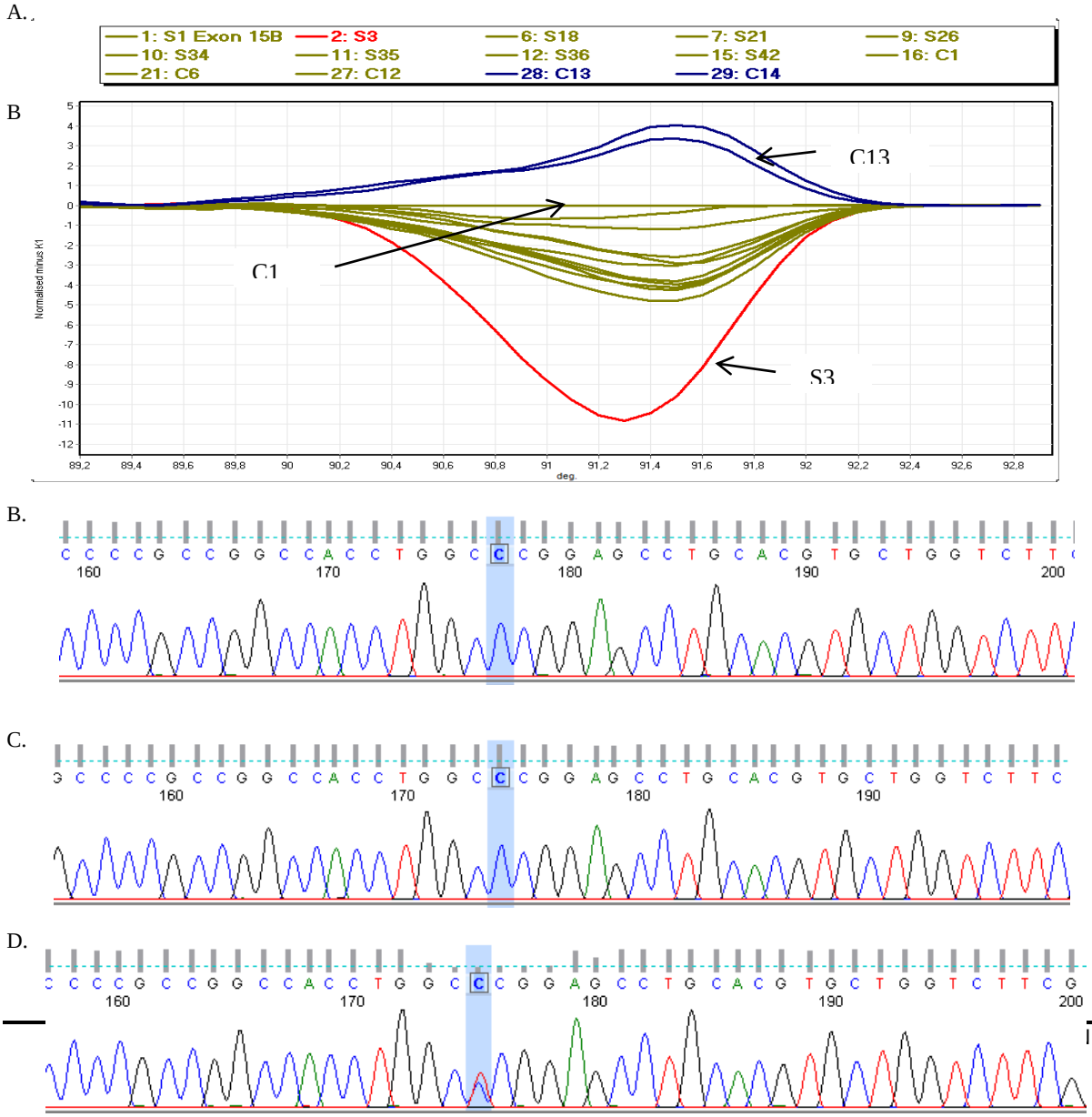
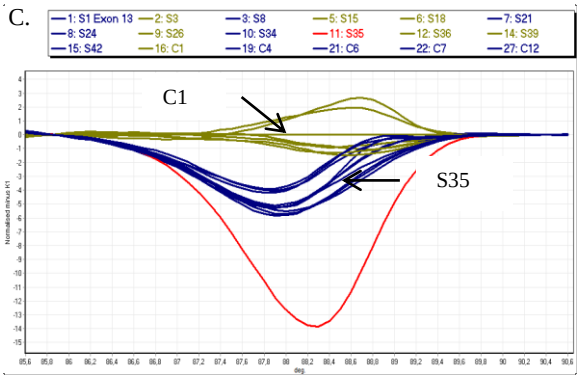
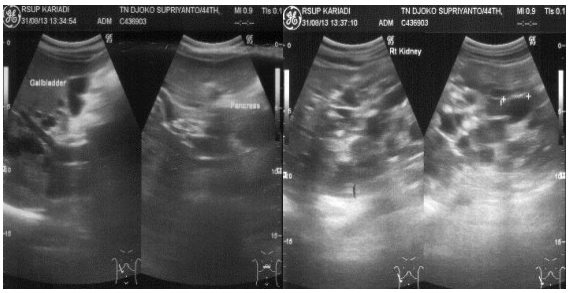


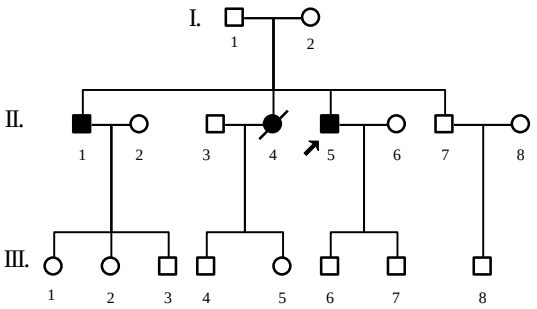
Fig. 2: Nonsense mutation c.3148G>GT (p.Glu1050*)
A. Analysis LR PCR – Nested PCR HRM showed S35 (sample) has aberrant graph.
B. Pedigree analysis for S35 (II.5). DNA samples were collected from II.5 and II.1 (S43).
C. Ultrasound showed polycystic liver (left) and kidney (right) disease for S35.
D. Ultrasound showed polycystic liver (left) and kidney (right) disease for S43.
E. S35 Sequencing (II.5) showed c.3148 G>GT: E1. Forward and E2. Reverse.
F. S43 Sequencing (II.1) showed c.3148 G>GT: E1. Forward and E2. Reverse.
G. C1 Sequencing (healthy control): G.1 Forward and G.2 Reverse.

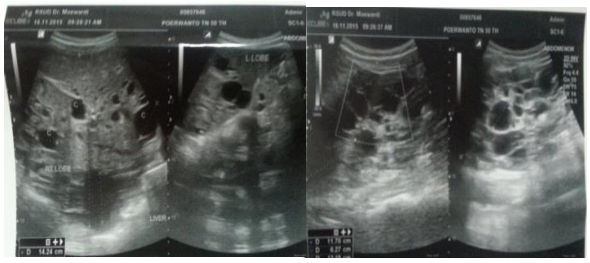
A. C.



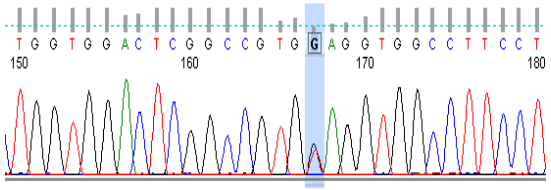


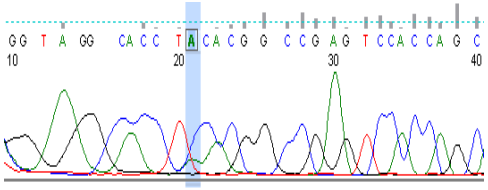
B. D.



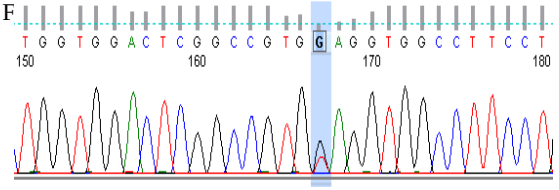


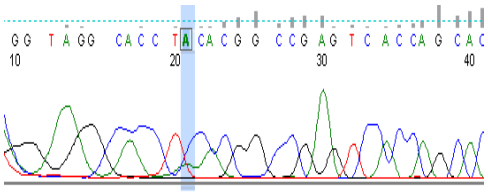
E. 1 E. 2



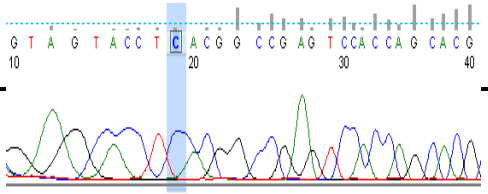


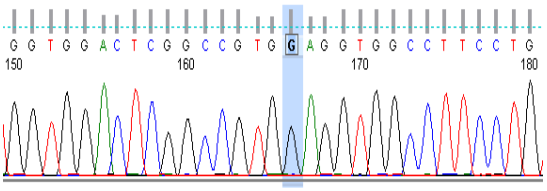
F. 1 F





G.1 G.2





DISCUSSION

Combination of LR PCR and Nested PCR HRM can be used to avoid amplification of pseudo genes. HRM sensitivity is influenced by sample purity, homogeneity of DNA samples concentration and GC-base content of the region of interest[20]. We performed LR PCR product purification to gain good sample purity. Amplification of *PKD1* is challenging because the sequence is GC rich[21]. Prediction for aberrant graph using HRM is also influenced by product size of the region of interest. Accuracy of HRM prediction is high when the product size is< 300 bp. Sensitivity and specificity will decrease when the product size is 400-1000 bp[22]. HRM products from the Bataille et al primer set (2011) which is used in this study have limitations because the product size is more than 300bp[19]. The HRM software analysis may also have a contribution to the sensitivity and specificity prediction. ABI HRM software v.2.0.1, for example, has limitations in detection of true-variation from pseudo-variation, which is the result of melting artefacts[23].

The family history sometimes does not need to be positive because some alleles are incompletely penetrant and approximately 2-5% of all mutations in ADPKD are thought to arise *de novo*[21]. Nonsense mutation c.3148G>GT leads to p.Glu1050*. The codon changes from CAG(Glu) to TAG(stopcodon(*), which may lead to truncation of PC1 protein, but more likely will result in degradation of the mRNA, by nonsense mediated decay. ADPKD is transmitted through autosomal dominant inheritance, so heterozygote mutations will cause the phenotype[15].

The characteristics of the patient with the c.3148G>GT mutation (S35) was male, 44 years and has 3 siblings, of whom two are affected (Fig. 2). He has experienced hypertension and uses oral antihypertensive (blood pressure 180/120 mmHg), history of haematuria, polycystic kidney disease, bilateral renal enlargement, liver cyst, liver enlargement and routine haemodialysis. His affected brother (S43) also has the c.3148G>GT mutation. The characteristics of S43 are: male, 50 years and has hypertension with antihypertensive drugs (blood pressure 130/90 mmHg), history of haematuria, polycystic kidney disease, bilateral renal enlargement, liver cyst, liver enlargement and he passed away after 2 years routine on haemodialysis. Levels of urea and creatinine for S35 and S43 pre haemodialysis were 167.1–14.1mg/dl and 178 – 10.4 mg/dl, respectively. Both of them have very similar characteristics (medical record). The affected sister (II-4) died of stroke and high blood pressure at age 43 and could not be studied.

This study also found 3 synonymous mutations; one of them is novel (c.3852C>CT) and is predicted to have a chance of exon skipping, due to loss of exonic splice enhancer sites (ESE). This opposes the general assumption that a synonymous mutation does not affect mRNA or protein level which will causes disease[23]. The other 2 synonymous mutations have been reported in NCBI dbSNP and the gnomAD database: c.4674G>GA: rs79884128 (highest frequency is 17.95%, found in the East-Asian population) and c.5847C>CT: rs80111665 (highest frequency is 5.41%, found in the East Asian population). The minor allele frequencies of both variants are high in at least one population, so these variants must be classified as benign. It is not relevant that in this case the highest frequencies are found in East Asian samples. The contribution of synonymous mutations to disease can be analysed on the mRNA level to determine the impact on mRNA splicing. Further studies for *PKD1* gene panel for whole exome sequencing (WES) using NGS are needed in more advanced laboratory.

CONCLUSIONS

The pathogenic mutation c.3148G>T with ADPKD phenotype was found in one family and in another patient the synonymous c.3852C>T mutation with possible effect on splicing which should be analysed on the mRNA level. Sanger sequencing should be carried out for all aberrant graph for future study, to elucidate certain mutation. The LR-PCR-HRM test needs to be extended for the entire *PKD1* gene using more advanced molecular testing such as NGS. Establishing molecular diagnosis is needed for genetic counselling in families with polycystic kidney disease and for early detection in relatives for early start of treatment. To the best of our knowledge this study is the first molecular investigation of ADPKD cases in Indonesia.

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