Molecular Defect of *PKD1* Gene Resulting in Abnormal RNA Processing in a Thai Family

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Abstract

Autosomal dominant polycystic kidney disease (ADPKD) is a common human autosomal disorder caused mainly by mutations of the *PKD1* gene. In analysis of *PKD1* transcripts by long RT-PCR and nested PCR procedures, we observed *PKD1*-cDNA fragments from three ADPKD siblings from the same family with a size approximately 250 base pairs (bp) shorter than normal. Further investigations showed that the *PKD1* transcripts from these patients had been abnormally processed, the nucleotide sequence of exon 43 containing 291 nt was missing from the transcripts, which would result in an abnormal polycystin-1 with an in-frame deletion of 97 amino acids. This splicing defect did not result from a mutation that disrupted the splice donor or acceptor sites adjacent to exon 43 or the branch sites in flanking introns but was most likely due to 20-bp deletion observed in intron 43. The intronic deletion was present in 8 affected members but absent in 11 unaffected members, corresponding with the results of genetic linkage analysis using 5 polymorphic markers in the *PKD1* region. Molecular diagnosis of *PKD1* in this family could, therefore, be carried out by genomic DNA amplification to directly detect the *PKD1* intronic deletion.

Key word : Polycystic Kidney Disease 1 (*PKD1*), *PKD1* Mutation, Intronic Deletion, Exon Skipping, RNA Processing Defect, Abnormal Splicing

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Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common human autosomal disorders, affecting approximately 1 per 1000 individuals. It is characterized by formation of multiple abnormal fluid-filled cysts in both kidneys, partly leading to end stage renal failure[1]. ADPKD is genetically heterogeneous with at least three different genes (*PKD1*, *PKD2*, and *PKD3*) responsible for similar phenotypes[2-4]. Abnormality of *PKD1*, which is located on chromosome 16p13.3, is the most common cause of ADPKD, accounting for approximately 85-90 per cent of cases[5], and appears to have a more severe effect with an early age of onset and end stage renal failure[6]. Recent studies have shown that *PKD1* probably occurs from two mutational events, germline and somatic mutations[7,8].

PKD1 has been isolated and characterized [9-12]. Its size is 54 kb consisting of 46 exons and its mRNA transcript is composed of 14,148 nucleotides (nt). The predicted protein product, polycystin-1, contains 4,302 amino acids and is proposed to play a role in cell-cell or cell-matrix interaction[11]. Approximately three-fourths of the sequence in the 5' region of *PKD1* is reiterated with about 95 per cent similarity to three highly homologous genes mapped on 16p13.1, whereas, about one-fourth of the sequence in the 3' region is unique[9]. This has complicated the characterization of *PKD1* mutations. Although it is believed that most of the mutations occur in the reiterated region, the majority of mutations identified to date are located within the 3' unique region[9,13-15] and some 82 mutations have been characterized[16]. The rate of mutation characterization has been slow and the number of mutation is too few for analysis of the correlation between type of mutation and variability of the *PKD1* phenotype.

Recently, a long reverse transcription-polymerase chain reaction (RT-PCR) method has been developed for amplification and isolation of the entire *PKD1* coding sequence from peripheral blood lymphocytes[17]. This method is useful not only for the characterization of mutations occurring especially in the reiterated region of *PKD1* but also for the analysis of *PKD1* RNA processing and transcripts, without acquisition of kidney tissue. In this report, we describe a deletion mutation in intron 43 of *PKD1* which resulted in abnormal RNA processing involving exon 43 skipping in the *PKD1* transcripts and also reported the development of a method for direct detection of this mutation in affected family members.

MATERIAL AND METHOD

ADPKD Family and Linkage Analysis

The family described in this study (PK009) was one of approximately 50 Thai ADPKD families that have been identified and followed-up in the Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University. ADPKD was diagnosed according to established clinical and genetic criteria, based on the finding by ultrasonic scanning of polycystic kidneys with at least one cyst in one kidney and two or more in the other[18], and the expression of the disease in members in consecutive generations of the family.

Blood samples (10-15 ml) were taken from ADPKD patients and family members with informed consent. Genomic DNAs were isolated from blood samples by standard method[19] for linkage analysis and PCR amplification. Linkage analysis and haplotype characterization were performed by examinations of five polymorphic markers linked to *PKD1* on chromosome 16p, namely, D16S85 (3' HVR), KG8, SM6, 16AC2.5, and SM7[2,20-23].

RNA Isolation, cDNA Synthesis, and PCR

The methods for RNA isolation, full-length cDNA synthesis, long PCR, and nested PCR have previously been described[17]. Briefly, RNA was isolated from the lymphocytes by TRIzol® reagent (Life Technologies), and used for full-length cDNA synthesis with oligo $(dT)_{12-18}$ primer and RNase H-free reverse transcriptase. *PKD1*-cDNA was amplified by long PCR using a pair of primers (TH1F/TH1B; Table 1) and ELONGASE™ Enzyme Mix (Life Technologies) containing thermostable *Taq* and *Pfu* polymerases. The length of PCR product was 13,634 bp (Fig. 1A).

Nested PCRs were performed by using 9 pairs of nested primers to generate 9 overlapping fragments. Sequences of nested PCR primers (SI9F/SI9B and WT5F/WT5B) used in this study are shown in Table 1. The others are available on request. The nested PCR products were analyzed by electrophoresis on 2 per cent LE agarose gel (FMC Corporation) in Tris-borate-EDTA (TBE) buffer. DNA fragments were stained with ethidium bromide, visualized on UV transilluminator, and photographed.

Table 1. Sequences of PCR primers for amplifications of *PKD1*.

Primer	Primer sequence (5'->3')	Nucleotide position	Location	PCR product size (bp)
Primers for long RT-PCR				
TH1F	CTGGGGACGGCGGGGCCATGCG	175-196[a]	5' UTR	13,634
TH1B	GGCCTGGGGCAAGGGAGGATGACAA	13808-13784[a]	3' UTR	
Primers for nested PCR				
SI9F	CTTCAGCACCAGCGATTACGACGTT	11533-11557[a]	Exon 40	1,650
SI9B	AGAAAGTAATACTGAGCGGTGTCCACTC	13182-13155[a]	Exon 46	
WT5F	TTGGCTGGGAGAGTCCTCACAATG	11556-11579[a]	Exon 40	817
WT5B	AGGGAGTCCACACAGGAAGACACG	12372-12349[a]	Exon 45	
Primers for genomic DNA amplification				
SI9.2F	CGGGCCTCTCGCTGCCTCTGCTCACCTCG	50143-50171[b]	Exon 42	563-758[c]
SI9.2B	ACGGACCACTGGCGCACGAAGCGTAGCTG	50822-50794[b]	Exon 44	
SI9.3F	CGGCCTCGCTGCTCTTCCTGCTTTTGGTC	50678-50706[b]	Exon 43	145
SI9.2B	ACGGACCACTGGCGCACGAAGCGTAGCTG	50822-50794[b]	Exon 44	

[a]The nucleotide positions are according to HUM*PKD1*A, GenBank Accession No. L33243(11).
[b]The nucleotide positions are according to HUM*PKD1*GEN, GenBank Accession No. L39891(10).
[c]Size of PCR product is variable due to numbers of variable repeated sequences in intron 42(14).

The nested PCR product, amplified with SI9F/SI9B, was digested with either *Taq* I plus *Bgl* I or *Pvu* II (New England Biolabs) for detailed analysis of cDNA deletion observed.

Amplification of Genomic DNA

Genomic DNA samples were amplified in a total volume of 25 µl containing 200 ng of genomic DNA, 400 nM of each primer (Table 1), 200 µM dNTP mixture, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.25 unit AmpliTaqGold™ (PE Applied Biosystems), 5 per cent DMSO, and 1.0 mM $MgCl_2$. The PCR was initiated at 95°C for 10 min, then conducted for 2 cycles at 94°C for 30 s, 62°C for 30 s, and 72°C for 30-60 s (depended on size of PCR product). It was then continued for 31 cycles with 2°C reduction of annealing temperature every 2 cycles until reaching the final annealing temperature at 48°C, followed by a terminal extension at 72°C for 10 min.

DNA Sequencing

The PCR product, separated on agarose-gel electrophoresis, was purified using QIAquick™ Gel Extraction Kit (QIAGEN). The purified DNA fragment was sequenced using ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 310 Automated DNA Sequencer (PE Applied Biosystems).

RESULTS

Linkage Analysis

From analyses of five polymorphic DNA markers in the *PKD1* region on chromosome 16p, 14 haplotypes (with 5 additional variants) were found to segregate in PK009 family and the abnormal *PKD1* allele was linked to haplotype A, which consisted of the following alleles: 2.1 kb for D16S85, 123 bp for KG8, 110 bp for SM6, 169 bp for 16AC2.5, and 88 bp for SM7 (Fig. 3).

Long RT-PCR and Nested PCR

Long RT-PCR and nested PCRs were applied to RNA samples prepared from three affected members (III-1, III-4, and IV-6) of PK009 family, the results of which were mostly normal. However, with nested PCR using SI19F/SI19B primer pair, while a single PCR product (1,650 bp) was observed in samples from normal individuals, products with two different sizes, normal (1,650 bp) and

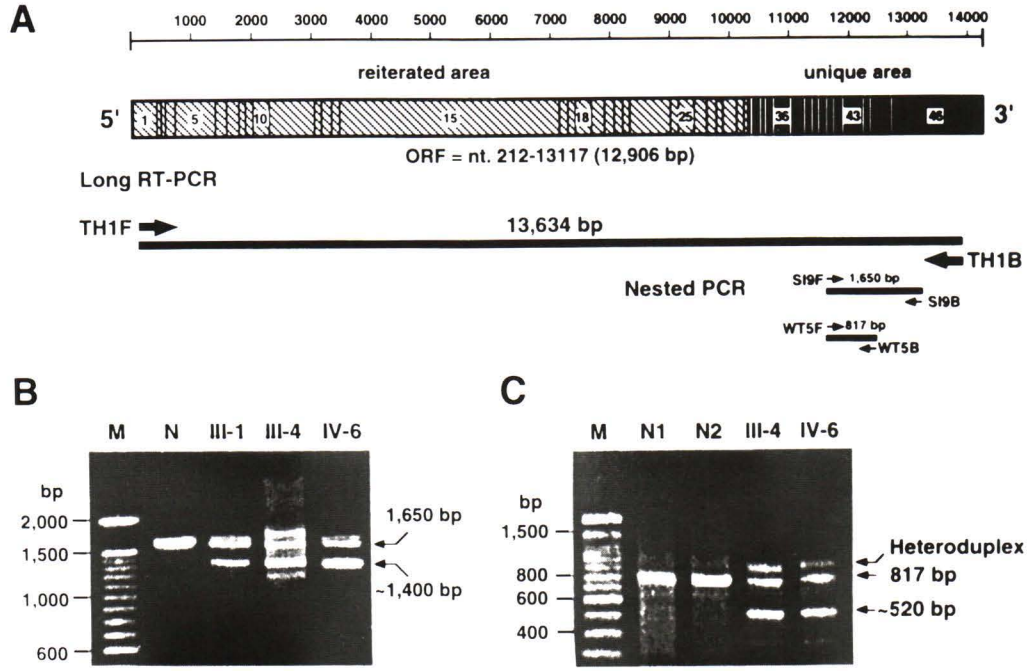

Fig. 1. **(A) Diagrammatic representation of the full-length *PKD1* mRNA (upper), long PCR product (middle), and nested PCR products (lower). The reiterated region in the *PKD1* mRNA (exons 1-32) is represented by the hatched area and the unique region (exons 33-46) by the blackened area. Sizes (in bp) and primer pairs (arrows) are shown above and below the long RT-PCR products (long solid horizontal line) and nested PCR products (short solid horizontal lines), respectively. (B) Nested PCR products amplified from long PCR product of *PKD1*-cDNA with the SI9F/SI9B primers from a normal individual (N) and three *PKD1* patients (III-1, III-4, and IV-6) of PK009 family. A single normal PCR product (1,650 bp) was observed in the normal sample while a normal and a shorter (~1,400 bp) products were found in three patients' samples. The uppermost band in the lanes of patients' samples is probably a heteroduplex of the normal and shorter products. Lane M is 100-bp DNA ladder. (C) Nested PCR products amplified from the long *PKD1*-cDNA samples by WT5F/WT5B primers of two normal individuals (N1 and N2) and the two patients (III-4 and IV-6) of PK009 family. A normal PCR product with the size of 817 bp was observed in the two normal samples but a normal (817 bp) and a shorter (~520 bp) fragments were detected in both patients' samples; the top band in both patients' samples was probably a heteroduplex of the normal and deleted DNA strands. Lane M is 100-bp DNA ladder.**

shorter (~1,400 bp), were detected in samples from the three affected members (Fig. 1B). In addition, an upper and fainter band of PCR product was also noticed. The presence of a shorter product may have resulted from a partial deletion of *PKD1* cDNA prepared from its mRNA transcript from one allele of *PKD1* in these patients, and upper band might be a heteroduplex of the normal and deleted DNA strands.

To locate the region with possible deletion, nested PCR products amplified by SI9F/SI9B primer pair from a normal individual and a patient (III-4) were digested with either *Taq* I plus *Bgl* I or *Pvu* II. It was found that the deleted region was of 300 bp, located between the first *Taq* I and *Bgl* I sites (or the fourth site of *Pvu* II) (data not shown).

A new pair of primers (WT5F/WT5B ; Table 1 and Fig. 1A) were designed to amplify DNA covering the deleted region. The result of the amplifications showed that two normal samples (N1 and N2) generated a single amplified product with the size of 817 bp, whereas, two patients' sam-

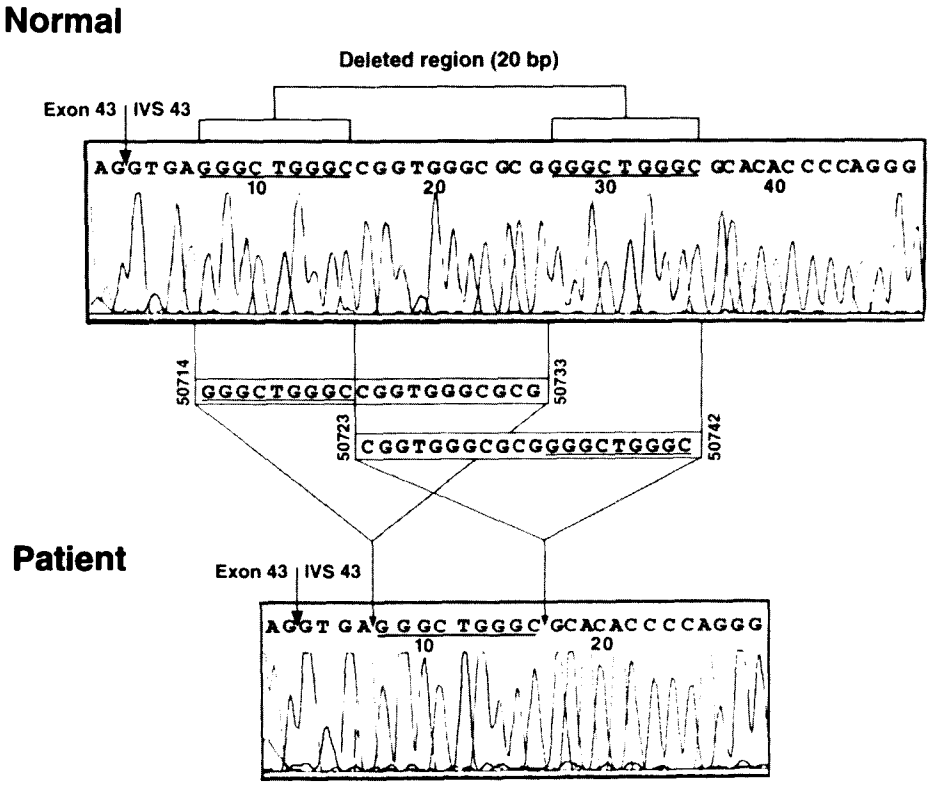

Fig. 2. Results of sequencing analyses of nested PCR products of intron 43 region amplified from genomic DNA samples of a normal control and patient III-4 of PK009 family by SI9.2F/SI9.2B primers. Comparison of the two nucleotide sequences shows a deletion of 20 bp in intron 43. The presence of two direct 9-nt repeat (GGGCTGGGC) (underlined) which are separated by 11 bp in the normal sequence of this intron makes many possibilities of the deletion breakpoint between the nucleotide positions 50714 and 50742 of *PKD1* (GenBank Accession No. L39891). Two examples of possibilities of the 20-bp deletion at the most 5' and 3' ends are shown.

ples (III-4 and IV-6) produced the normal amplified product (817 bp) and a shorter fragment (~520 bp) together with the upper heteroduplex band (Fig. 1C).

Sequencing Analysis of the Shorter Nested-PCR Product

The 520 bp fragment was purified from agarose-gel electrophoresis and subjected to direct DNA sequencing. There was a deletion of 291 bp which corresponded to the total exon 43 sequence of *PKD1* while complete sequences of exons 42 and 44 were still present (data not shown). This finding indicated that the deletion was most likely due to the skipping of exon 43 from the *PKD1*-mRNA transcript.

Analysis of the *PKD1* Gene

The skipping of exon 43 from *PKD1*-mRNA transcript might have occurred from a number of defects in the *PKD1* gene, viz. complete absence of the exon, mutation at the exon-intron junctions, or mutation at the A branch site in the flanking introns 42 and 43. To identify the precise mutation, genomic DNA in this region of *PKD1* was sequenced. Since the genomic region surrounding exon 43 was difficult to amplify by PCR due to the presence of 34-nt repeat polymorphism in intron 42[14], the primary genomic DNA amplification was performed by using SI9F/SI9B primer pair which had been used for nested PCR of *PKD1* cDNA (Table 1). A PCR product with the length of 2,351-2,546 bp was produced and used in nested PCR with SI9.2F/SI9.2B

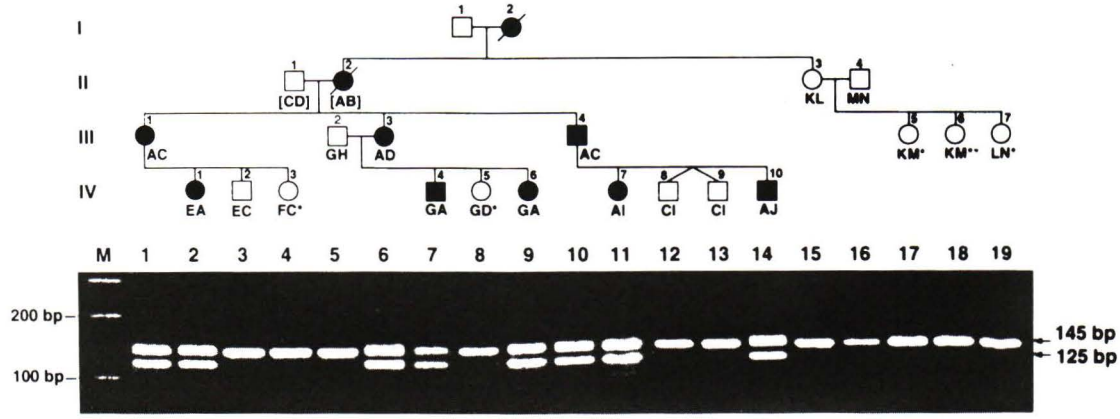

Fig. 3. Detection of the 20-bp deletion in intron 43 of *PKD1* by amplification of genomic DNA samples from members of PK009 family with SI9.3F/SI9.2B primers. Pedigree of the family is shown and haplotyes in the region of *PKD1* on chromosome 16p13.3, determined by using 5 polymorphic DNA markers, are indicated under the symbols. Genomic DNA samples from 19 family members, 8 from affected (filled symbols) and 11 from unaffected (blank symbols) members, were analyzed. DNA samples of the unaffected members produced only the product of the wild-type allele (145 bp) but those of affected members generated the products of both wild-type and mutant (125 bp) alleles. The mutant allele segregated with the haplotype A in the family. The haplotypes with one or two asterisks are variants of the ones without. Lane M is 100-bp DNA ladder.

primer pair specific to sequences in exons 42 and 44 (Table 1). A PCR product with the size of 563-758 bp was generated and sequenced. There was a deletion of 20 bp in intron 43 (Fig. 2), while sequences in other regions were normal. Since there are two 9-bp repeats (GGGCTGGGC) situated 11 bp apart in intron 43 of *PKD1*, it was not possible to locate precisely the deletion breakpoints in this abnormal gene.

Direct Detection of the 20-bp Deletion in Intron 43 in Members of PK009 Family

To perform direct detection of the 20-bp deletion in intron 43 in members of PK009 family, a primer specific to the sequence of exon 43 (SI9.3F) was used together with a primer specific to the sequence of exon 44 (SI9.2B) (Table 1). With this primer pair, genomic DNA sample with normal intron 43 would generate PCR product of 145 bp while that with deleted intron 43 of 125 bp. When genomic DNA samples from all members of PK009 family were amplified, DNA samples of 11 normal members showed only the normal size product (145 bp), whereas, those of 8 affected members displayed 145 bp and 125 bp products (Fig. 3). Heteroduplex band was sometimes observed. The presence of deleted intron 43 in affected members and its absence in unaffected members of PK009 family corresponded with the presence and absence of haplotype A which linked to the abnormal *PKD1* allele (Fig. 3).

DISCUSSION

An analysis of *PKD1*-mRNA transcripts from three patients in PK009 family showed that the truncated *PKD1* cDNA observed in the initial nested PCR screening (Fig. 1B and C) was due to skipping of the entire 291 nt sequence of exon 43, while the sequences of exon 42 and 44 were still intact in the abnormal transcript. Further analysis of *PKD1*-genomic DNA by direct sequencing revealed a 20-bp deletion in intron 43 of the patient's *PKD1* gene (Fig. 2) while the sequences of other critical regions, such as splice donor and acceptor sites close to exon 43 and branch sites in the flanking introns, were not changed. Thus, the observed 20-bp deletion in intron 43 was most likely to be the cause of exon 43 skipping in the patient's mRNA transcript. A PCR method for amplification of DNA region covering

intron 43 was used to examine the deletion in DNA samples from 19 members of PK009 family. The expected 20-bp deletion in intron 43 was found in all samples from 8 affected members but was not detected in the 11 unaffected members (Fig. 3). In addition, the deletion segregated in complete linkage to the haplotype A as characterized by using 5 polymorphic DNA markers in the *PKD1* region (Fig. 3). All the evidence clearly supported the 20-bp deletion in intron 43 of *PKD1* as being the disease mutation in this family.

Two 9 bp direct-repeat sequences (GGGC TGGGC) separated by 11 nucleotides were present in *PKD1* intron 43 (Fig. 2). Thus, the deletion might have occurred from a misalignment between the two direct repeat sequences in this region of two *PKD1* alleles and interchromosomal recombination during meiosis. Since the length of deleted nucleotides (20 bp) was equal to the sum of one repeat (9 bp) and the joining part (11 bp), and one repeat sequence still remained in the deleted allele, the exact position of the recombination or deletion breakpoint could not be determined. As the deletion did not involve the nearby exon, it would not be detected by exon analysis of genomic DNA.

Mutations causing exon skipping usually involve either splice donor or splice acceptor site at 3' and 5' consensus sequences of the intron. Two reported mutations of *PKD1* resulting in exon 39 and exon 44 skipping are IVS39+1G>C and IVS44+1G>C substitutions, respectively[9,24]. However, the intron 43 deletion leading to exon skipping reported here did not affect those functionally important sites. One explanation for the 20 bp-deletion in intron 43 causing exon 43 skipping may be that the truncated intron is too short for proper spliceosome formation and correct RNA splicing process. The minimal length of intron in human genes for correct *in vivo* splicing is not known. In an *in vitro* study to determine a minimal intron length using rabbit beta-globin gene constructs in HeLa cells, it was found that the correct splicing required six 5' and twelve or more 3' intron nucleotides with at least 80 inner nucleotides[25]. The shortest *PKD1* intron is intron 19 with a length of 66 bp[11]. The length of the mutated intron 43 is 55 bp.

Deletions of 18 and 20 bp in intron 43 of *PKD1* have been reported[26]. In each case, two different transcripts, either with deleted-intron sequence retained or with a 66-nt deletion due to activation of a cryptic 5' splice site, as well as the transcripts with exon 43 skipping, were produced. These findings are different from this study where the major defective transcript contained exon 43 skipping. It should be noted that in this study fresh peripheral blood lymphocytes were used for RNA isolation while lymphoblastoid cell lines were used in the previous work.

The deletion of 291 nucleotides of exon 43 in *PKD1*-mRNA transcript will lead to an in-frame deletion of 97 amino acids at positions 3904-4001 in polycystin-1. This deleted peptide region is located between the second half of the 7th transmembrane (TM) domain and the first one-third of the 9th TM domain of the proposed model of polycystin-1[27]. Since the deletion of this region has resulted in the disease phenotype, it is of importance to the function of polycystin-1.

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ความผิดปกติของยีน *PKD1* ที่ทำให้เกิดความผิดปกติในกระบวนการตัดต่ออาร์เอ็นเอในครอบครัวผู้ป่วยไทย

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โรคไตเป็นถุงน้ำชนิด autosomal dominant มีสาเหตุใหญ่จากมิวเตชั่นของยีน *PKD1* จากการศึกษา mRNA ของยีน *PKD1* ด้วยวิธี long reverse transcription–polymerase chain reaction (RT–PCR) และ nested PCR คณะผู้วิจัยพบว่า *PKD1*–cDNA จากพี่น้อง 3 คน ที่เป็นโรคนี้จากครอบครัวเดียวกัน มีขนาดสั้นลงกว่าปกติประมาณ 250 คู่เบส การศึกษาในรายละเอียดพบว่า *PKD1*–mRNA ของผู้ป่วยมีความผิดปกติในกระบวนการตัดต่ออาร์เอ็นเอ ทำให้ลำดับนิวคลีโอไทด์ของ exon 43 ซึ่งมีขนาด 291 นิวคลีโอไทด์ขาดหายไป คาดว่าทำให้โปรตีน polycystin–1 ผิดปกติมีกรดอะมิโนหายไปจำนวน 97 ตัว ความผิดปกติในกระบวนการตัดต่ออาร์เอ็นเอในผู้ป่วยนี้ไม่ได้เกิดจากมิวเตชั่นที่ทำลาย splice donor หรือ acceptor site ที่อยู่ติดกับ exon 43 หรือ branch sites ใน intron ที่ขนาบข้าง แต่น่าจะเป็นผลจากการขาดหายของลำดับนิวคลีโอไทด์จำนวน 20 คู่เบสใน intron 43 การขาดหายของดีเอ็นเอใน intron นี้ ตรวจพบในสมาชิกครอบครัวที่เป็นโรคจำนวน 8 คน แต่ไม่พบในผู้ที่ไม่เป็นโรค 11 คน ตรงกับผลการตรวจด้วยวิธี linkage analysis โดยใช้ polymorphic markers ในบริเวณใกล้ยีน *PKD1* จำนวน 5 markers การวินิจฉัยโรคในครอบครัวนี้สามารถทำได้โดยตรงด้วยวิธี genomic DNA amplification เพื่อตรวจการขาดหายของดีเอ็นเอใน intron ของยีน *PKD1*

คำสำคัญ : ยีน Polycystic kidney disease 1 (*PKD1*), มิวเตชั่นของยีน *PKD1*, การขาดหายของอินทรอน, การข้ามเว้นของเอ็กซอน, ความผิดปกติในกระบวนการตัดต่ออาร์เอ็นเอ, การเชื่อมต่ออาร์เอ็นเอที่ผิดปกติ

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