Gene 550 (2014) 131–135

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Methods Paper

Development and validation of a whole genome amplification long-range PCR sequencing method for ADPKD genotyping of low-level DNA samples

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ARTICLE INFO

Article history:
Received 22 April 2014
Received in revised form 17 June 2014
Accepted 7 July 2014
Available online 8 July 2014

Keywords:
ADPKD
PKD genes
Whole genome amplification
Gene variations
Long-range PCR
Sequencing

ABSTRACT

Autosomal dominant polycystic kidney disease (ADPKD) is caused by mutations in two large genes, *PKD1* and *PKD2*, but genetic testing is complicated by the large transcript sizes and the duplication of *PKD1* exons 1–33 as six pseudogenes on chromosome 16. Long-range PCR (LR-PCR) represents the gold standard approach for *PKD1* genetic analysis. However, a major issue with this approach is that it requires large quantities of genomic DNA (gDNA) material limiting its application primarily to DNA extracted from blood. In this study, we have developed a whole genome amplification (WGA)-based genotyping assay for *PKD1* and *PKD2*, and examined whether this approach can be applied to biosamples with low DNA yield, including blood, buccal cells and urine. DNA samples were amplified by multiple displacement amplification (MDA) and a high-fidelity DNA polymerase followed by LR-PCR and exon-specific amplifications of *PKD1* and *PKD2* respectively, and Sanger sequencing. This method has generated large amounts of DNA with high average product length (>10 kb), which were uniformly amplified across all sequences assessed. When compared to the gDNA direct sequencing method for six ADPKD samples, a total of 89 variants were detected including all 86 variations previously reported, in addition to three new variations, including one pathogenic mutation not previously detected by the standard gDNA-based analysis. We have further applied WGA to ADPKD mutation analysis of low DNA-yield specimens, successfully detecting all 63 gene variations. Compared to the gDNA method the WGA-based assay had a sensitivity and specificity of 100%. In conclusion, WGA-based LR-PCR represents a major technical improvement for PKD genotyping from trace amounts of DNA.

1. Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is the most common inherited kidney disease, affecting approximately 1 in 1000 individuals in the United States and 12.5 million patients worldwide. It is characterized by bilateral kidney cyst development and progressive chronic kidney disease, leading to end-stage renal disease. ADPKD is caused by mutations in two genes, with *PKD1* accounting for 75%–85% of the cases, and *PKD2* that is responsible for the remainder of cases. Genetic testing plays an increasingly important role in the diagnosis of patients with an uncertain renal phenotype, particularly in the absence of a known family history, and in the evaluation of family members who are considering kidney donation to affected individuals (Harris and Rossetti, 2010).

ADPKD genetic testing is complicated by the large transcript sizes, genetic heterogeneity and the duplication of *PKD1* exons 1–33 as six human homologues (HGs) on chromosome 16. Long-range PCR (LR-PCR) represents the gold standard approach for *PKD1* genetic analysis. ADPKD molecular genetic assays typically requiring large amounts (~1.5 μg) of genomic DNA (gDNA) template. Therefore, for many genetic studies, the amount of gDNA starting material is limited especially in applications utilizing valuable clinical samples, such as, fetal cells or renal epithelial cells. Moreover, even highly sensitive analytical methods such as PCR are constrained by the limited amount of DNA template because LR-PCR is required for the amplification of *PKD1* distinctive sequences (Rossetti et al., 2012; Tan et al., 2012). Moreover, the variety of clinical samples used in ADPKD mutation investigation (e.g.,

Abbreviations: ADPKD, autosomal dominant polycystic kidney disease; PKD1, polycystic kidney disease 1; PKD2, polycystic kidney disease 2; HGs, human homologues; WGA, whole genome amplification; MDA, multiple displacement amplification; LR-PCR, long-range polymerase chain reaction; gDNA, genomic DNA.

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http://dx.doi.org/10.1016/j.gene.2014.07.008
0378-1119/

preimplantation genetic diagnosis, stereotactic needle aspirate biopsy, urinary sediment) has grown rapidly and requires improved amplification procedures.

The concept of whole genome amplification (WGA) has arisen recently as the method for procuring abundant DNA from valuable and often limited clinical specimens (Lasken, 2009; Luthra and Medeiros, 2004). Successful genetic analysis using WGA DNA as a template has been reported for several applications, including prenatal diagnosis, forensic science and other research fields (Hawkins et al., 2002; Paunio et al., 1996; Tate et al., 2011). However, the feasibility of this method for ADPKD mutation analysis has not been evaluated.

In this study, we have developed a whole genome amplification LR-PCR-based assay for the sequencing of *PKD1* and *PKD2*, using a commercially available multiple displacement amplification (MDA) protocol, and examined whether this assay can be applied to biosamples with low DNA yield, including buccal cells and urine.

2. Materials and methods

2.1. Study subjects

Study subjects were participants in The Rogosin Institute ADPKD Data Repository (http://clinicaltrials.gov identifier: NCT00792155, December 23, 2013). This is a single-center longitudinal study of genotype and phenotype characteristics of individuals with ADPKD. Six subjects were randomly selected from the repository for this study in addition to two other patients for whom buccal cells and urine samples were available. All subjects have undergone PKD genotyping by the Molecular Pathology Research Laboratory of Weill Cornell Medical College (WCMC) (Tan et al., 2012). The study was approved by the Institutional Review Board Committees at WCMC, and all subjects provided written informed consent.

2.2. Whole genome amplification

Genomic DNA (gDNA) was extracted from peripheral blood lymphocytes (PBLs), buccal cells and urine sediments using standard procedures (Koptides et al., 1998; Tan et al., 2012; van Noord, 2003; van Wieren-de Wijer et al., 2009), and treated with 400 mM KOH/10 mM EDTA (denaturation buffer) and 400 mM HCl/600 mM Tris–HCl (neutralization buffer), respectively, to reduce possible DNA contamination. MDA was then performed using the REPLI-g kit (Qiagen Inc., Germantown, MA). Briefly, 10 ng of DNA from each specimen was first denatured at RT for 3 min and neutralized with modified buffer. WGA was then performed in a total volume of 50 μL for 16 h, using random priming and strand displacement synthesis at 30 °C in the presence of ø29 DNA polymerase according to the kit's instructions (Qiagen). The reaction was terminated by heating to 60 °C for 10 min. The WGA-generated DNA (WGA-DNA) was then analyzed by 0.5% agarose gel with ethidium-bromide staining, followed by purification with Agencourt AMPURE XP beads (Beckman Coulter, Beverly, MA) and quantification with Quant-iT™ PicoGreen® dsDNA Reagent (Quant-It, Invitrogen Corporation, Carlsbad, CA). The purified WGA-DNA samples were stored at −20 °C for later use.

2.3. PCR amplification and sequencing

Genomic and WGA-DNA *PKD1* and *PKD2* sequences were amplified by LR-PCR and exon-based PCR, respectively, according to Tan et al. (2012), with some primer modifications (exons 35–45). For *PKD1*, the entire coding region, 5′ and 3′ untranslated regions, and the exon–intron boundaries were amplified in nine distinct LR-PCRs (Gene 1, Gene 2–7, Gene 8–12, Gene 13–15, Gene 15–21, Gene 22–26, Gene 27–34, Gene 35–41, and Gene 40-3′UTR), using PCR primers (Sigma-Genosys Ltd, St. Louis, MO) anchored in either the rare mismatched region with the HGs or the single-copy region of *PKD1*. The LR-PCR primer sequences for *PKD1* are shown in Table 1. LR-PCR was performed using the GeneAmp® High Fidelity PCR System (Applied Biosystems, Foster City, CA), as previously described (Tan et al., 2012). Briefly, 60 ng of genomic DNA was amplified in a final volume of 25 μL, containing 200 μmol/L deoxyribonucleotide triphosphate, 0.2 μmol/L of each primer, 0.5 mol/L betaine, 5% dimethyl sulfoxide (except for exon 1 with an extremely high content of GC, for which 10% dimethyl sulfoxide was used), manufacturer's supplied buffer, and 2 U of enzyme (Applied Biosystems). The LR-PCR products were then amplified separately using the Biometra T-3000 thermalcycler (Biometra GmbH, Goettingen, Germany), as published previously (Tan et al., 2012). *PKD2* sequences were amplified as previously described using modified primers (Table 2) (Lasken, 2009; Tan et al., 2009, 2012).

PCR products were then subjected to Sanger sequencing. The LR-PCR products of *PKD1* were purified with the Qiaquick PCR purification kit (Qiagen Inc., Germantown, MA), quantified, and sequenced with 45 pairs of walking primers located at least 50 bp away from intron–exon junctions, using Big Dye Terminator Chemistry with Ampli Taq-FS DNA Polymerase (Applied Biosystems) on an ABI 3100 Genetic Analyzer with sequence primers published before (Tan et al., 2009). Sequencing data (ABI file) were then analyzed by Mutation Surveyor software version 4.0 (Soft Genetics, State College, PA) for automatic variation calling first, followed by careful inspection of the electropherograms for quality assurance purposes.

Table 1
Long-range PCR primers for *PKD1*.

Exons	Primer	Sequence	Tm (°C)	Fragment size (bp)
1	Gene 1 F	CGCAGCCTTACCATCCACCT	64.6	2278
	Gene 1 R	TCATCGCCCCTTCCTAAGCA	65.2	
2–7	2-7 F	CCCCGAGTAGCTGGAACTACAGTTACACACT	68.5	4041
	2–7 R	CGTCCTGCTGTGCCAGAGGCG	68.1	
8–12	8–12 F	ACGTCTGCGAGCTGCAGCCC	70.7	3893
	8-12 R	CTGCAGGGACAGGCGTCAGTGA	70.4	
13–15[a]	13–15 F	TGGAGGGAGGGACGCCAATC	68.9	4391
	13–15 R	GTCAACGTGGGCCTCCAAGT	64.7	
15–21[a]	15-21 F	CTGTGGGCCAGCAGCAAGGT	68.2	4350
	15–21 R	ACACAGGACAGAACGGCTGAGGCTA	69.3	
22–26[a]	22–26 F	CCTGGGTCATGCAGAGCCACAG	69.6	3301
	22–26 R	GCTTAAAGGGGAATGGCTTAAACCCG	69.5	
27–34	27–34 F	CGGGTCACCGGTTGTGGCA	71	3916
	27–34 R	ATGAGGCTCTTTCCACAGACAACAGAGGTT	70.5	
35–41	35-41 F	CAAGAGGCTCAAGAAACTGCCCG	68.4	2632
	35-41 R	GGGCTGTGGAAGCCGCCTA	67.9	
40-3′UTR[a]	40-3'UTR F	GTGGCGCCGAACCAGAGC	67.7	2909
	40-3'UTR R	CTGAAGCCAGCAGCCTTAGCAG	65.8	

[a] Modified primer as compared to the previously published sequence (Tan et al., 2012).