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SYMPOSIUM PAPER

Modification of primers for GRHPR genotyping: avoiding allele dropout by single nucleotide polymorphisms and homology sequence

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Abstract Mutation of primer site for genotyping by polymerase chain reaction (PCR) may cause allele dropout and other genotyping failures. Primary hyperoxaluria type 2 (PH2) is a rare inherited disease caused by overproduction of endogenous oxalate due to mutations in the glyoxylate/hydroxypyruvate reductase (GRHPR) gene. Here, to avoid allele dropout and primer annealing to multiple sites, and given the discrepancy in intron length between GRHPR gene data, we updated the primers used in the sequence assay of the GRHPR gene. These redesigned primers show potential in reducing detection failure of GRHPR mutations. In addition, we performed a single nucleotide polymorphism (SNP) linkage analysis of the GRHPR gene using direct sequencing with PCR amplification of specific alleles (DS-PASA). Using this technique, we sequenced four common SNPs between intron E and exon 6, which show linkage disequilibrium (LD) consisting of three types of haplotypes, similar to data from the HapMap SNP database.

Keywords Single nucleotide polymorphism · Allele dropout · Glyoxylate/hydroxypyruvate reductase · Genotyping · Haplotype · Linkage disequilibrium

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Introduction

Allele dropout is the failure to detect one of the germ line alleles in a patient by genotyping. A cause for allele dropout using PCR-based methods is the presence of a polymorphism within a primer-binding site. In cases, in which two alleles differ in a region of interest, dropout of a single allele possibly leads to incorrect identification of wild type or mutant homozygotes. Previous studies have evaluated possible intronic SNP-primer mismatches in tests for Hepatocyte Nuclear factor-1 alpha [1], Wilson's disease [2], the Apolipoprotein AV gene [3], congenital long QT syndrome [4] and the cadherin 1 gene [5].

PCR amplification of specific alleles (PASA [6]), a technique also known as the amplification refractory mutation system (ARMS [7]), uses primers designed to match and selectively amplify different SNP alleles. The double-ARMS method was developed to identify haplotypes composed of two SNPs [8, 9]. Direct haplotype-specific DNA sequencing was used to determine haplotypes within the intron 2 sequence of the fructan-6-fructosyltransferase gene in *Lolium perenne* L. due to its sensitivity to 3′ end mismatches in the sequence primer [10].

Primary hyperoxaluria type 2 (PH2, OMIM 260000) is an inherited autosomal recessive disorder caused by overproduction of endogenous oxalate, resulting in urolithiasis, nephrocalcinosis, and renal failure. The disease originates from mutations in the GRHPR gene, which encodes for the glyoxylate/hydroxypyruvate reductase enzyme. The GRHPR gene is composed of nine exons and is located in the pericentromeric region of chromosome 9 [11]. A total of 14 mutations have been reported in this gene [11–15]. Further, a great number of GRHPR mutants have been reported as homozygotes. In addition, Bhat et al. [16] reported a discrepancy between leukocyte mRNA and GRHPR gene sequences.

Here, we report primers for GRHPR genotyping, which were redesigned based on the complete human genome sequence and SNP database. Following the identification of an SNP within the published exon 6 sense primer, we investigated the presence of an SNP linkage near exon 6 by direct sequencing with PASA (DS-PASA).

Materials and Methods

Identification of SNPs in GRHPR primers

The human GRHPR gene sequence was obtained from the Entrez database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene). Data on SNPs in the GRHPR gene were obtained from the SNP GeneView Report on GRHPR glyoxylate reductase/hydroxypyruvate reductase [Homo sapiens].

DNA extraction

Peripheral blood was collected in ethylenediaminetetraacetic acid (EDTA) from seven healthy volunteers at the Hamamatsu University school of Medicine (HUSM). Genomic DNA was extracted and purified using the QIAamp DNA Blood Kit (QIAGEN, Inc., CA). All protocols were approved by the Institutional Review Boards of HUSM. Informed consent was obtained from volunteers in accordance with the Institutional Review Boards of HUSM.

PCR and sequence

The primers, which are listed in Table 1, were synthesized by Hokkaido System Science Co., Ltd. (Japan). PCR reactions were carried out in a 20-μL volume containing 1 μL genome DNA template, 1.5 mM $MgCl_2$, 0.5 μM sense primer, 0.5 μM antisense primer, 0.8 mM dNTP mix, 1× PCR buffer, and 1 μL AmpliTaq Gold (Applied Biosystems, CA). PCR reactions were performed using the GeneAmp PCR System 9700 (Applied Biosystems), and first initiated at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at the primer melting temperature for 30 s, and extension at 72°C for 1 min, with final elongation at 72°C for 7 min. For the first PCR reaction of exon 4/5 and 7/8 amplifying about 2 Kb fragment, the 30 cycles were modified to 94°C for 1 min, 58°C for 1 min, and 72°C for 3 min. For the second PCR reaction, a 1-μL aliquot from a 1/50 dilution of the first PCR reaction was used. PCR products were purified using the Wizard PCR Preps DNA Purification System (Promega, CA) prior to cycle sequencing using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The DNA sequence was analyzed on an ABI 3100 DNA sequencer (Applied Biosystems).

Results

SNPs within published GRHPR primers

SNP database analysis identified SNPs within published sense primers [11] for exon 6 and 8 (Fig. 1a). To eliminate possible allele dropout, we designed primers, which do not contain the SNP site (Table 1). Figure 2a shows a polymorphism (rs309459) is located within the published exon 6 sense primer. A polymorphism (rs309458) on exon 6 was sequenced using published sense and redesigned exon 6 primers (Fig. 1b). Although the polymorphism was identified using redesigned primer sets, detection failed after altering the sequence of the redesigned sense primer to that of the published sense primer. Results from the PCR reaction, which used the published exon 6 sense primer with an annealing temperature of 62°C, showed allele dropout, which may lead to misdiagnosis. In contrast, allele dropout was avoided using redesigned primers for GRHPR exon 6.

Homologous region on intron D

The NCBI sequence data for CH471071, which includes the GRHPR gene, contains a number of sequences homologous to the published antisense primer of GRHPR exon 4 [11] (Fig. 1c). To avoid primers annealed to multiple sites, amplification of exon 4 was performed using redesigned antisense primers (Table 1). Compared to published primers, sequence analysis results for GRHPR exon 4 using the redesigned primers show a decrease in the failure ratio from 2/5 to 0/5.

Length of intron D and intron H

Comparison of the NCBI sequence data of AF146689 (GRHPR gene) with that of CH471071 revealed a length disparity in introns D and H of the GRHPR gene (Fig. 1c). To determine the length of intron D after the first PCR reaction, the redesigned PCR first primers were used to amplify exons 4 and 5. In addition, due to the difficulty in amplifying a 5 Kb fragment between exons 8 and 9, we redesigned the published exon 8/9 first primers [11] to amplify exons 8 and 9 separately. Further, the redesigned primers for exon 9 were used to determine the complete sequence of exon 9.

Additional GRHPR primer modifications

Alignment of the published first sense primer of exon 1, final primers of exon 1 and the first antisense primer of

Table 1 Primer sets used for GRHPR gene amplification

Exon	First PCR primer	Second PCR primer
1	**AGTGGCACGAGGCGCACCC**	**TACTGTCACTCCCCGAGCAC**
	CAATCTCCCCTCTCCAAAGC	**CTCCGAGACTCCCCAAAACT**
2	GCCAGGATTCCCAGCTGG	**ACAGGTGTGCGGCTCCTG**
	CTATCTGCCCGCTCGGGC	**CTGCAGAGCAGCCAGGAGCC**
3	GCCCTGAGGTGAACCCGG	
	CCTGAATGGCCGAGGGATATG	
4	**CCCTGAACCCTGATCTTCC**	GGCAGGCAGATCAAAGAGGG
	GCATGTGGGTCTTCTCAGC	**CACCGTCACCTGGGCTGG**
5	**CCCTGAACCCTGATCTTCC**	CATCTTGGTCCAAGGCTGG
	GCATGTGGGTCTTCTCAGC	AGACACGTGGTGCCAGGG
6	**GCTGGCAGTTTGGCAGTGC**	**GTGTCCCTACCCTTTGCG**
	GGGTAAGTCCCTGTGCTCC	**CTGAGTGGGCCAGTGTCCAGG**
7	GGCCTTCAGGAAGCATCTTGG	AGCAAGGGGCTGGTCTCC
	TAGGGACAGAGACCCAGC	TCCAGATGGCAGCAGTGG
8	GGCCTTCAGGAAGCATCTTGG	CAGCTAGAAGGAGGCAGG
	TAGGGACAGAGACCCAGC	**CATGCGCAAGAGGACTGCTCC**
9	**CTTACCGGCAAACCCAAGCC**	**GCTGAAGGCTGCTGAACC**
	CACACTGCAGCCTTGAACTGGC	**CCATATCCACTTGTTAGC**
A1	GCTGGCAGTTTGGCAGTGC	GTCAAATGGGGTCTGAGACTGC
	GGGTAAGTCCCTGTGCTCC	CTGCCTGGAATTCCGCTG
A2	GCTGGCAGTTTGGCAGTGC	GTCAAATGGGGTCTGAGACTGC
	GGGTAAGTCCCTGTGCTCC	CTGCCTGGAATTCTGCTG

Exons 1–9 are updated GRHPR primer sets. *Bold* indicates primer redesigned from published primer [11]. A1 and A2 primers were used to analyze SNP linkage between intron E and exon 6. *Underlines* show SNP position of rs309458

exon 2 [11] on the GRHPR gene was not achieved using the BLAST 2 SEQUENCES (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). Given these results, primers were redesigned as shown in Table 1. In addition, the published second antisense primer of exon 2 [11] was redesigned due to its extremely close proximity to exon 2, which resulted in sequencing failure at the exon boundary site. The updated primer sets for the PCR-based genotyping of GRHPR are shown in Table 1.

Analysis of SNP linkage by allele dropout

The GRHPR gene region between intron E and exon 6 contains four common SNPs (>10% minor allele frequency). To determine the linkage of these SNPs, we performed direct sequencing with PCR amplification of specific alleles (DS-PASA) by designing A1 and A2 primers (Table 1) containing the rs309458 SNP (Fig. 2a) in exon 6. The A1 primers were used to sequence the allele containing polymorphism G on the rs309458 SNP, which corresponds to SNP C on the second antisense A1 primer (Table 1), whereas A2 primers were used to sequence the allele containing polymorphism A on the rs309458 SNP, which corresponds to SNP T on the second antisense A1 primer (Table 1). PCR amplification using A1 and A2 primers was done following the method described in Sect. "Materials and methods", with an annealing temperature of 60°C. Examination of the four SNP linkages in seven healthy volunteers revealed three different alleles among sequences (Fig. 2b). Sequencing distinguished the "-T-T-G-G-" from "-C-C-A-A-" allele due to an SNP difference in the second antisense primer. These results show that determination of allele-specific sequences is possible due to allele dropout by an SNP, which possibly occurs when the annealing temperature exceeds the melting temperature of the primer containing the SNP near the 3′ tail by several degrees. Further, the four identified SNPs, which are found in the HapMap SNP database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=snp), form three different haplotypes (Fig. 2c; [17]), and potentially produce 16 different haplotypes. The presence of a common SNP linkage among seemingly unrelated subjects suggests shared SNP ancestry among humans (Fig. 2d).

Discussion

A recent study evaluated the frequency of human SNPs and their effect on high-throughput genotyping [18]. In the present study, we investigated SNPs in published primers, which are used for GRHPR genotyping, and found SNPs in the sense primers of exons 6 and 8 (Fig. 1a). Depending on conditions, sequence analysis revealed allele dropout using a published exon 6 sense primer (Fig. 1b). Further, Bhat et al. [16]

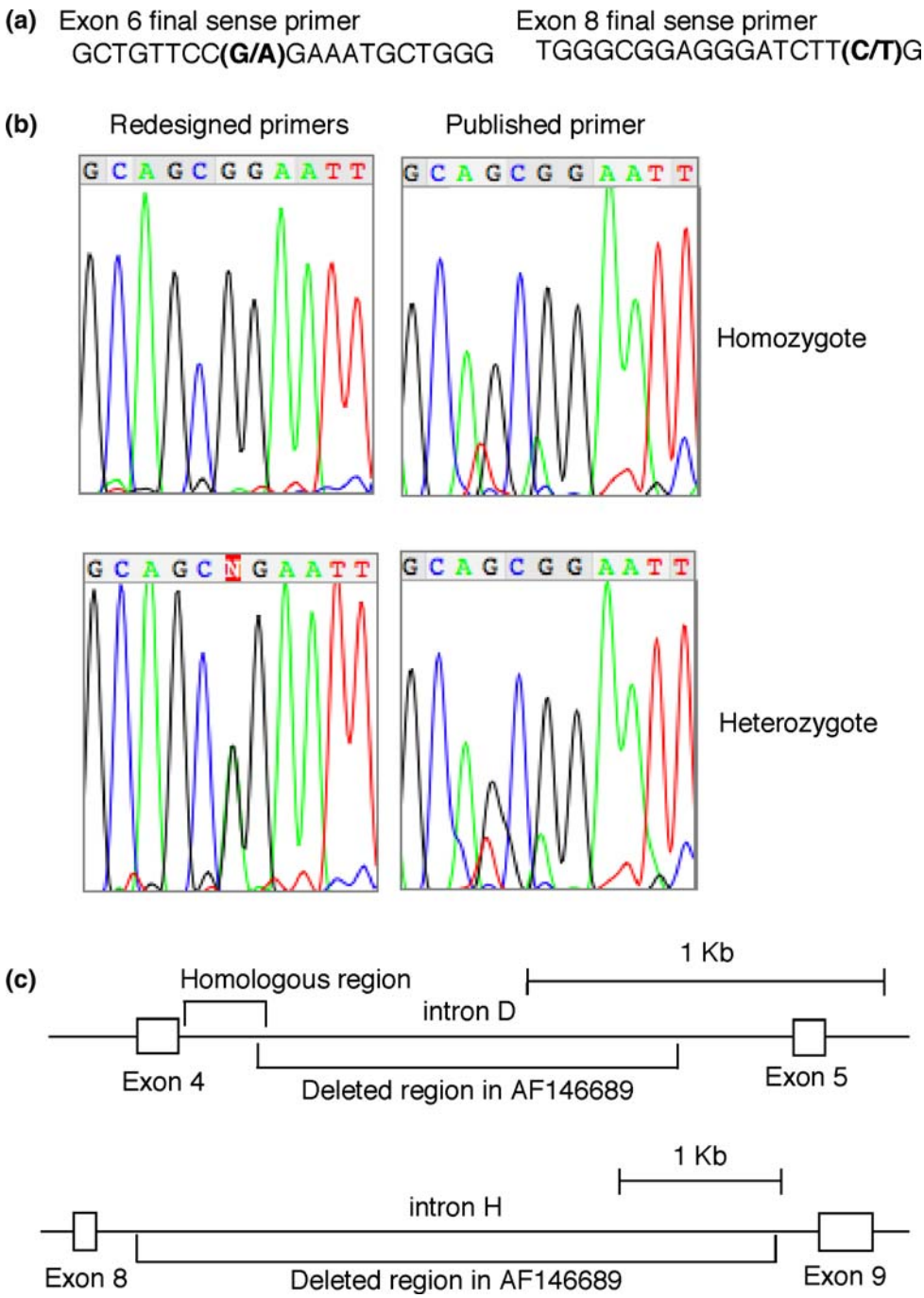

Fig. 1 Allele dropout on GRHPR exon 6 and structure of the GRHPR gene. **a** SNPs in primers. **(G/A)** and **(C/T)** represent the location of the SNP relative to primer sequences, with the *first letter* representing the nucleotide used in the published primer [11]. **b** Allele dropout in exon 6. *Left* Sequence of GRHPR exon 6 generated using redesigned primers (Table 1). *Right* Sequence of GRHPR exon 6 generated using the published exon 6 final sense primer [11] modified from redesigned exon 6 sense primers. **c** GRHPR gene structure near introns D and H. The NCBI sequence data for CH471071, which includes the GRHPR gene, contains a number of sequences homologous to a portion of GRHPR intron D, shown as '*Homologous region*'. In contrast to the CH471071 sequence data, the NCBI sequence data for AF146689 (GRHPR gene) shows a deletion in GRHPR introns D and H, shown as "*Deleted region in AF146689*". Exons and introns are represented by *boxes* and *lines*, respectively

reported discrepancy in the exon 6 polymorphism between the GRHPR gene and the leukocyte cDNA, which contained the 576G and both 567G and 567A polymorphisms, respectively. These findings suggest that this discrepancy occurred from allele dropout using the exon 6 sense primer, and that allele dropout secondary to intronic SNP-primer mismatch obfuscates the detection of GRHPR mutation. In addition, they found the discrepancy between heterozygous leukocyte cDNA and homozygous lever cDNA [16]. It might be monoallelic expression on GRHPR gene [19]. Definitive diagnosis of PH2 generally requires liver biopsies [20], which potentially burdens the patient. Although this approach is unnecessary when using genetic testing, results must be interpreted with caution due to the risk of mutation detection failure. To avoid known SNPs within primers and primer sequence may anneal to multiple genome sites, a similar approach to the primer design described in the present study is recommended for PCR-based assays. Our results also suggest that evaluation of the gene and SNP databases prior to using published primers is crucial for genotyping.

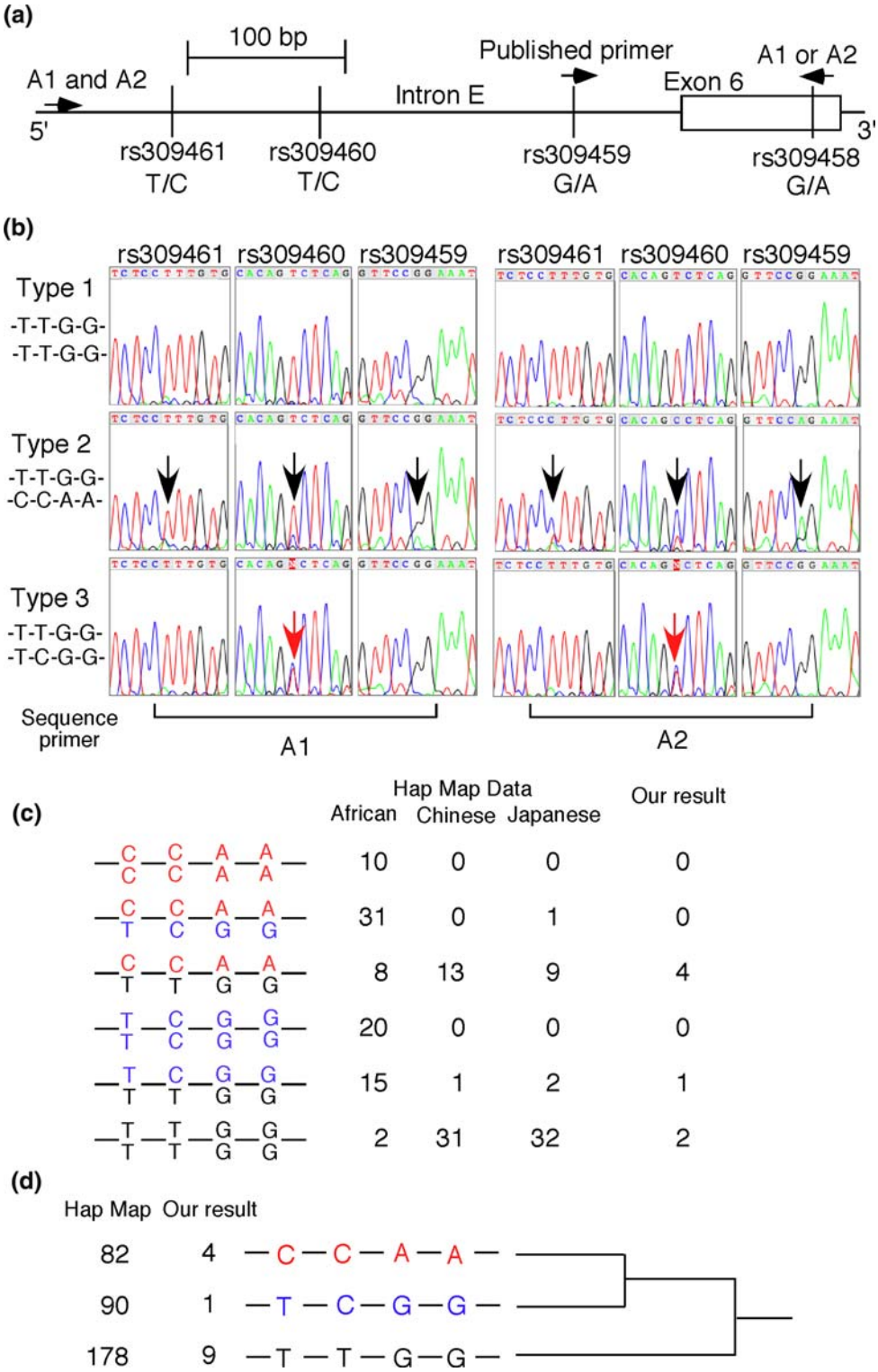

Fig. 2 Analysis of SNP linkage by allele dropout. **a** Common GRHPR SNPs between intron E and exon 6. Four common SNPs (>10% minor allele frequency) were observed between intron E and exon 6. The reference SNP IDs and SNP bases are shown. *Arrows* show positions of A1 and A2 second primers, as well as the published exon 6 final sense primer [11]. These primer sequences are written on Table 1 or Fig. 1a. Exons and introns are represented by *boxes* and *lines*, respectively. **b** Direct sequencing with PCR amplification of specific alleles was used to analyze SNP linkage within the GRHPR gene region from intron E to exon 6. The three sequences are respectively labeled as Type 1, 2 and 3. 'T/C-T/C-G/A-G/A' represents SNP of rs309461, rs309460, rs309459 and rs309458. *Black* arrows show distinct SNPs within the sequence. *Red* arrows show polymorphically sequenced SNPs. **c** SNP linkage of the GRHPR gene between intron E and exon 6. Genotyping data, which shows linkage for the four common SNPs from results in HapMap data as well as seven healthy volunteers, are shown. **d** Genealogical tree of GRHPR gene SNPs between intron E and exon 6. SNPs mapped to the genealogical tree relate to the three haplotypes identified from data in this region

Linkage disequilibrium (LD) analysis generally yields satisfactory results due to the typical correlation between SNPs in close proximity, whose linkages form a haplotype map. The LD relationship also requires empirical determination across the genome by evaluating a high number of polymorphisms in population samples. Further, a large-scale analysis suggests that SNPs are clustered rather than randomly distributed throughout the genome [17, 18]. Here, we report that determination of SNP linkage is possible using DS-PASA. This technique determines haplotypes without the need for pedigree analysis or cloning.

In conclusion, we have updated the primers for the PCR-based genotyping of GRHPR. These redesigned primers show potential in reducing detection failure of GRHPR mutations. In addition, we determined SNP linkage by DS-PASA, which is a possibly effective technique in the identification of contiguous SNP linkage.

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