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ORIGINAL ARTICLE

An evaluation of the performance of the PAP-PCR method in detecting UGT1A1 gene polymorphisms

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Abstract

Background Gilbert's syndrome (GS) is a hereditary disorder caused by mutations in the UGT1A1 gene, leading to unconjugated hyperbilirubinemia. Accurate detection of UGT1A1 gene polymorphisms is crucial for diagnosis and management of GS. This study evaluated the clinical application of pyrophosphorolysis-activated polymerization PCR (PAP-PCR) method in dection of UGT1A1 gene polymorphisms.

Methods and Results Whole-blood samples from 53 patients with GS were genotyped using PAP-PCR for UGT1A1 variations, including UGT1A1*60 (c.-3279T > G), UGT1A1*28 ([TA]6 > [TA]7), UGT1A1*6 (c.211G > A, G71R), UGT1A1*27 (c.686C > A, P229Q), UGT1A1*63 (c.1091C > T, P364L), and UGT1A1*7 (c.1456T > G, Y486D). For each locus, all cases were verified using the direct sequencing to assess PAP-PCR precision. One mutation type per locus was selected to investigate reproducibility and detection limits. The UGT1A1 gene polymorphism detected using PAP-PCR showed that, among the 53 patients, 83.02% presented missense mutations at UGT1A1*60 (c.-3279T > G), 54.72% had heterozygous or homozygous insertions in the TATA box in the promoter, 52.38% had the UGT1A1*6 variant (c.211G > A, G71R). The results obtained using the PAP-PCR and direct sequencing methods were almost consistent among all patients with UGT1A1 gene variants. However, there were different results in the promoter region variants among 2 patients in which PAP-PCR showed [TA]7 homozygosity, whereas direct sequencing revealed [TA]7/[TA]6 heterozygosity. This inconsistent result had been confirmed to be caused by differences in DNA polymerase activity.

Conclusions PAP-PCR could effectively detect UGT1A1 gene polymorphisms associated with Gilbert's syndrome. And it had exhibited superior accuracy in the analysis of TA repeat sequences compared to direct sequencing method. Additionally, due to its reduced program complexity, easy execution, and simple standardization, PAP-PCR may be a highly favorable option for clinical diagnosis.

Keywords Gilbert's syndrome (GS) · Pyrophosphorolysis-Activated Polymerization PCR (PAP-PCR) · Uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1)

Abbreviations

UGT1A1	Uridine diphosphate glucuronosyltransferase 1A1
UDP-GT	Uridine diphosphate glucuronosyltransferase
GS	Gilbert's syndrome
PBREM	Phenobarbital-responsive enhancer module
PAP-PCR	Pyrophosphorolysis-activated polymerization PCR
CAP	College of American Pathologists
RS	Reference Single Nucleotide Polymorphism

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Introduction

The uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) gene is located at 2q37.1, and consists of five exons, and spans a total of 533 amino acid. UGT1A1 encodes uridine diphosphate glucuronosyltransferase (UDP-GT) family 1 member A1. It is responsible for the biotransformation of unconjugated bilirubin to conjugated bilirubin, and the metabolism of endogenous toxic bilirubin [1]. UDP-GT deficiency leads to congenital jaundice disorders, primarily Gilbert's syndrome (GS), with rare instances of Crigler–Najjar syndrome [2], with clinical symptoms showing unconjugated bilirubin elevation in newborns and intermittent or persistent jaundice in adults. UGT1A1 gene polymorphisms form the molecular genetic basis of Gilbert's syndrome, and these can be used to aid diagnosis. In addition, genetic variation in UGT1A1 affects pharmacokinetics of over 50 drugs [3–7].

UGT1A1 genetic variations involve various forms of mutations, including insertions, deletions, and single-nucleotide polymorphisms, resulting in numerous individual differences in sequences [2]. A total of 160 mutants of this gene have been identified to date. According to internationally accepted nomenclature, the wild type is labeled as UGT1A1*1, and mutants are labeled as *2, *3, *4, *5, etc. There are three main forms of genetic polymorphisms: (i) Missense mutations in exon regions, e.g., UGT1A1*6 (c.211G > A, G71R), UGT1A1*27 (c.686C > A, P229Q), UGT1A1*63 (c.1091C > T, P364L), and UGT1A1*7 (c.1456T > G, Y486D). Among these, UGT1A1*6 (G71R) at the 211 position on exon 1 is the most common genotype [6–9]; this has been reported to reduce UDP-GT activity to 30%–70% of its normal value% [8, 10]. (ii) TA insertion mutations on the TATA box which are manifested as dinucleotide (TA) inserted into the TATA box approximately 25–35 bp upstream of the UGT1A1 gene promoter, causing wild-type A[TA]6TAA to mutate into polymorphisms such as A[TA]7TAA (UGT1A1*28) or A[TA]8TAA (UGT1A1*37) [11], and reducing UDP-GT activity to 30% of its normal value [12]. (iii) The c.-3279T > G mutant in the phenobarbital-responsive enhancer module (PBREM) region, has been strongly linked with A[TA]7TAA [13, 14].

Undoubtedly, accurate and convenient methods for detecting UGT1A1 genotype are important in clinical settings [8]. Genotyping technologies commonly used today include Illumina sequencing, fragment analysis, gel sizing, pyrosequencing, array-based technologies, pharmacoscan genotyping, direct sequencing, and fluorescent PCR. Among the above methods, direct sequencing and fluorescent PCR are most commonly used in clinical practice. Direct sequecing is considered as the gold standard for detcing genetic variation loci, but its operation is complicated and it is difficult to be standardized. Therefore, it is not suitable for large-scale clinical application. Sissung et al. compared the capabilities of the above eight methods to determine genotype of the UGT1A1(TA)n repeat polymorphism, they found that fluorescent PCR provided unambiguous results without the need for post-processing of the data [9].

Unlike direct sequencing, PCR is simple to operate and easy to standardize. In this context, we aimed to evaluate the clinical application of the pyrophosphorolysis-activated polymerization PCR (PAP-PCR) method for detecting UGT1A1 gene polymorphism with comparison to the direct sequencing methods.

Materials and methods

Subjects of the study

Data from 53 patients who were clinically diagnosed with Gilbert's syndrome at Peking University People's Hospital between January 2022 and December 2023 were collected. The data showed that all patients presented intermittent or persistent unexplained mild elevation of unconjugated bilirubin which lasted for over 3 months, without evidence of active hemolysis. All patients had normal levels of alanine transaminase, aspartate aminotransferase, and serum albumin, with no viral hepatitis or biliary obstruction. Whole blood samples from these patients (hereinafter referred to as cohort G) were stored in a refrigerator at −80 °C. All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Peking University People's Hospital.

Whole-genome DNA extraction

Peripheral blood genomic DNA from cohort G was extracted using nucleic acid extraction reagents. The kit was purchased from Shanghai Keyee Biotechnology Co. Ltd. The basic principle of nucleic acid extraction is to utilize heat treatment in combination with a DNA-releasing reagent to obtain DNA and quench impurities. The procedure was carried out according to the instructions of the kit. Extracted DNA was used directly for PCR amplification or stored at −20 °C.

Detection of UGT1A1 gene polymorphism using the PAP-PCR method

The principle of the PAP-PCR method is that, in the presence of pyrophosphate, Taq DNA polymerase catalyzes

reverse reaction of DNA synthesis, resulting in template-dependent DNA degradation from the 3′ end; this is known as pyrophosphorolysis. Degradation from the 3′ end in a labeled probe releases fluorescence; such a probe can then be used as a primer for PCR amplification. The assay reagent used was the UGT1A1 Gene Polymorphism Detection Kit (fluorescent PCR method), which was purchased from Shanghai Keyee Biotechnology Co., Ltd., and the amplification instrument used was ABI 7500. The UGT1A1 gene polymorphic loci detected by PAP-PCR are shown in Supplementary Table 1. All sites tested are associated with GS.

Validation of the PAP-PCR method for detecting UGT1A1 gene polymorphisms

Performance validation of the UGT1A1 Gene Polymorphism Detection Kit (Fluorescent PCR Method) (Shanghai Keyee Biotechnology Co., Ltd.) was conducted to evaluate whether the kit meets the analytical performance requirements in clinical laboratories. The reagents lot number was 202,212,001. Reference standards of S1 (*1/*6) and S2 (*28/*28) was selected to verify the reproducibility of the kits for *6 and *28, and the test was repeated three times, with three replicates on each occasion. Reference standards of S3, including the synthetic positive fragments (*60 for TG, *27 for CC, *63 for CT, *7 for TG), were selected and repeated three times, with three replicates each time, to validate the kit in detecting sites *60, *27, *63, and *7. Reference standards of S4 (*6 for AA, *28 for 6/6TA,) and S5 (*60 for TG, *27 for AC, *63 for CC, *7 for TG), with a DNA concentration of 10 ng/μl, were selected to verify the limit of detection for *6, *28, *60, *27, *63 and *7.

Direct sequencing method for detecting UGT1A1 gene polymorphism

The primers used in this study were based on the human UGT1A1 gene sequence (AF297093.1) in GenBank. Primers were designed using the software primer 5.0 to amplify the enhancer region, promoter region, and exon 1–5 regions of UGT1A1. The primer sequences are shown in Supplementary Table 2. The amplified products were sequenced by Beijing Hope Group Biotechnology Co. The sequencing instrument was a ABI 3500 Series Genetic Analyzer (ABI Prism 3130XL Genetic Analyzer Applied Biosystems sequencing), and the sequencing primers were the upstream primers for PCR amplification. The amplified products of exon 1 region were sequenced forward and reverse.

Statistical analysis

SPSS 25.0 statistical software was applied to statistically analyze the data, including consistency rates, CV percentages. GraphPad Prism 9 software was used for statistical plotting analysis.

Results

Validation of the performance of the PAP-PCR method in detecting UGT1A1 gene polymorphism

The genotype *1/*6 (S1, AG 6/6TA), *28/*28 (S2, GG 7/7TA) and the synthetic fragment S3 (*60 for TG, *27 for CC, *63 for CT, and *7 for TG) were used to test the reproducibility of the reagents. All replicates for those specimens matched the expected results [100% (9/9)] (Table 1). The coefficient of variation (CV%) values for the mutant Cts were calculated, which were < 5% (the maximum CV% was 3.87%), respectively.

S4 and S5 containing each of the loci (*6, *28, *60, *27, *63, *7) were used at a concentration of 10 ng/μl. A total of 9 replicates were tested, and the results were in perfect agreement with the theoretical genotypes [100% (9/9)] (Table 2). This indicated that the performance of the kit was stable and reliable, and met the requirements for clinical use.

Results of detection of UGT1A1 gene polymorphism using the PAP-PCR method

Polymorphism of the UGT1A1 gene in the 53 patients is shown in Fig. 1 and Table 3, and Supplementary Table 3 presents the raw data. The following results were determined: 8 of 53 patients (8/53, 15.09%) had heterozygous variation at a single locus; 7 of 53 patients (7/53, 13.21%) had homozygous variation at a single locus. 13 of 53 patients (13/53, 24.53%) exhibited heterozygous variations at two loci; 8 of 53 patients (8/53, 15.09%) exhibited homozygous variations at two loci; and 1 of 53 patients exhibited homozygous variation at one locus and heterozygous variation at the other locus. The remaining 16 patients (16/53, 30.19%) had more than two of the six polymorphic mutations (including homozygous mutations at a single site). Most notably, eight patients had exclusively homozygous mutations at two loci; both were *28 and *60, suggesting that these two loci may be partially linked, which was consistent with previously reported results [14]. When we independently analyzed individuals in the cohort G, we found that 53 patients carried at least one mutation. Among these, 83.02% (44/53) had UGT1A1*60 (c.-3279T > G) mutation; 52.83% (28/53) had insertion mutations in the TATA box; and 50.94% (27/53) had a mutation in UGT1A1*6 (c.211G > A, G71R) in exon 1. The total proportion of the above three variants was 98.11% (52/53); the only remaining case was a rare p.Y486D homozygous mutation.

Table 1 Consistency and repeatability validation results for the UGT1A1 kit

Sample number	RS	UGT1A1	Results	Genotypes	Consistency rate	CV(%)
S1	rs4148323(G)	*1	AG	AG	100% (9/9)	0.51%
	rs4148323(A)	*6				0.49%
	rs3064744([TA]6)	*1	[TA]6[TA]6	[TA]6[TA]6	100% (9/9)	2.51%
	rs3064744([TA]7)	*28				/
S2	rs4148323(G)	*1	GG	GG	100% (9/9)	1.37%
	rs4148323(A)	*6				/
	rs3064744([TA]6)	*1	[TA]7[TA]7	[TA]7[TA]7	100% (9/9)	/
	rs3064744([TA]7)	*28				3.87%
S3	rs4124874(T)	*1	TG	TG	100% (9/9)	2.13%
	rs4124874(G)	*60				2.06%
	rs35350960(C)	*1	AC	AC	100% (9/9)	1.59%
	rs35350960(A)	*27				1.47%
	rs34946978(C)	*1	CC	CC	100% (9/9)	1.81%
	rs34946978(T)	*63				/
	rs34993780(T)	*1	TG	TG	100% (9/9)	1.31%
	rs34993780(G)	*7				1.60%

RS is reference single nucleotide polymorphism. *1 is UGT1A1*1, wild type; *6 is UGT1A1*6, c.211G > A, G71R; *28 is UGT1A1*28, [TA]7; c.-40_-39insTA; *60 is UGT1A1*60, c.-3279T > G; *27 is UGT1A1*27, c.686C > A, P229Q; *63 is UGT1A1*63, c.1091C > T, P364L; *7 is UGT1A1*7, c.1456T > G, Y486D

Table 2 Consistency and repeatability validation of the limit of detection of the UGT1A1 kit

Sample number	RS	UGT1A1	Results	Genotypes	Consistency rate
S4	rs4148323(A)	*6	AA	AA	100% (9/9)
	rs4148323(G)	*1			
	rs3064744([TA]6)	*1	[TA]6[TA]6	[TA]6[TA]6	100% (9/9)
	rs3064744([TA]7)	*28			
S5	rs4124874(T)	*1	TG	TG	100% (9/9)
	rs4124874(G)	*60			
	rs35350960(C)	*1	AC	AC	100% (9/9)
	rs35350960(A)	*27			
	rs34946978(C)	*1	CC	CC	100% (9/9)
	rs34946978(T)	*63			
	rs34993780(T)	*1	TG	TG	100% (9/9)
	rs34993780(G)	*7			

RS is reference single nucleotide polymorphism. *1 is UGT1A1*1, wild type; *6 is UGT1A1*6, c.211G > A, G71R; *28 is UGT1A1*28, [TA]7; c.-40_-39insTA; *60 is UGT1A1*60, c.-3279T > G; *27 is UGT1A1*27, c.686C > A, P229Q; *63 is UGT1A1*63, c.1091C > T, P364L; *7 is UGT1A1*7, c.1456T > G, Y486D

Results of detection of UGT1A1 gene polymorphism using direct sequencing

All patients were sequenced using the direct sequencing method, and the sequencing results are shown in Supplementary Table 4. For the first sequencing, Taq DNA polymerase was used for PCR amplification and the products were analyzed using the direct sequencing method. If the sequencing quality was lower than that of PAP-PCR, or was otherwise inconsistent, a repeat PCR amplification was carried out in which Taq DNA polymerase was replaced with KOD DNA polymerase (TOYOBO, Japan). Sample number 1 was not tested for *60 and *28 due to insufficient sample volume. Sample 9 and sample 20 were tested twice, but the results were inconsistent.

The sequencing results showed that the positive rate of mutation was as high as 98.11% (52/53) at the three loci: *28, *60, *6. However, the detection rates of *27 (5/53,

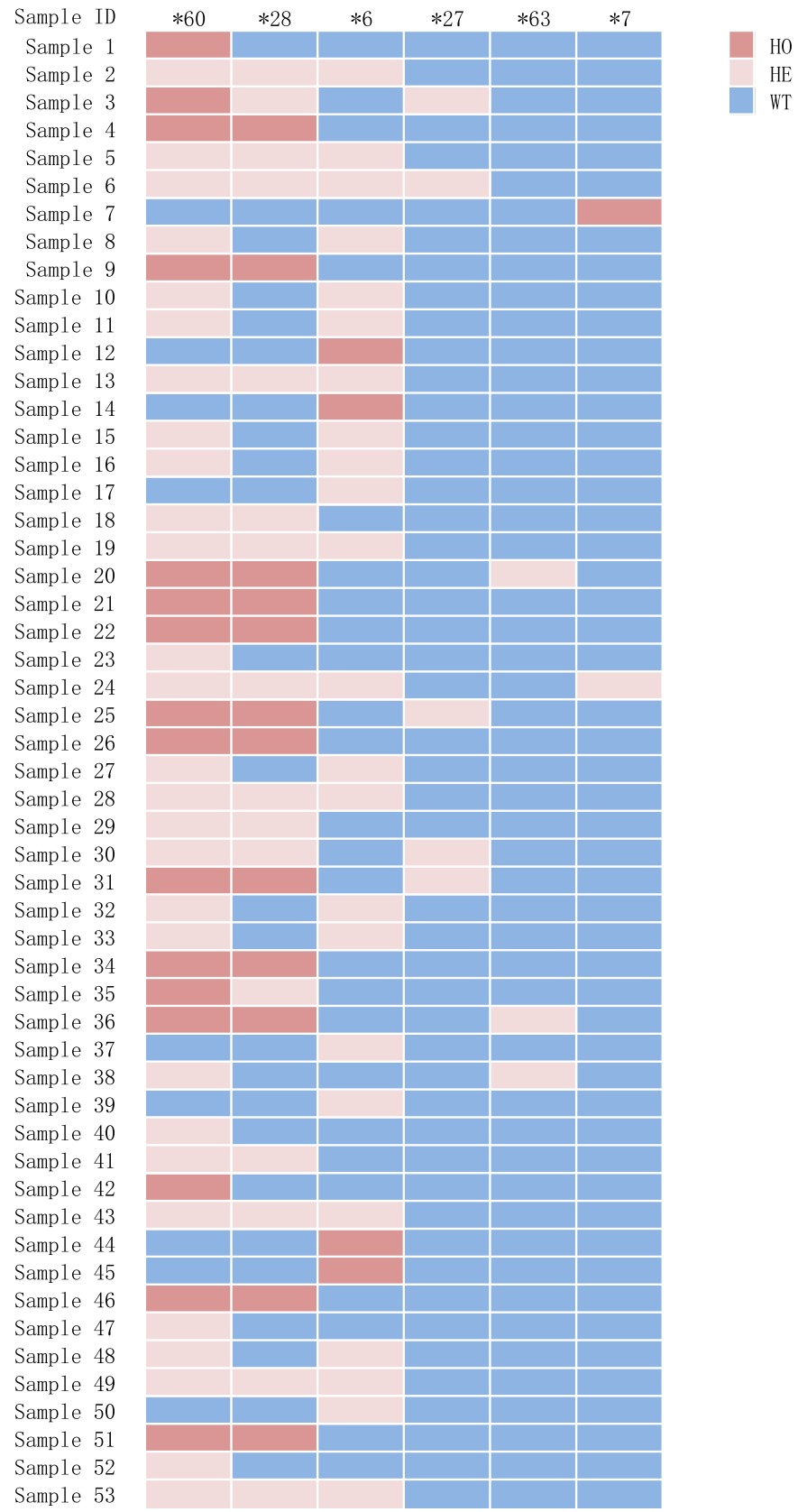


Fig. 1 UGT1A1 gene polymorphism detected using PAP-PCR. *6 is UGT1A1*6, c.211G > A, G71R; *28 is UGT1A1*28, [TA]7; c.-40_-39insTA; *60 is UGT1A1*60, c.-3279T > G; *27 is UGT1A1*27, c.686C > A, P229Q; *63 is UGT1A1*63, c.1091C > T, P364L; *7 is UGT1A1*7, c.1456T > G, Y486D. WT represents wild type, HE represents heterozygous variation, HO represents homozygous variation

Table 3 Results obtained using PAP-PCR for distribution of UGT1A1 gene polymorphism in 53 patients

Genotype	Enhancer *60(%)	Promoter *28(%)	Exon 1 *6(%)	Exon 1 *27(%)	Exon 4 *63(%)	Exon 5 *7(%)
Wild type	9 (16.98)	25 (47.17)	26 (49.06)	48 (90.57)	50 (94.34)	51 (96.22)
Heterozygous variation	28 (52.83)	16 (30.18)	23 (43.40)	5 (9.43)	3 (5.67)	1 (1.89)
Homozygous variation	16 (30.19)	12 (22.64)	4 (7.55)	0 (0)	0 (0)	1 (1.89)

*6 is UGT1A1*6, c.211G > A, G71R; *28 is UGT1A1*28, c.-40_-39insTA; *60 is UGT1A1*60, c.-3279T > G; *27 is UGT1A1*27, c.686C > A, P229Q; *63 is UGT1A1*63, c.1091C > T, P364L; *7 is UGT1A1*7, c.1456T > G, Y486D

9.4%), *63 (3/53, 5.66%) and *7 (2/53, 3.77%) were relatively low. Direct sequencing also detected heterozygous variations in other two loci (Supplementary Table 4), one was c.1198A > C (sample 13), the other was c.182C > G (samle 19).

Comparison of PAP-PCR and direct sequencing methods for detection of single-base mutations in UGT1A1

All patients, both PAP-PCR and the direct sequencing method were used to detect genomic polymorphisms in the PBREM region and exon regions of the UGT1A1 gene. The results of PAP-PCR and the direct sequencing method show that the numbers and genotypes of single-nucleotide mutations were consistent (Fig. 2). In total, 15 of the 53 patients (28.30%) had UGT1A1*60 (c.-3279T > G) homozygous variants, and 28 of 53 patients (52.83%) had UGT1A1*60 (c.-3279T > G) heterozygous variants, while 1 patient was not detected in the promoter region by direct sequencing due to insufficient sample volume. In the direct sequencing results, 4 cases (4/53, 7.54%) had the UGT1A1*6 (c.211G > A, G71R) homozygous mutation, 23 cases (23/53, 43.40%) had UGT1A1*6 (c.211G > A, G71R) heterozygous mutation. 4 cases (4/53, 7.54%) exhibited UGT1A1*27 (c.686C > A, P229Q) heterozygous mutation, 3 cases (3/53, 5.66%) showed UGT1A1*63 (c.1091C > T, P364L) heterozygous mutation, 1 case had UGT1A1*7 (c.1456T > G, Y486D) homozygous mutation, and 1 case showed UGT1A1*7 (c.1456T > G, Y486D) heterozygous variation.

Comparison of PAP-PCR and direct sequencing methods for detection at UGT1A1*28

The PAP-PCR and direct sequencing methods were used to detect UGT1A1 gene polymorphisms in the promoter region. These results are shown in Fig. 2. Both methods revealed that 24 patients (24/53, 45.28%) were wild-type in the promoter region, while the other 28 patients (28/53, 52.83%) had the variants, and 1 patient was not detected in the promoter region by direct sequencing due to insufficient sample volume. However, these two methods produced different results in 2 of the 28 patients (2/28, 7.14%), with PAP-PCR showing [TA]7 homozygous mutation and direct sequencing showing [TA]7/[TA]6 heterozygous mutation. The retesting using PAP-PCR method confirmed the [TA]7 homozygous variants. When using high-fidelity DNA polymerases, the direct sequencing method produced the same results with PAP-PCR method.

Discussion

UGT1A1 gene polymorphisms diminish activity of UDP-GT to a range of 10% to 60% compared to the wild-type. The degree of UDP-GT activity diminution directly correlates with an elevated likelihood of developing disorders associated with bilirubin metabolism will develop [15–17]. Furthermore, the UGT1A1 genetic variations may lead to drug metabolism disturbances or exacerbate hyperbilirubinemia [18].

The objective of this study was to evaluate the utility of PAP-PCR for detecting UGT1A1 gene polymorphisms with comparison to the direct sequencing methods. PAP-PCR has been used to detect circulating tumor DNA in metastatic uveal melanoma [19] and has effectively detected Y chromosome sequences in free fetal DNA from maternal plasma for non-invasive prenatal testing [20], showing its reliability and versatility in detecting somatic mutations across various clinical scenarios [21]. Although fluorescent PCR has been reported to provide clear results, there have been no reports on the use of PAP-PCR for detecting UGT1A1 gene polymorphisms [9].

The prevalence rates of GS-associated UGT1A1*60 (rs4124874), UGT1A1*6 (rs4148323), UGT1A1*28 (rs3064744) mutations in East Asia are 32.75%, 15.45% and 7.57%, respectively [22]. In the present study, among the 53 patients clinically diagnosed with GS, the mutation rate for any of UGT1A1*60, UGT1A1*6 or UGT1A1*28 was notably high at 98.11%, with 83.02% (44/53) having a mutation in UGT1A1*60, 52.83% (28/53) carrying a mutation in UGT1A1*6, and 50.94% (27/53) exhibiting a mutation in UGT1A1*28. These results suggest that UGT1A1*60, UGT1A1*6 and UGT1A1*28 mutations are associated with

clinical elevation of unconjugated bilirubin and warrant further investigation.

Compared with the direct sequencing method, the fluorescent PCR-based PAP-PCR method for detecting UGT1A1 gene polymorphism has the following advantages: (1) it is easy to standardize and automate; (2) the fluorescent PCR kit has fewer, simpler operating steps and shorter detection and turnaround times (TAs); (3) there are many PCR-trained medical technicians who can support better performance of the kit; (4) the qPCR method involves closed-tube amplification and detection, which effectively prevents contamination of PCR products. Because of these advantages, fluorescent PCR-related techniques and products have been one of the fastest-growing, most successful, and most widely used diagnostic techniques of the past 30 years.

We used both PAP-PCR and direct sequencing methods to analyze the PBREM and exon regions of UGT1A1 in 53 patients. For the six loci (*60, *28, *6, *27, *63, *7), we initially observed discrepancies in the promoter region mutations in two patients (Sample 9 and 20, Table 4). PAP-PCR showed [TA]7 homozygous mutation, while direct sequencing showed [TA]7/[TA]6 heterozygous mutation. After retesting with high-fidelity DNA polymerases in direct sequencing, it was conformed that the mutations were both [TA]7 homozygous variants. Therefore, PAP-PCR demonstrated the same detection capability as direct sequencing methods in our study. We speculate that the low concentration of TA repeat sequences may affect the specificity of Taq DNA polymerase, leading to replication errors. PAP-PCR is more reliable than direct sequencing for detecting UGT1A-1gene polymorphisms.

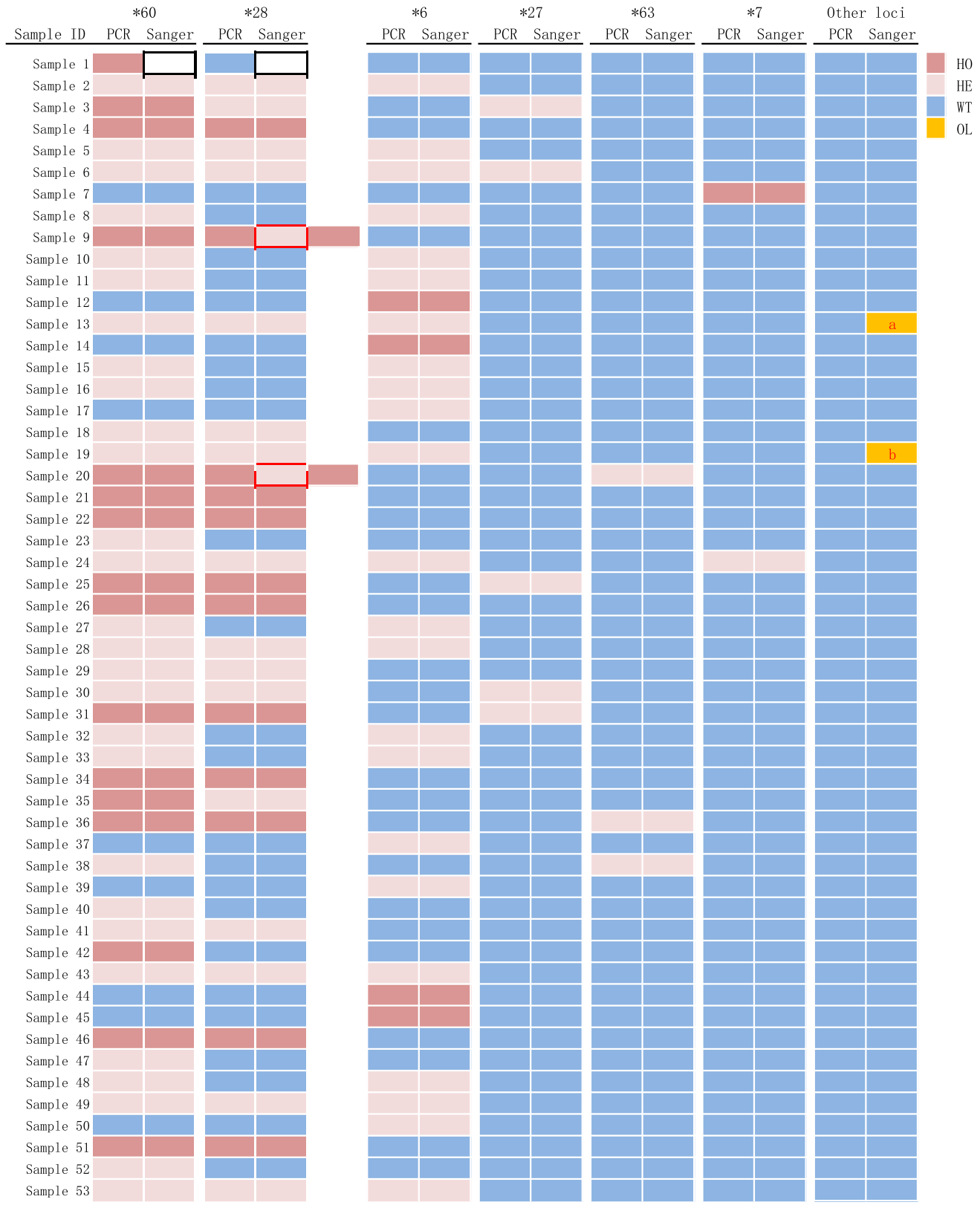
Additionally, direct sequencing detected heterozygous variations at two other loci (Supplementary Table 4), c.1198A > C in sample 13, and c.182C > G in sample 19. However, in the two patients, PAP-PCR had already detected three heterozygous mutations (*60, *28, *6), which did not affect clinical diagnosis. In our study, using PAP-PCR to detect mutations at these six loci can help diagnose the vast majority of GS.

In accordance with the international standard for medical laboratory accreditation ISO 15189 [23], and the relevant requirements of the College of American Pathologists (CAP), new detection systems need to be performance-verified before clinical application to ensure that the test performance claimed by the manufacturer can be reproduced. In this study, the performance of PAP-PCR for detecting UGT1A1 gene polymorphisms was verified in accordance with the requirements of ISO15189-related documents, such as Application Requirements in the Field of Molecular Diagnostics of Accreditation Criteria for the Quality and Competence of Medical Laboratories [24], and Guidance on the Performance Verification for Molecular Diagnostic Procedures. Our results showed that the reproducibility, accuracy [25], and limit of detection obtained using the PAP-PCR method for detecting UGT1A1 gene polymorphisms were all as expected, and so the method can be used for clinical testing.

In this study, UGT1A1 gene mutations were detected in all 53 samples of the cohort G, a finding which further confirmed that UGT1A1 variations are associated with GS. Two methods were used to detect UGT1A1 gene polymorphisms, and the results obtained were identical after correction, indicating that the PAP-PCR method was highly accurate in detecting UGT1A1 gene polymorphisms. Tests of reproducibility and limits of detection confirmed the stability and reliability of the method. Compared with the direct sequencing method, PAP-PCR was found to be more accurate in analyzing the numbers of repeats of repetitive sequences, delivered better performance, and exhibited the advantages of fewer steps, more convenient operation, and greater ease of standardization, which make it suitable for promotion in clinical laboratories.

Conclusions

PAP-PCR could effectively detect UGT1A1 gene polymorphisms associated with Gilbert's syndrome. Compared with the direct sequencing method, PAP-PCR can analyze the numbers of TA repeats more accurately and effectively in two separate inspections. In addition to its advantages of fewer steps, its convenient operation and easy standardization make it suitable for clinical consideration.


*60
*28
*6
*27
*63
*7
Other loci
Sample ID
PCR
Sanger
HO
HE
WT
OL
a
b

◂**Fig. 2** Comparison of UGT1A1 gene polymorphisms detected using the PAP-PCR and direct sequencing methods. *60 is UGT1A1*60, c.-3279T > G; *28 is UGT1A1*28, [TA]7; c.-40_-39insTA; *6 is UGT1A1*6, c.211G > A, G71R; *27 is UGT1A1*27, c.686C > A, P229Q; *63 is UGT1A1*63, c.1091C > T, P364L; *7 is UGT1A1*7, c.1456T > G, Y486D. WT represents wild type, HE represents heterozygous variation, HO represents homozygous variation, OL represets other loci, a is c.1198A > C heterozygous variation, b is c.182C > G heterozygous variation, blank represents not detected

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-025-10391-9.

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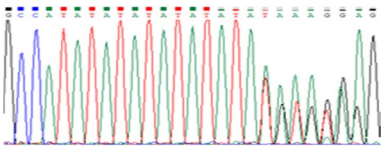
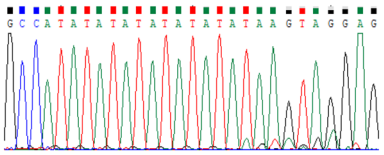
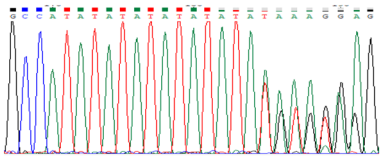
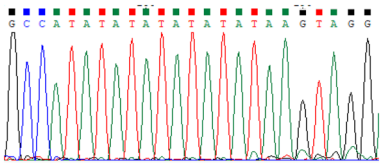
Author contributions Nan Wu, Xiangsha Kong, Bo Feng, Hongsong Chen, Huiying Rao contributed to the study conception and design. Material preparation, data collection and analysis were performed by Nan Wu, Xiangsha Kong, Zixiang Huang, Yijun Shi and Xiaofang Liu. The first draft of the manuscript was written by Xiangsha Kong and all authors commented on previous versions of the manuscript. The final

Table 4 Results of retesting conducted after the two methods first produced inconsistent results for two patients

Sample number	PAP-PCR	Direct Sequencing	
1st 9	[TA]7 homozygous	[TA]7/[TA]6 heterozygous	
2nd 9	[TA]7 homozygous	[TA]7 homozygous	
1st 20	[TA]7 homozygous	[TA]7/[TA]6 heterozygous	
2nd 20	[TA]7 homozygous	[TA]7 homozygous	

manuscript was revised by Nan Wu, Xiaofang Liu, and Feng Liu. All authors read and approved final manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interests The authors declare no competing interests.

Ethical approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Peking University People's Hospital (No.2024PHB579-001).

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