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Original Article

Detection of the jaundice-related G71R mutation in the UGT1A1 gene by denaturing high performance liquid chromatography (DHPLC)

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Abstract

Background The G71R mutation in the UGT1A1 gene has been associated with neonatal jaundice and other cases of hereditary, unconjugated hyperbilirubinemia in several Asian populations. Currently, DNA sequencing is the only method available to identify the mutation, which can be time- and labor-intensive, particularly for such projects as population-based genetic studies. A relatively new method, denaturing high performance liquid chromatography (DHPLC), is increasingly used to detect various mutations.

Objective The aim of the present study was to investigate the ability of DHPLC to detect the G71R mutation, in comparison with the gold standard of sequencing analysis.

Methods Seventy-two infants were enrolled. Following genomic DNA extraction, exon 1 of the UGT1A1 gene was amplified by polymerase chain reaction (PCR). Afterwards, the G71R mutation was simultaneously, and blindly, determined in all subjects by DHPLC and sequence analysis. The performance of the DHPLC analysis, compared to the sequence analysis, was assessed in terms of sensitivity and specificity.

Results DHPLC detected the G71R mutation in 31 individuals. Of these, 26 were heterozygous and 5 were homozygous for the mutation. This method did not find the mutation in 41 other individuals. Sequence analysis produced identical results for all individuals.

Conclusion DHPLC analysis is capable of detecting the G71R mutation in the UGT1A1 with a degree of sensitivity and specificity (100% each) that is comparable to sequencing analysis. **[Paediatr Indones. 2009;49:1-6]**.

Keywords: *G71R mutation, UGT1A1 gene, DHPLC, sequencing, neonatal jaundice*

The UGT1A1 gene, which encodes bilirubin uridine diphosphate glucuronyl transferase, is the only enzyme responsible for bilirubin conjugation in hepatocytes.[1,2] Defects in this gene have been associated with hereditary unconjugated hyperbilirubinemia.[3-5]

G71R is a well-known mutation in exon 1 of UGT1A1. It is a G-to-A transition at nucleotide 211 that results in the substitution of glycine with arginine at codon 71. This mutation is a genetic risk factor for the development of neonatal jaundice in East Asian populations, including Japan, China, Korea, and Taiwan. The role of G71R in other Asian populations remains unclear. The neonates carrying G71R tend to develop more severe and prolonged jaundice compared to those without G71R and neonates with breast milk jaundice.[6-12] Moreover, the mutation exacerbates hyperbilirubinemia when it appears in combination with other genetic, hemolytic disorders, such as glucose-6-phosphate dehydrogenase

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(G6PD) deficiency and thalassemia.[13-15] Previously, DNA sequencing has been the only method used for identifying the G71R mutation. However, it is very costly, inefficient and laborious; especially in large studies. Additionally, the detection method, restriction fragment length polymorphism (RFLP), is not suitable for detecting this mutation.[16]

Denaturing high performance liquid chromatography (DHPLC) is a relatively new technique for detecting DNA mutations. Recently, it has been used to screen mutations in various genes with an impressive degree of sensitivity and specificity.[17-19] The aim of this study was to investigate the ability of the DHPLC method to detect the G71R mutation, compared to the gold standard of DNA sequencing.

Methods

Subjects

Infants born in the Neonatal Unit of the Kobe University Hospital were enrolled in this study, after informed consent was obtained from the parents. A 2 ml venous blood sample was drawn from each subject and mixed with the anticoagulant, ethylene diamine tetra-acetate (EDTA).

DNA extraction

Genomic DNA was extracted from peripheral blood leukocytes using the SepaGene® DNA extraction kit (Sanko Junyaku, Tokyo, Japan). Extracted DNA samples were stored at -20 °C until the time of analysis.

PCR amplification

Exon 1 of the UGT1A1 gene was amplified by polymerase chain reaction (PCR) using the forward primer, 5´-GCA GAG GGG ACA TGA AAT AG-3´, and the reverse primer, 5´-TTG TTG TGC AGT AAG TGG GA-3´, as previously reported.[20] PCR was carried out with a PC-701 thermal cycler (Astec, Tokyo, Japan) in 30 μl of reaction mixture 200 ng genomic DNA in 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.15 μM primers, 1 U FastStart Taq DNA polymerase (F. Hoffmann-La Roche Ltd., Diagnostics Division, Basel, Switzerland). The PCR conditions consisted of an initial denaturation at 94°C for 7 minute, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute, extension at 72 °C for 1 minute, and a final extension period at 72°C for 7 minute. The PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide.

DHPLC analysis

Each PCR product was denatured at 95°C for 5 minutes and then slowly re-annealed by ramping the temperature down to 25°C over 45 minutes, to allow heteroduplex formation.[21,22] Five μl samples was loaded onto an automated DHPLC system, the WAVE® Nucleic Acid Fragment Analysis System, equipped with a DNASep® cartridge (Transgenomic, Inc., Omaha, NE). The analysis was conducted at a column temperature of 62°C, which was adjusted automatically by the system. The gradient was generated by changing the concentration of buffer B (0.1 M triethylamine acetate, 25% acetonitrile) relative to that of buffer A (0.1 M triethylamine acetate). The following gradients were used: 50–45% buffer A and 50–55% buffer B, for a DNA loading step of 0.5 minute, 45–36% buffer A and 55–64% buffer B, for a linear separation step of 5 minute, 36–0% buffer A and 64–0% buffer B, for a clean-off step of 0.7 minute, and 0–50% buffer A and 0–50% buffer B, for an equilibration step of 1 minute. The flow rate was 0.9 ml/minute. A heteroduplex peak observed in the chromatogram indicated the presence of a heterozygous mutation, while a homoduplex peak suggested either the wild type state or a homozygous mutation. To distinguish a homozygous mutation state from the wild type, samples with homoduplex peaks were subjected to a second DHPLC analysis. The procedures and conditions were the same as mentioned above, but each sample was mixed with a known wild type sample during the heteroduplex formation step. The presence of a heteroduplex or a homoduplex peak in second analysis suggested the presence of homozygosity or no mutation, respectively.

DNA sequencing analysis

Sequencing analysis was performed simultaneously, and blindly, with DHPLC analysis, using BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer

Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The sequencing reaction product was electrophoresed on a genetic analyzer (ABI PRISM 310; Applied Biosystems) and then analyzed using DNA Sequencing Analysis Software (Applied Biosystems).

Assessment of DHPLC analysis performance

When two different procedures were used to measure the same characteristics, two statistical approaches can be used to determine the degree of similarity between the two procedures. If neither was a gold standard, kappa statistics were used, but if one can function as a gold standard, then calculations of sensitivity and specificity can be performed using diagnostic tests.[23] In the present study, DNA sequencing was the gold standard for analysis of mutations. Therefore, the results of the two methods were compared with a diagnostic test in a 2x2 format.[2, 24]

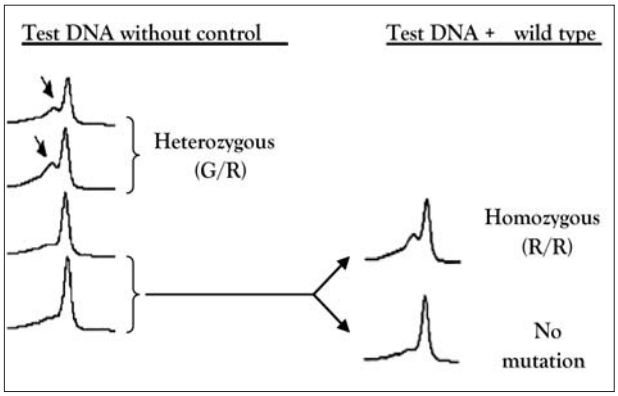

Figure 1. Chromatographic profiles showing the G71R mutation. In step 1, without mixing with control DNA, the presence of a heteroduplex peak indicated a heterozygous mutation (G/R), indicated by the arrow. In step 2, tested DNA samples with homoduplex peaks (identified in step 1) were reanalyzed by mixing with a wild type DNA control. The presence of a heteroduplex or homoduplex peak in this step suggested homozygosity (R/R) or no mutation (G/G) present, respectively.

Results

Seventy-two infants were enrolled in the study. DHPLC analysis detected G71R mutation in 31 subjects, including 5 subjects with homozygous mutations and 26 with heterozygous mutations. The results of the DNA sequencing analysis were identical to those of DHPLC for all subjects. Thus the sensitivity and specificity of the DHPLC analysis were both 100%.

Representative results of the DHPLC and DNA sequencing analysis are shown in **Figures 1** and **2**, respectively.

The results of the two methods used for detecting the G71R were represented as a 2x2 matrix **(Table 1)**. In this table, all subjects with mutations were placed into one group, regardless of the state of the mutation (homozygous or heterozygous).

Table 1. 2x2 matrix summarizing the results of mutation detection by DHPLC and sequencing analysis

		DNA SEQUENCING		
		Mutation	No mutation	Total
DHPLC	Mutation	31	0	31
	No mutation	0	41	41
	Total	31	41	72

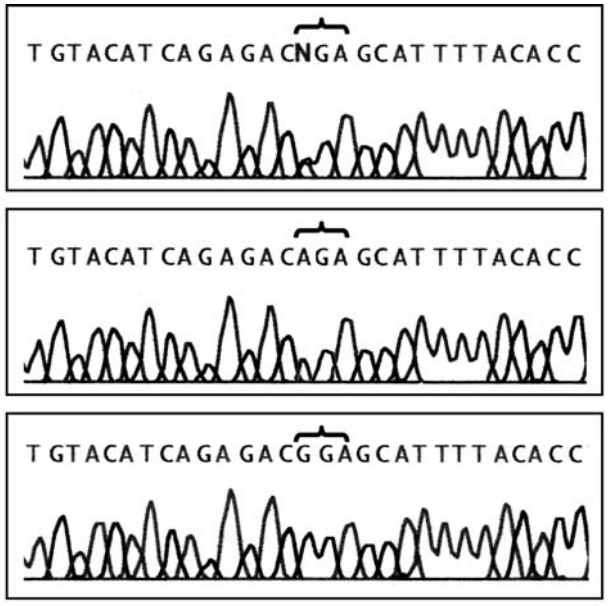

Figure 2. Representative results of the sequencing analysis of the G71R mutation (GGA→AGA). The upper, middle and lower figures show the heterozygous, homozygous, and wild type mutation states, respectively.

Discussion

DHPLC detects mutations based on the formation of heteroduplexes which are due to the presence of mismatched nucleotides in the two DNA strands being amplified. The heteroduplexes are thermally less stable, and run faster, than homoduplexes along the DNA separation column used in DHPLC system. This enables the separation of heteroduplexes from homoduplexes on the chromatogram.[17-19]

This study showed that the DHPLC analysis was as accurate as the DNA sequencing analysis in detecting the G71R mutation; and each had a sensitivity and specificity of 100%. The same results have been reported by previous studies for various genes, including the retino blastoma (RB1),[19] neurofibromatosis type 1 (NF1),[26] coagulation factor 9 (F9),[26] breast cancer (BRCA1 and BRCA2)[27-29] and connexin (GJB2) genes.[30] In general, the sensitivity and specificity of DHPLC have both been shown to be consistently above 90%.[18,19]

DHPLC analysis has a number of advantages over sequencing analysis. Its operational cost is approximately ten times lower than that of the sequencing analysis.[18,19] The DHPLC process is also less laborious, with only one post-PCR step – the heteroduplex formation reaction. The analysis is fast - less than 10 minutes per sample - and the results can be monitored in real time. Both DHPLC and sequencing analyses are highly automated, allowing the simultaneous analysis of multiple samples.[18,19,21,22]

To date, there are no reports on the use of other methods, such as single stranded conformational analysis (SSCA) or denaturing gradient gel electrophoresis (DGGE), to detect the G71R mutation. SSCA is relatively costly, involves laborious procedures, and sometimes requires the use of radioactive substances. DGGA, apart from being long and tedious, it requires an electrophoretic media with specific properties. Moreover, previous studies have demonstrated that the accuracy of DHPLC analysis was consistently higher than that of SSCA or DGGE.[18,19] The PCR-RFLP method, despite being more cost-effective, is inappropriate for detecting the G71R mutation because the mutation does not affect existing restriction enzyme sites, and does not allow formation of artificially-introduced ones.[16]

Unlike DNA sequencing, DHPLC is a detection method, and is not used for identifying the mutation. There are two possible methodological approaches that can be applied to mutation analysis. Firstly, DHPLC can be used to scan for unknown mutations within DNA fragments. The origin and location of the detected mutations are then identified by DNA sequencing. Using this approach, sequencing analysis would be done only on the DNA fragment with the mutation. In second approach, DHPLC analysis is performed to investigate the presence or absence of a known mutation in DNA fragments, as was the case in our study. This approach has been commonly used to determine the allele frequency of a certain mutation in control subjects, or to study distribution of a mutation in a population.[21,22]

Successful DHPLC analysis depends on two factors, i.e. the quality of the PCR product and the optimized conditions of the DHPLC analysis. The first factor is influenced by primer design, optimization of PCR mixture and PCR thermal cycling. The second factor relies on appropriate heteroduplex formation and the determination of an optimum temperature for the analysis.[18,19,21,22]

To this point, the G71R mutation has been widely studied in the Eastern Asian population including Japan, Korea, China and Taiwan.[6-15] In these populations; the presence of the mutation increases the risk for developing neonatal jaundice and other hyperbilirubinemia-related disorders. Such conditions are also common in other Asian populations, but there is a lack of data from these groups. Applying DHPLC analysis, which is less costly and less laborious than the sequencing analysis, could therefore facilitate studies on the role and distribution of the G71R mutation in Asian populations where data is lacking, such as in Indonesia. Such studies would be beneficial to both genetic counselors, and physicians assessing the prognosis of affected patients.

In conclusion, we demonstrate that the DHPLC method of detecting the G71R mutation has an accuracy similar to that of the DNA sequencing analysis.

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