Detection of His1069Gln mutation in Wilson disease by bidirectional PCR amplification of specific alleles (BI-PASA) test

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Abstract. Wilson disease (WD) is an autosomal recessive disorder of hepatic copper metabolism caused by mutations in a gene encoding a copper-transporting P-type ATPase, *ATP7B*. The majority of known mutations affecting this gene are frequent in different populations, which may help to introduce rapid diagnostic procedures based on direct DNA analysis into routine clinical practise. The His1069Gln mutation in exon 14 is the most frequent one, accounting for 30–60% of all mutations in Caucasian patients. The aim of the present work was to introduce DNA-based direct analysis into routine molecular screening for the above mutation in Slovak WD patients and to assess its frequency in patients as well as in a control population. Twenty seven clinicaly diagnosed patients from twenty five families, twenty relatives of index patients and three hundred and six control DNA samples were tested using two different DNA-based methods: the earlier described amplification created restriction site (ACRS) for Alw21I in combination with nested PCR and the amplification refractory mutation system (ARMS). In 18 of 25 unrelated patients (72%), the mentioned genetic defect was present in at least one copy. In ten of them (40%), the above mutation was detected in homozygous and in eight individuals (32%) in heterozygous state. In seven WD patients (28%), this mutation was not detected. The allele frequency of His1069Gln in Slovak patients with WD was 56%, which was higher as reported in other populations. In a control group of 306 random DNA samples (612 alleles), the His1069Gln mutation was observed in 3 samples (carrier frequency 1%; allele frequency 0.49%). These frequencies correspond to figures observed in different population of European origin. Taken together, we have provided further evidence that the His1069Gln mutation is the prevalent *ATP7B* mutation in central-european WD patients.

Although both methods used in this study worked in our hands reliably, there are in every-day use some drawbacks and limitations inherent to them (PCR reactions in two tubes, possibility of star activity or not complet digestion by restriction endonuclease, etc.). Therefore we developed a simpler, cost effective and rapid DNA diagnostic test based on bidirectional amplification of specific alleles (BI-PASA), which enables detection of homozygotes (wild and mutant) and heterozygotes, respectivelly, in one PCR reaction. The test was highly sensitive and specific, yielding no false-positive or false-negative results. Its reliability and discriminating power was tested on samples of 27 WD patients and 120 random control DNA's, previously genotyped by above mentioned methods. Comparing results of BI-PASA with ACRS and ARMS tests showed 100% concordance.

Key words: Wilson disease — BI-PASA test — ARMS test — ACRS test — His1069Gln mutation

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Introduction

Wilson disease (WD), also known as hepatolenticular degeneration, is an autosomal recessive disorder of copper transport characterized by progressive copper accumulation in the liver and the central nervous system (OMIM 277900). The worldwide prevalence of WD is estimated to be 1 : 30,000, with a corresponding gene and carrier frequency of 0.56% and 1.1%, respectively (Scheinberg and Sternlieb 1984; Danks 1989; Brewer et al. 1992; Hoogenraad 1996; Schilsky 1996; Gollan and Gollan 1998; Ferenci 1999). Patients usually present with hepatic or neuropsychiatric symptoms, or both (Ferenci et al. 2001; Mareček 1996). Since biochemical markers of impaired copper metabolism can be misleading, diagnosis may be difficult in the absence of typical symptoms and in asymptomatic siblings. Early diagnosis is, however, critical, since treatment due to time prevents life-long neurological disability and/or liver cirrhosis. Recently the gene for WD was identified as a copper transporting ATPase, designated as *ATP7B* (Bull et al. 1993; Petrukhin et al. 1993; Tanzi et al. 1993). Subsequently identification of disease-causing mutations led to substantial progress in the early diagnosis and successful treatment of the disease. To date, more than 300 disease causing mutations have been identified in WD patients from different populations (www.medgen.ualberta.ca). One of the most common mutations, His1069Gln, accounts for 30–60% of the WD chromosomes in Caucasian patients (Czlonkowska et al. 1997; Maier-Dobersberger et al. 1997; Shah et al. 1997; Ha-Hao et al. 1998; Ivanova-Smolenskaya et al. 1999; Cauza et al. 2000; Tarnacka et al. 2000; Caca et al. 2001; Firneisz et al. 2002; Vrábelová et al. 2002). Up to present, no data are available from the population of Slovakia. Here we present results of molecular screening for the above mutation in a sample of Slovak patients with WD. For mutation detection, two different DNA-based methods were used: restriction fragment length polymorphism (RFLP) analysis using nested PCR with introduction of a Alw21I restriction site (nested ACRS/RFLP) (Maier-Dobersberger et al. 1997), and amplification refractory mutation system (ARMS) (Newton et al. 1989; Nichols et al. 1989). Furtheron to overcome some limitations and drawbacks inherent to both methods, we developed a new single tube and one step method for rapid and reliable detection of this mutation, based on bidirectional amplification of specific alleles (BI-PASA) (Liu et al. 1997).

Materials and Methods

Patients

27 patients with classical symptoms of WD and their 20 relatives (6 parents, 8 siblings and 6 their offspring) from 25 unrelated Slovak families were examined. Patients involved in this study included 12 females and 15 males with mean age of onset of disease 12 years. Diagnosis was based on the presence of Kayser–Fleischer rings, lower serum ceruloplasmin level (<0.22 g/l), low copper serum concentrations (<11 μmol/l) and accompanying neurological and/or liver symptoms. In addition, a total of 306 DNA samples from randomly choosen healthy controls were tested. Blood samples and data on diagnosis were provided by 3rd Department of Internal Medicine, Dérer's Faculty Hospital Bratislava, and Departments of Clinical Genetics in Slovakia. This research was approved by local Ethic Committee and all participants gave informed written consent.

DNA samples

High-molecular-weight DNA was extracted from EDTA anticoagulated whole peripheral blood by phenol-chloroform extraction method according to standard protocols.

Detection of His1069Gln mutation

In initial phase, the modified nested PCR-based ACRS technique (Maier-Dobersberger et al. 1997), and ARMS (Newton et al. 1989; Nichols et al. 1989) were used to detect the His1069Gln mutation. Previously published primers (Petrukhin et al. 1993; Maier-Dobersberger et al. 1997) were modified and designed with the assistance of the Primer3 express software program (www-genome.wi.mit.edu/cgibin/primer/primer3_www.cgi) and produced by Merck (VBC Genomics). The modified primer sequences are shown in Table 1.

The nested PCR-based ACRS technique used was similar to that described by Maier-Dobersberger et al. (1997), however, modified in a few essential points. After amplification of exon 14 of *ATP7B* by using the intronic primers P1 and P2, a second PCR was performed using the first PCR product (340 base pairs) as template. In the second PCR intronic primer P1 was used paired with mismatch primer P5 to create an Alw21I specific restriction site in wild allele, but not in mutated one. Primers P1 and P5 produce 136 base pairs long fragment. This difference was used to detect the mutation by digesting the product by restriction enzyme Alw21I. Patients homozygous for the His1069Gln mutation show only single 136 base pairs long fragment, heterozygous carriers two fragments, 136 and 121 base pairs long, respectively, and patients with no His1069Gln mutation a single 121 base pairs long fragment. PCR was performed in a total volume of 25 μl containing 200 ng genomic DNA (first PCR) and 100× diluted first PCR product (second PCR); 1.5 mmol·l^{-1} $MgCl_2$; 200 μmol·l^{-1} dNTPs; 0.12 μmol·l^{-1} of each primer; 0.5 U Taq DNA polymerase (Invitrogen). Twenty eight and twenty five amplification cycles were performed in the first and the second PCR, respectively. Each cycle consisted of

Table 1. Oligonucleotide sequences used in ACRS, ARMS and BI-PASA test. Mismatch in P5 primer and the nucleotides in P3 primer specific for the mutant allele and in P4 and P6 primers specific for the normal allele are marked by bold and underlined

Methods		Primers	Sequence
PCR of exon 14	1st PCR	P1	5' GGG CAG CTA GGA GAG AAG GAC 3'
		P2	5' AGT TCT GCC TCA GGA GTG TGA CT 3'
ACRS	2nd PCR	P1	5' GGG CAG CTA GGA GAG AAG GAC 3'
		P5	5' CGG AGG CCA GCA GTG A**G**C 3'
ARMS	PCR with specific mutant allele	P1	5' GGG CAG CTA GGA GAG AAG GAC 3'
		P2	5' AGT TCT GCC TCA GGA GTG TGA CT 3'
		P3	5' GGA GGC CAG CAG TGA ACA **A** 3'
ARMS	PCR with allele specific primers for normal allele	P1	5' GGG CAG CTA GGA GAG AAG GAC 3'
		P2	5' AGT TCT GCC TCA GGA GTG TGA CT 3'
		P6	5' GGA GGC CAG CAG TGA ACA **C** 3'
BI-PASA	PCR with allele specific primers for normal and mutant allele	P1	5' GGG CAG CTA GGA GAG AAG GAC 3'
		P2	5' AGT TCT GCC TCA GGA GTG TGA CT 3'
		P3	5' GGA GGC CAG CAG TGA ACA **A** 3'
		P4	5' GAC TGC CAC GCC CAA GGG **G** 3'

denaturation at 94°C/1 min; annealing at 54°C/30 s; extension at 72°C/25 s for thirty cycles. Nested PCR product was digested with restriction enzyme Alw21I for 6 h at 37°C, and the fragments electrophoresed through 9% not denaturing polyacrylamide gel (29 : 1).

Subsequently an ARMS test was designed as a simpler diagnostic assay. ARMS test consisted of two complementary reactions – one containg a primer specific for the mutant allele and the other one containg primer for the wild-type allele (both have a common second primer). The first reaction was performed using primers P1, P2 and P3. The second one was performed using primers P1, P2 and P6. Primers P1 and P2 represent an internal control, which amplifies the entire exon 14, and produce 340 base pairs long fragment. Primers P3 and P6 are allele specific for the mutant sequence and for the wild one, respectively. Primers P1 and P3, P1 and P6 produce a 135 bp long fragment, respectively. Genotyping was based on the presence of amplification product in one (patients with two copies or no copy of His1069Gln mutation) or in both reactions (compound heterozygous or carriers of the mentioned mutation). ARMS test was performed in a total volume of 25 μl containing 200 ng genomic DNA; 1.2 mmol·l^{-1} $MgCl_2$; 50 μmol·l^{-1} dNTPs; 0.12 μmol·l^{-1} of primers P1 and P2, and 0.06 μmol·l^{-1} of P3 (mutation allele-specific amplification) or P6 (normal allele-specific amplification); 0.75 U Taq DNA polymerase. Conditions for PCR were: 94°C/1 min; 54°C/30 s; 72°C/25 s for thirty cycles.

Results

A total of 27 WD patients and their 20 relatives (6 parents of patients, homozygous for His1069Gln, 8 siblings and 6 offspring) were tested. In 18 of 25 unrelated patients (72%), the His1069Gln was present in at least one copy (proportion 56% in all WD mutations). In ten patients (40%), the above mutation was detected in homozygous and in eight ones (32%) in heterozygous state. In seven patients (28%), this mutation was not detected. Our results are similar to those reported for Central, Eastern and Northern-European populations (Thomas et al. 1995; Czlonkowska et al. 1997; Maier-Dobersberger et al. 1997; Ivanova-Smolenskaya et al. 1999; Caca et al. 2001). In five of fourteen asymptomatic siblings of index patients and their offspring, the mutation was present in one copy and in nine the above mutation was not identified. In a control group of 306 DNA samples from randomly chosen controls (612 alleles), the His1069Gln mutation was observed in 3 samples (carrier frequency 1%; allele frequency 0.49%). These results are strikingly similar to those described in other populations (Scheinberg and Sternlieb 1984; Brewer et al.1992; Hoogenraad 1996; Schilsky 1996; Gollan and Gollan 1998; Ferenci 1999; Firneisz et al. 2002).

Although both methods used were reproducible and precise, in every-day use there are some drawbacks and limitations inherent to them. The most serious limitation lies in using restriction digestion in nested-PCR-based ACRS technique. Star activity or not-complete digestion (due to some contamination) may lead to false-positive or false-negative results. Further on, both methods require performing two PCR reactions, and in addition nested-PCR is prone to increased risk of contamination by PCR products. Therefore a faster and simpler PCR based on BI-PASA (Liu et al. 1997) was developed. BI-PASA PCR is a method by which two allele-specific amplifications oc-

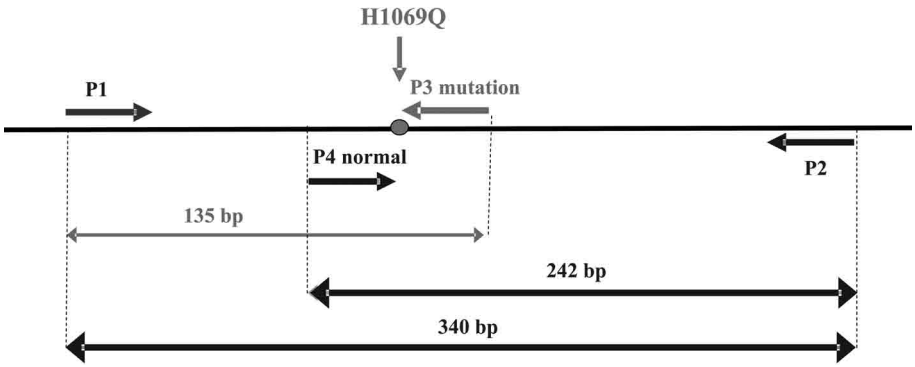

Figure 1. Scheme of BI-PASA test. BI-PASA PCR is performed with four primers. Two outer primers P1 and P2 produce 340 base pairs fragments. P1-P2 fragment is always produced and serves as a positive control. P3 and P4 primers are each allele specific, one for the mutant allele (P3) and the other for the normal allele (P4). Depending on the zygosity, BI-PASA produces two or three overlapping fragments. P1-P3 (135 base pairs) and P2-P4 (242 base pairs) are both present in a heterozygous samples, but only P2-P4 fragment is produced in homozygous normal and only P1-P3 is produced in homozygous mutant samples.

cur simultaneously in one PCR reaction. In BI-PASA test, one of the allele is amplified by allele specific reaction in one direction while the second allele is amplified by allele specific reaction in the opposite directions. Two outer (P1 and P2) and two inner allele-specific (P3 and P4) primers are required, which determine three fragments of different length: primers P1-P2 a 340 bp control fragment (present in all samples), primers P1-P3 a 135 bp fragment (in the presence of the mutant allele), and primers P2-P4 a 242 bp fragment (in the presence of wild allele). An overview of the test principle is given in Figure 1. After optimization of reaction condition and designing the sequences of the primers, the optimal PCR protocol was introduced. BI-PASA amplification was performed in a total volume of 25 μl containing 200 ng genomic DNA; 1.5 mmol·l^{-1} $MgCl_2$; 200 nmol·l^{-1} dNTPs; 5% DMSO, 0.06 μmol·l^{-1} of primers P1 and P2; 0.12 μmol·l^{-1} of primers P3 and P4; and 0.75 U of Taq DNA polymerase. Conditions for PCR were 94°C/1 min; 54°C/30 s; 72°C/25 s for thirty cycles.

Amplifications were performed on a GeneAmp PCR System 9700 or GeneAmp PCR System 2700 (Applied Biosystems).

Discussion

Direct gene analysis is being increasingly used as a diagnostic strategy for many diseases. New methods are needed, however, because all current techniques for gene analysis are time consuming, expensive, or inefficient. The His1069Gln point mutation found in 72% of Slovak patients with WD seems high enough (56% of all WD mutations) to merit screening by a single mutation test. The occurrence rate in our patients is higher as reported in other populations (Czlonkowska et al. 1997; Maier-Dobersberger et al. 1997; Shah et al. 1997; Ha-Hao et al. 1998; Ivanova-Smolenskaya et al. 1999; Cauza et al. 2000; Tarnacka et al. 2000; Caca et al. 2001; Firneisz et al. 2002; Vrábelová et al. 2002). The highest rates of occurrence of His1069Gln mutation were reported in WD patients from Poland (Czlonkowska et al. 1997) (73%) and Eastern Germany (Ferenci et al. 2001) (63%). High proportion of this mutation in our WD patients may be due to the small number of patients tested in our sample.

In initial phase of the present work, the His1069Gln mutation was tested using two earlier described DNA based techniques: a nested PCR-based ACRS technique (Maier-Dobersberger et al. 1997), and an ARMS test (Newton et al. 1989; Nichols et al. 1989). However, both methods described in this paper are equally specific and powerful (comparing the results showed 100% concordance), ACRS requires two rounds of PCR reactions (nested PCR), restriction digestion of PCR product (star activity or incomplete digestion may interfere with the results) and PAGE electrophoresis. On the other hand, in an ARMS test two PCR reactions need to be set up using the same template DNA. Therefore we have developed more rapid, simpler, highly sensitive and specific test for screening the above mutation based on a BI-PASA, which identifies this mutation both in homozygous and heterozygous samples in one PCR reaction. Since its first description (Liu et al. 1997), BI-PASA test has been used for the detection of point mutations, because method is easy to perform and does not require specific PCR materials and equipment. This method allows genotyping without a need for nested PCR and restriction enzyme digestion of PCR products . In addition, BI-PASA overcomes the possible

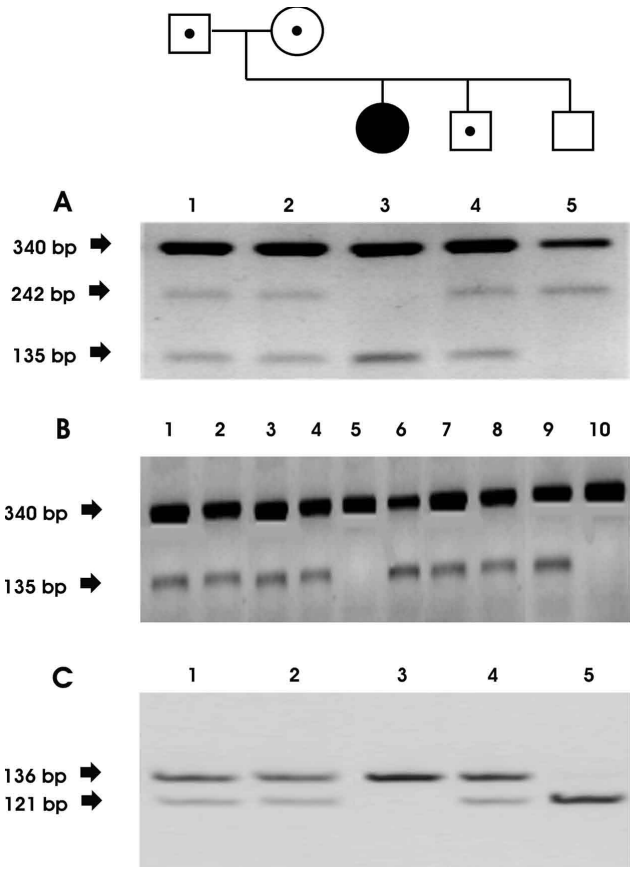

Figure 2. Detection of His1069Gln mutation by BI-PASA (A), ARMS (B) and nested PCR-based ACRS tests (C). **A.** In lanes 1, 2 and 4 are heterozygous carriers, in lane 3 is homozygous for the above mutation, and in lane 5 is healthy subject. **B.** Lanes 1, 3, 5, 7 and 9 represent results of ARMS PCR performed with primer for the normal specific allele; lanes 2, 4, 6, 8 and 10 with primer for the mutant specific allele. In lanes 1, 2; 3, 4; and 7, 8; are heterozygous carriers; in lanes 5, 6 is homozygous for the mentioned mutation; in lanes 9, 10 is healthy subject. **C.** His1069Gln homozygous patients show uncut single fragment (136 base pairs); the fragments from heterozygous carriers or compound heterozygous patients are partially digested and show two bands (136 base pairs and 121 base pairs); the fragments from healthy subjects or patients with other mutation show a single band (121 base pairs). In lanes 1, 2 and 4 are heterozygous carriers; in lane 3 is affected homozygous; in lane 5 is unaffected subject.

contamination of PCRs by the products resulting from the first round of amplification. After designing the primers and optimization of reaction and cycling conditions for BI-PASA test, a total of 27 WD patients and 120 random DNA samples previously screened for the above mutation by nested PCR-based an ACRS and an ARMS assay were tested. The results observed by this technique were completely identical with those found by the above two methods. Figure 2 demonstrates the results of testing His1069Gln mutation in patients and his family members using all three mentioned methods. However, all three methods used in this study give fully comparable results, BI-PASA test seems to be simpler and more rapid, yielding no false-positive or false-negative results.

The results presented here represent a starting point of direct DNA diagnosis in the majority of WD patients in Slovakia. DNA analysis performed with either one of the method described in this work is able to eliminate the difficulties existing in diagnosis of WD in case of asymptomatic stage of this disease as well as in cases of carriers, either healthy or compound heterozygous. In addition, molecular analysis supplies effective prenatal and postnatal screening possibility for WD that is critical in case of this disorder, while it is a treatable genetic defect. In conclusion, the high occurrence rate of His1069Gln mutation in Slovak patients resembles the rate of occurrence of this mutation in other Central and Eastern European countries (Czlonkowska et al. 1997; Maier-Dobersberger et al. 1997; Ha-Hao et al. 1998; Ivanova-Smolenskaya et al. 1999; Cauza et al. 2000; Tarnaczka et al. 2000; Caca et al. 2001; Firneisz et al. 2002; Vrábelová et al. 2002). Presented results could support the hypothesis that the original mutation event occurred long years ago somewhere in Eastern Europe and that the present day frequencies of this mutation observed in the area of Eastern and Central Europe are the consequences of the founder effect (Cauza et al. 2000; Firneisz et al. 2002).

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