

P.Gly61Glu and P.Arg368His Mutations in CYP1B1 that Cause Congenital Glaucoma may be Relatively Frequent in Certain Regions of Gilan Province, Iran

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Abstract

Purpose: To perform a population-based screening of four *CYP1B1* mutations—p.Gly61Glu, p.Arg368His, p.Arg390His, and p.Arg469Trp—in the province of Gilan, Iran. Previous studies have shown that *CYP1B1* is a cause of disease in approximately 70% of Iranian patients with primary congenital glaucoma (PCG), and that these four mutations constitute the majority of *CYP1B1* mutated alleles. The carrier frequencies may even justify premarital screenings.

Methods: DNA was extracted from the blood samples of 700 individuals recruited in a population-based epidemiology study in Gilan. Screenings were performed using polymerase chain reaction protocols based on restriction fragment length polymorphism or the amplification-refractory mutation system. For confirmation, the DNA of individuals with mutations was sequenced using the Sanger protocol.

Results: Five individuals carried the p.Gly61Glu mutation, and seven carried the p.Arg368His mutation. The p.Arg390His and p.Arg469Trp mutations were not observed in any of the 700 individuals screened. The mutations were not geographically randomly distributed in Gilan; four of the p.Gly61Glu-harboring individuals were from Talesh, and six of the p.Arg368His-harboring individuals were from the eastern regions of Gilan.

Conclusion: The frequency of individuals who carry either p.Gly61Glu or p.Arg368His is relatively high in Gilan, and notably high in certain localities within Gilan. We suggest further screenings be performed to definitively assess the need for implementing measures to encourage screening for p.Gly61Glu and p.Arg368His before marriage in Talesh and the eastern regions of Gilan, respectively. Finally, our assessment showed that regional frequencies of *CYP1B1* mutations do not necessarily mirror national frequencies.

Keywords: CYP1B1; p.Gly61Glu; p.Arg368His; p.Arg390His; p.Arg469Trp; Gilan; Iran

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INTRODUCTION

Glaucoma comprises a heterogeneous group of optic neuropathies whose defining features are a

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specific pattern of visual field loss and a characteristic glaucomatous appearance of the optic nerve head that reflects degeneration of this nerve.^[1,2] Increased intraocular pressure (IOP) due to the deterred outflow of the aqueous fluid through the trabecular meshwork at the iridocorneal angle in the anterior chamber of the eye is a strong risk factor for developing glaucoma. It is the major cause of irreversible blindness worldwide.^[3] It is sub-classified on the basis of etiology as primary or secondary. In the secondary forms, the glaucoma phenotype presents along with other manifestations of a syndrome. The primary forms are classified on the basis of the anatomy of the anterior chamber drainage angle and age of onset as primary congenital glaucoma (PCG; OMIM 231300), primary open-angle glaucoma, and primary angle-closure glaucoma. PCG, the subject of this report, is the most severe form of glaucoma.^[4] It is characterized by an anatomical defect (trabeculodysgenesis) in the trabecular meshwork and an age of onset in the neonatal period or before the age of 3 years.^[5] PCG manifests with increased IOP, corneal edema, excessive tearing, photophobia, enlargement of the globe (buphthalmos), corneal opacity, and optic nerve damage. It occurs in both sporadic and familial patterns. In familial cases, inheritance is usually autosomal recessive, and sometimes associated with incomplete penetrance or variable expressivity.^[5-8] While less common than the adult-onset forms, PCG is an important cause of childhood blindness.^[9,10] The incidence of PCG is geographically and ethnically variable, estimated at 1:10,000 in Western countries and higher in inbred populations, such as in Saudi Arabia (1:2500).^[6,11]

Genetic analyses of recessive PCG-affected families have identified four associated loci, GLC3A, GLC3B, GLC3C, and GLC3D.^[12-16] The causative genes in two of the loci have been identified, namely, *CYP1B1* in GLC3A and *LTBP2* in GLC3D that encode cytochrome P450 family 1 subfamily B polypeptide 1 and latent transforming growth factor- β binding protein 2, respectively. *CYP1B1* is a member of a family of enzymes that catalyze oxidative reactions, and *LTBP2* is an extracellular matrix protein. Recently, mutations in *TEK* that encodes tunica interna endothelial cell kinase have been reported in patients with both sporadic and autosomal-dominant familial PCG.^[8] The gene may affect vasculogenesis functions. Mutations in *CYP1B1* are by far the most common known cause of PCG. Nevertheless, the proportion of patients with PCG whose disease is attributable to *CYP1B1* is different among populations. The numbers range from 20% in Japanese, 50% in Brazilian, and 90% in Saudi Arabian populations, to 100% in Slovakian Roma populations.^[1,6,17,18] More than 130 putative PCG-causing mutations distributed in the coding regions of *CYP1B1* have been documented.^[19] The degree of heterogeneity of *CYP1B1* mutations in different populations is quite variable.^[20]

The most thorough genetic analysis of *CYP1B1* mutation screening in Iranian patients with PCG was reported in 2007.^[21] An important finding of that study was that PCG incidence was not evenly distributed in Iran, and that incidence was relatively high in the west and northwest of Iran. Among the 104 patients screened, 63 (60.6%) had familial disease. Notably, *CYP1B1* mutations were found in approximately 70% of the patients, suggesting that this gene significantly contributes to the PCG burden in Iran. *CYP1B1* mutations were observed in approximately 75% of the familial and 61% of the sporadic cases. Although 19 different disease-associated mutations were identified, four of these constituted 77.3% of the mutated alleles and were observed in 70 (67.3%) of the patients screened. Among patients with *CYP1B1* mutations, 93% harbored at least one of these mutations. The four common mutations caused p.Gly61Glu, p.Arg368His, p.Arg390His, and p.Arg469Trp.

The findings summarized here and expanded upon in an earlier publication suggest that the frequency of PCG-unaffected individuals in Iran who harbor one allele of the four aforementioned *CYP1B1* mutations may be considerable, particularly in regions with a high prevalence of the disease. A correspondingly high frequency of marriages would be expected to occur between carriers of these mutations, thereby predisposing the couples to have PCG-affected offspring. Here, we report the results of a population-based screening of the four common *CYP1B1* mutations in the province of Gilan. Gilan, which has a population of approximately two million and is located in the northwestern region of Iran, was the origin of a relatively large fraction of patients with PCG recruited in the earlier study.

METHODS

This research was performed in accordance with the Declaration of Helsinki and was approved by the ethics board of the University of Tehran and the Ophthalmic Research Center of Shahid Beheshti University of Medical Sciences.

Subjects

Seven hundred individuals (358 male and 342 female) were included in the present study. They constituted approximately one-fourth of all individuals (2587) and all non-consanguineous individuals recruited in a cross-sectional population-based survey that included adults aged 50 years and over from clusters in urban and rural areas and that was performed to assess the frequency of avoidable blindness in the province of Gilan.^[22] As the study had been designed in the framework of a clinical epidemiologic survey, the option of obtaining blood samples was not always available.

The geographic distribution of the 700 individuals is shown in Figure 1.

CYP1B1 Mutation Screening

DNA was extracted from the peripheral blood leukocytes of each of the 700 individuals by using standard phenol-chloroform protocols. Briefly, the four target mutations were initially screened in the DNA of the individuals by using either restriction fragment length polymorphism (RFLP) or amplification-refractory mutation system (ARMS) protocols. For confirmation, the DNA of individuals in whom mutations were detected were sequenced using the Sanger dideoxynucleotide termination protocol. C.182G>A that causes p.Gly61Glu

is positioned in exon 2 of *CYP1B1*, and c.1103G>A that causes p.Arg368His, c.1169G>A that causes p.Arg390His, and c.1405C>T that causes p.Arg469Trp are positioned in exon 3. An 830-bp exon 2 fragment that includes c.182G>A and an 872-bp exon 3 fragment that includes the mutation sites of the remaining three mutations were amplified using polymerase chain reaction (PCR) with the primers shown in Table 1. The p.Gly61Glu and p.Arg390His mutations were screened using RFLP; the restriction enzymes TaqI and HhaI, respectively, were used for detecting these two mutations. The unmutated exon 2 amplicon contains two TaqI recognition sites. The c.182G>A mutation that causes p.Gly61Glu creates a novel recognition site, and digestion by TaqI results in an altered electrophoretic pattern of the digested products. The c.1169G>A mutation that causes p.Arg390His disrupts the single HhaI recognition site present in the wild-type exon 3 amplicon, also resulting in an altered electrophoretic pattern. C.1103G>A that causes p.Arg368His and c.1405C>T that causes p.Arg469Trp were screened using the ARMS. The common and allele-specific primers used for detecting these mutations are presented in Table 2. PCR amplification of the wild-type allele will proceed only in the presence of the primer specific for the unmutated sequence, and amplification of the mutated allele will proceed only in the presence of the primer specific for the mutated sequence. Primers for the amplification of a control irrelevant DNA fragment were included in all the ARMS reactions. In the reaction for detecting p.Arg368His and p.Arg469Trp, the control primers amplified, respectively, a 474-bp fragment from *BOX11* and a 310-bp fragment from *SMAD3*.

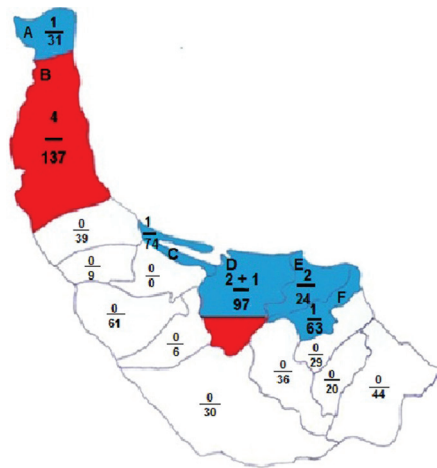


Figure 1. Prevalence of p.Gly61Glu and p.Arg368His mutations of *CYP1B1* in the districts of Gilan province. The various districts of Gilan are shown, and the districts with observed mutations are colored and labeled with letters: (A), astara; (B), talesh; (C), anzali; (D), rasht; (E), Astaneh Ashrafieh; and (F), Lahijan. P.Gly61Glu and p.Arg368His are observed in the districts colored red and blue, respectively. Both mutations are found in Rasht. Numerators indicate the number of individuals in whom a mutation was observed, and the denominators indicate the number of individuals screened in each district.

Statistical Analysis

To present the prevalence of the mutations, the 95% confidence interval (CI) was derived using the exact binomial method. The cluster effect was considered by incorporating the design effect into the calculations.

Region	Forward primer	Reverse primer
exon 2	5'-GGCCATTTGTCCAGAGAGTC-3'	5'-GAACTCTCCGTTGTGGCTGA-3'
exon 3	5'-AATTTAGTCACTGAGCTAGATAGCC-3'	5'-TATGGAGCACACCTCACCTGATG-3'

Position	c.1103G>A (p.Arg368His)	c.1405C>T (p.Arg469Trp)
Common primer (forward)	5'-AATTTAGTCACTGAGCTAGATAGCC-3'	5'-AATTTAGTCACTGAGCTAGATAGCC-3'
Wild-type allele-specific primer (reverse)	5'-GTCACCCATACAAGGCTGAC-3'	5'-AGTTCTTCGCCAATGCAACG-3'
Mutant-type allele-specific primer (reverse)	5'-GTCACCCATACAAGGCTGAT-3'	5'-AGTTCTTCGCCAATGCAACA-3'

ARMSL, amplification-refractory mutation system

All statistical analyses were performed using STATA version 14.0 (STATA Corp., Texas, USA).

RESULTS

On the basis of the electrophoretic patterns, five individuals with the p.Gly61Glu mutation and seven with the p.Arg368His mutation were identified [Figures 2a, b, and 3a]. Four carried the p.Gly61Glu

mutation in the heterozygous state and one was homozygous for the mutation. Consistent with the genetic data, eye examination data recorded on the homozygous individual during the course of the epidemiologic study included a diagnosis of unilateral glaucoma in the right eye, which had an IOP of 38 mmHg and had almost complete blindness. All seven individuals with the p.Arg368His mutation were heterozygous carriers. Sanger sequencing confirmed the electrophoresis results

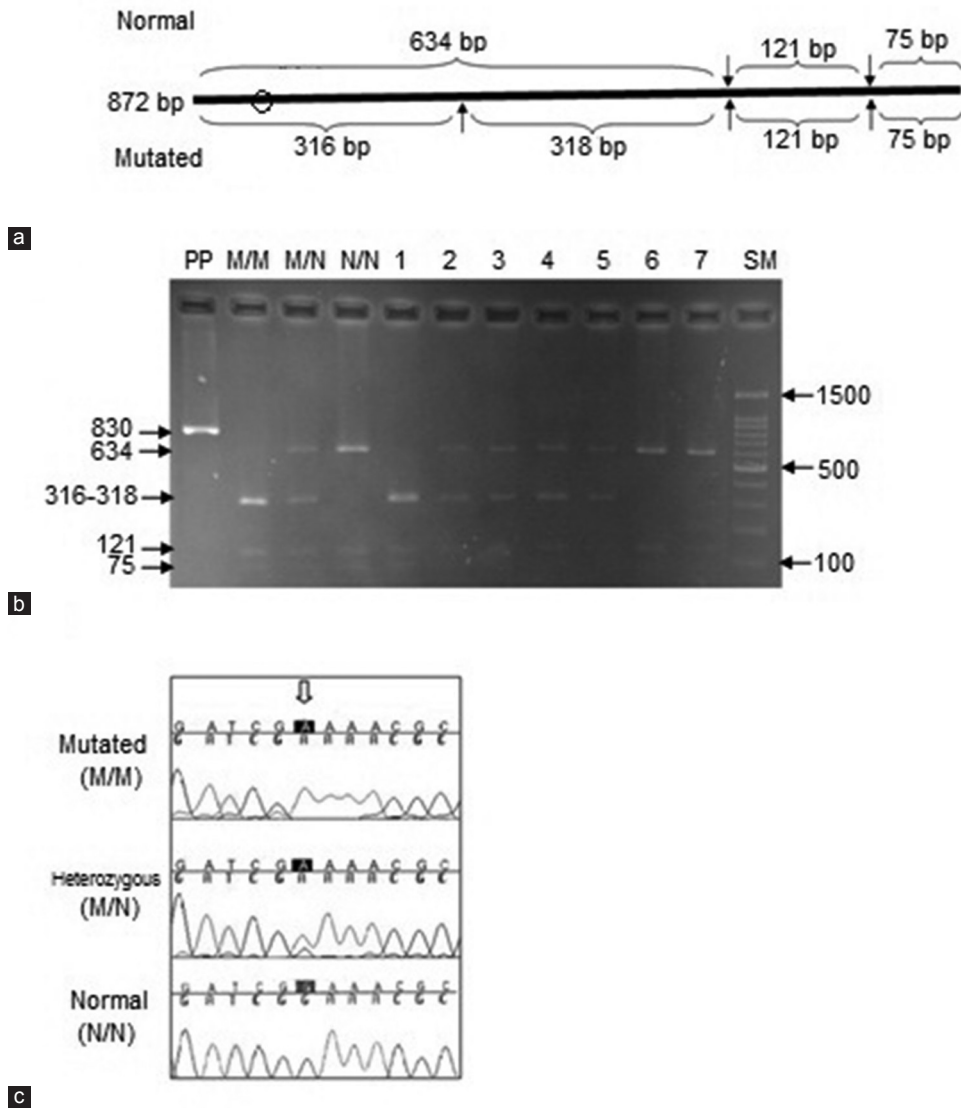


Figure 2. P.Gly61Glu mutation in *CYP1B1* caused by a mutation in exon 2 of the encoding gene. (a) Schematic diagram of the polymerase chain reaction amplicon that includes part of exon 2 of *CYP1B1*. TaqI recognition sites in the amplicon containing normal and mutated exon 2 sequences are shown by arrows above and below the amplicon, respectively. The size of DNA digestion products predicted for the two types of alleles are shown. The circle shows the position of the start of exon 2 within the polymerase chain reaction amplicon. (b) PCR products relevant to screening p.Gly61Glu. PP, polymerase chain reaction amplicon containing the exon 2 fragment without restriction enzyme treatment; M/M, digestion pattern of the amplicon of an individual homozygous for p.Gly61Glu; M/N, digestion pattern of the amplicon of an individual heterozygous for p.Gly61Glu; N/N, digestion pattern of the amplicon of an individual homozygous for the normal sequence; lanes 1–7: representative electrophoretic patterns of TaqI digestion products of individuals from Gilan screened for p.Gly61Glu: lane 1, M/M; lanes 2–5, M/N; lanes 6 and 7: N/N; SM, size markers. (c) DNA sequence chromatograms showing the c.182G>A mutation in *CYP1B1* that causes p.Gly61Glu. Top: homozygous mutated; middle: heterozygous genotype; bottom: homozygous normal. M, mutated allele; N, normal allele.

in all 12 individuals with mutations [Figures 2c and 3b]. Seven of the 12 individuals with mutations were male, and five were female. The p.Arg390His and p.Arg469Trp mutations were not observed in any of the 700 individuals screened [Figure 4]. These findings suggest that the frequency of individuals in Gilan who carry the p.Gly61Glu, p.Arg368His, p.Arg390His, and p.Arg469Trp mutations are 0.007 (95% CI: 0.002-0.017),

0.010 (95% CI: 0.004-0.020), 0 (95% CI: 0-0.004), and 0 (95% CI: 0-0.004), respectively. The frequency of individuals with any of the four mutations (12/700) was 0.017 (95% CI: 0.009-0.030). A closer look at the origin of the individuals with the mutations revealed that they were not randomly distributed in the province [Figure 1]. Four of the carriers of the p.Gly61Glu mutation were from Talesh district, and one was from another locality. As 137 individuals from Talesh were screened, this indicates a carrier frequency of 0.029 (95% CI: 0.008-0.073) in Talesh as compared to 0.002 (95% CI: 0.000-0.010) outside of Talesh. Six carriers of the p.Arg368His mutation were from the northeast of Gilan. As 268 individuals from this region were screened, this indicates a carrier frequency of 0.022 (95% CI: 0.008-0.048) as compared to 0.002 (95% CI: 0.000-0.013) outside the region. Some of these figures are also presented in Table 3.

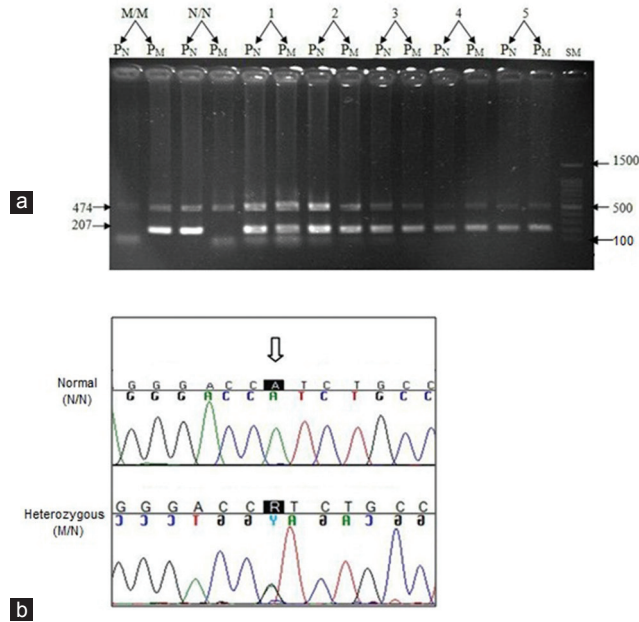


Figure 3. P.Arg368His mutation in *CYP1B1* caused by a mutation in exon 3 of the encoding gene. (a) Electrophoretic patterns of polymerase chain reaction products relevant to screening p.Arg368His by using the amplification-refractory mutation system. Two patterns are presented for each individual, one representing products obtained using the primer specific for the normal allele (P_N) and another representing products obtained using the primer specific for the mutated allele (P_M). The 474-bp fragment is the control fragment. The 207-bp fragment is from *CYP1B1*. M/M, electrophoretic pattern of polymerase chain reaction products of an individual homozygous for p.Arg368His; N/N, electrophoretic pattern of polymerase chain reaction products of an individual homozygous for the normal allele; samples 1-5: electrophoretic patterns of five individuals from Gilan screened for p.Arg368His, whose electrophoretic patterns are consistent with a heterozygous genotype for the mutation. (b) DNA sequence chromatograms showing the c.1103G>A mutation in *CYP1B1* that causes p.Arg368His. Top: homozygous normal; bottom: heterozygous genotype. M, mutated allele; N, normal allele.

DISCUSSION

Our findings suggest that the frequencies of carriers of two of the *CYP1B1* mutations screened, p.Gly61Glu and p.Arg368His, are indeed relatively high in the province of Gilan. At the 95% confidence level, at least 0.9% of the individuals carry either one or the other of these mutations. Notably, each of these mutations was clustered in specific localities within Gilan. This likely reflects mating patterns within the province. At the 95% confidence level, at least 0.8% of individuals from Talesh carry the p.Gly61Glu mutation. The figure may be as high as 7%. With respect to the p.Arg368His mutation, at the 95% confidence level, at least 0.8% of individuals from the eastern regions of Gilan carry this mutation, and the figure may be as high as 4.8%. These figures warrant the need for further screenings in larger cohorts to obtain more reliable estimates on carrier frequencies and to definitively assess the need for implementing measures to encourage screening of p.Gly61Glu and p.Arg368His before marriage in Talesh and the eastern regions of Gilan, respectively. In this regard, the PCR-based RFLP and ARMS protocols developed for screening in this study are relatively inexpensive and easy to implement in clinical laboratories. Interestingly, the premarital screening of the β -globin encoding gene, which started in Iran in 1997, resulted in a more than 85% reduction in thalassemia major (from approximately 1200 to 200) within 8 years of implementation. The incidence of this disease in Iran

Table 3. Frequency of p.Gly61Glu and p.Arg368His *CYP1B1* mutations in Gilan

	p.Gly61Glu		p.Arg368His	
	All of Gilan	Talesh region	All of Gilan	Eastern Gilan
Number of individuals screened	700	137	700	268
Frequency of individuals with mutations	5/700=0.007	4/137=0.029	7/700=0.010	6/268=0.022
95% CI of frequency of individuals with mutations	0.002-0.017	0.008-0.073	0.004-0.020	0.008-0.048

CI, confidence interval

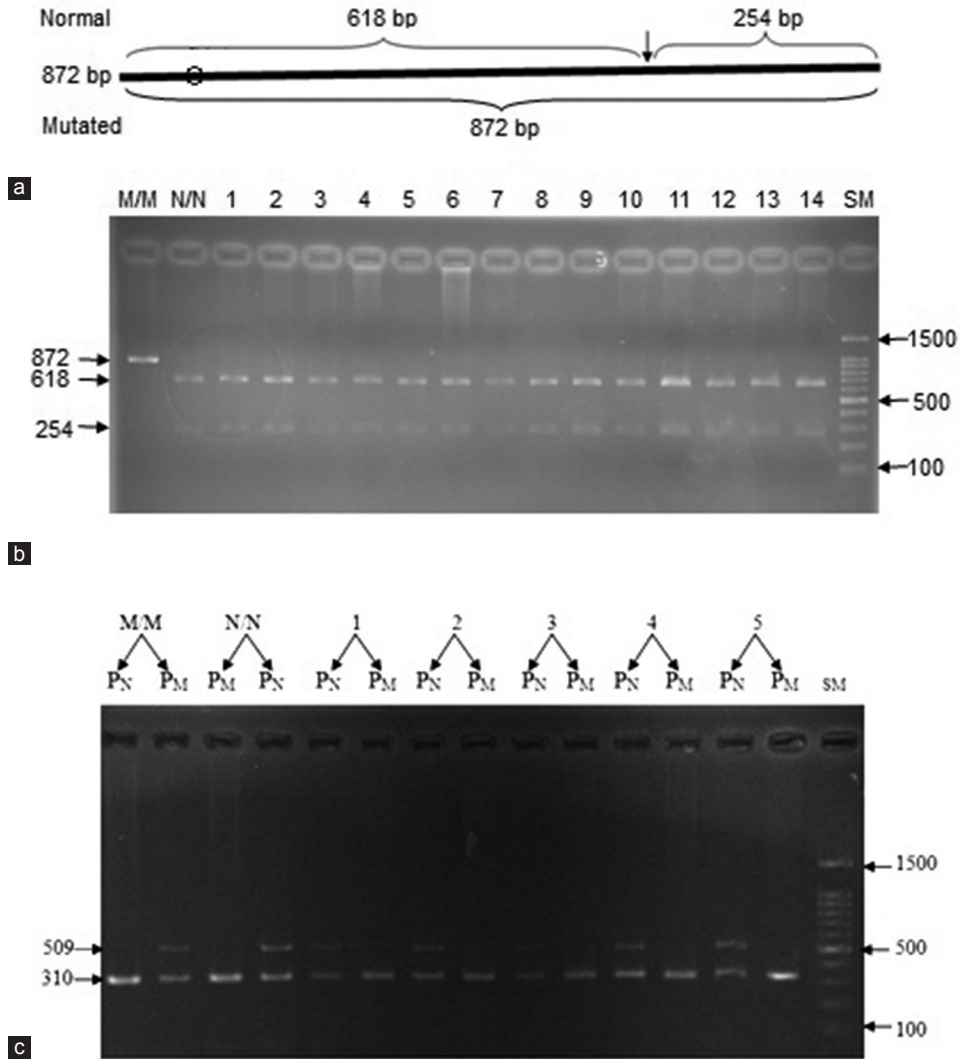


Figure 4. P.Arg390His and p.Arg469His mutations in *CYP1B1* caused by a mutation in exon 3 of the encoding gene. (a) Schematic diagram of the polymerase chain reaction amplicon that includes exon 3 of *CYP1B1*. HhaI recognition site that is present only in the amplicon containing the normal c.1169G>A (p.Arg390His) sequence is shown by an arrow above the amplicon. The size of DNA digestion products predicted for the two types of alleles are shown. The circle shows the position of the start of exon 3 within the PCR amplicon. (b) Polymerase chain reaction products relevant to screening p.Arg390His. M/M, digestion pattern of the amplicon of an individual homozygous for p.Arg390His; N/N, digestion pattern of the amplicon of an individual homozygous for the normal sequence; lanes 1–14: representative electrophoretic patterns of HhaI digestion products of individuals from Gilan screened for p.Arg390His; SM, size markers. The electrophoretic patterns of all 14 individuals indicate homozygous normal genotypes. (c) Electrophoretic patterns of polymerase chain reaction products relevant to screening p.Arg469Trp by using the amplification-refractory mutation system. Two patterns are presented for each individual, one representing products obtained using the primer specific for the normal allele (P_N) and another representing products obtained using the primer specific for the mutated allele (P_M). The 310-bp fragment is the control fragment. The 504-bp fragment is from *CYP1B1*. M/M, electrophoretic pattern of polymerase chain reaction products of an individual homozygous for p.Arg469Trp; N/N, electrophoretic pattern of polymerase chain reaction products of an individual homozygous for the normal allele; samples 1–5: electrophoretic patterns of five individuals from Gilan screened for p.Arg469Trp, whose electrophoretic patterns are consistent with a homozygous genotype for the normal allele.

further decreased to 150 by 2014.^[23,24] Comparable studies with respect to thalassemia and cystic fibrosis have been obtained in other populations.^[25,26] In the study on cystic fibrosis, which is more comparable to our study, the frequency of carriers of the mutation screened was 0.013 (95% CI: 0.007-0.016).

In terms of prevalence, the earlier screening on 104 Iranian patients with PCG recruited throughout the country showed that p.Gly61Glu was by far the most common *CYP1B1* mutation. It was found in 30 patients (28.8%), and it constituted 32.4% of the mutated *CYP1B1* alleles identified. P.Arg390His was

identified in 20 patients (19.2%) and constituted 23.0% of the mutated alleles. P.Arg368His and p.Arg469Trp each constituted about 10% of the mutated alleles. The relative frequencies of the four types of mutated alleles in Gilan do not exactly mirror their frequencies at the national level; p.Arg390His and p.Arg469Trp were not observed and p.Arg368His was more frequent in Gilan. This disparity is not unexpected as random mating is not a feature of marriages in Iran. This finding emphasizes that local CYP1B1 mutation screenings may also be appropriate in other provinces.^[27] Finally, one or two specific mutations were observed to constitute the vast majority of CYP1B1 mutations in various populations, including Slovakian Roma, Indonesian, Saudi Arabian, and Moroccan.^[21] In Saudi Arabia, where the prevalence of PCG is 1:2500 and where disease in approximately 90% of the patients is accounted for by CYP1B1 mutations, p.Gly61Glu constitutes approximately 75% of the CYP1B1 mutated alleles. Simple premarital screenings in these countries could reduce the worldwide burden of PCG and childhood blindness.

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Conflicts of Interest

There are no conflicts of interest.

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