ONLINE FIRST

Mutation Screening of the CYP1B1 Gene Reveals Novel and Recurrent Pathogenic Variants in Pakistani Primary Congenital Glaucoma Patients

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ABSTRACT

OBJECTIVE: To identify the pathogenic alleles in primary congenital glaucoma patients for early cure of the disease
METHODOLOGY: A cross-sectional descriptive study was carried out after approval from the ethical committee of SIOVS from December 2022 to December 2023 at Sindh Institute of Ophthalmology & Visual Sciences, Hyderabad. The consanguineous pedigree consisting of more than one affected was included, and the pedigree consists only one affected or secondary cause of vision loss was excluded. After getting informed consent, ten cc blood samples from all available participants in the pedigree were drawn, and DNA was extracted. The ARMS Assay and Sanger sequencing methods were adapted to analyze the CYP1B1 gen.
RESULTS: In the present study, one novel c.1187C>T, p.Pro396Leu and one reported c.1169G>A, p.Arg390His allele in CYP1B1 gene were found in two isolated pedigrees enrolled from Sindh Pakistan. ARMS Assay method and the Sanger sequencing method were adopted to detect pathogenic variants. Bioinformatics tools were used to analyze the pathogenesis of identified alleles and compare phenotype-genotype correlation.
CONCLUSION: The findings of novel and frequently reported mutations have a significant role in advancing genetic testing protocols, enabling more accurate targeting of diagnoses and identified alleles that may be added to existing repositories of the genetic database.

KEYWORDS: CYP1B1, Primary congenital glaucoma, consanguineous pedigree, PCG mutation, Sequencing/diagnostic testing

INTRODUCTION

Glaucoma is a heterogeneous disorder that is the second leading cause of blindness and globally affecting 65 million people (1). Primary congenital glaucoma (PCG) is a rare type of glaucoma usually inherited as an autosomal recessive disorder and affects infant's children. It is caused by increased intraocular pressure, leading to damaged optic nerve and vision loss. PCG is frequently found in certain ethnic and geographical groups, whereas consanguineous marriages are the most common (2).

The CYP1B1 (Cytochrome P450 family 1 subfamily B Member 1) gene was the first causative gene to develop PCG (3). The CYP1B1 gene encodes 543 amino acids and has 5218 nucleotide base pairs (4). The CYP1B1 gene provides instructions to play a role in metabolizing various substances in the body, including certain hormones and toxins. The gene expressed in an interior chamber of the eye, a mutation in the CYP1B1 gene, caused blockage of the drainage canal blockage to increase eye pressure and damage blood vessels and the optic nerve (5). The CYP1B1 gene has been found in many families with an autosomal inherited form of PCG. Other genes implicated in PCG include the LTBP2, MYOC, and FOXC1 genes. CYP1B1 gene is mapped on chromosome 16 and consists of three exon and intron regions; to date, more than 150 mutations, including point and frameshifts, have been reported worldwide with the phenotype of Primary congenital glaucoma. Individual Patients with genetic variation in CYP1B1gene showed variable disease presentation in the same or different ethnic groups. CYP1B1 gene protein is associated with the cytochrome p450 enzyme family and is involved in the metabolism of various substrates (6), including steroids and retinoids, and acts as a morphogen during development (7).

The prevalence of PCG varies across worldwide populations, but it is estimated to affect 1 in every 10,000 to 20,000 live births. It's worth noting that the prevalence of both PCG and POAG may vary depending on several factors, including age, ethnicity, family history, and environmental factors. Therefore, the prevalence may be higher or lower in specific populations or regions. The highest prevalence was 1 in 1250 in a subpopulation of Slovakia (8), and the lowest was 1 in 20000 in Western countries(9). Consanguinity, or the practice of marrying close relatives, has been linked to an increased risk of primary congenital glaucoma. In consanguineous marriages, there is a higher likelihood of inheriting autosomal recessive genetic mutations, including those associated with PCG. Consanguinity is often observed in populations where cultural or social practices encourage such unions.

The findings of novel and frequently reported mutations have a significant role in the advancement of genetic testing protocols, enabling more accurate and targeted diagnoses for PCG in Pakistani families. Additionally, the findings contribute to genetic counselling, empowering healthcare professionals to offer informed guidance and support to patients affected by the disease.

METHODOLOGY

Ascertainment and clinical evaluation of families

A cross-sectional descriptive study was carried out after approval from the Sindh Institute of Ophthalmology & Visual Sciences (SIOVS) Hyderabad's ethical committee from December 2022 to December 2023 at SIOVS. The consanguineous pedigree consisting of more than one affected was included, and the pedigree consists only one affected or secondary cause of vision loss was excluded. In the present study, eight consanguineous families were enrolled from the Eye Hospital of Sindh Institute of Ophthalmology and Visual Sciences; each family consists of more than one affected individual. Each subjected individual underwent a clinical examination held by a clinical ophthalmologist and relevant supporting staff; the main parameters associated with PCG were recorded, including corneal diameter, corneal capacity, visual acuity, IOP (intraocular pressure), CDR (cup to disc ratio) and trabeculectomy. After getting informed consent, ten ccs of Venous blood sample was drawn in a 50ml falcon tube containing 200ul of 0.5M EDTA. DNA of all available participants in the pedigree was extracted through an optimized organic method (10), and 25ng dilution was prepared for PCR amplification.

ARMS Assay PCR

The ARMS Assay method was adopted to exclude the most frequently reported mutation associated with the phenotype of PCG in the Pakistani population. The CYP1B1 gene was retrieved from the NCBI database (Reference Sequence Gene ID=NG_000104.4. Primer1 software tool was used to design Tetra ARMS Assay primers (http:// primer1.soton.ac.uk/primer1.html) (11). The synthesized Primers were critically checked for GC content, melting temperature, heterodimers, homodimers and hairpin loops by the freely available tool Oligocalc software (http://biotools.nubic.northwestern.edu/OligoCalc.html) (12) and oligoAnalyzer software (https://www.idtdna.com/pages/tools/oligoanalyzer) (11). To analyze the specific amplification of the desired template and band size on agarose gel, In-silico PCR of the UCSC genome browser (https://genome.ucsc.edu/cgi-bin/hgPcr) tool was used.

Mutation Screening and Bioinformatics Analysis

The sequencing primers of the CYP1B1 gene were designed using the freely available Primer3 tool software and optimized on different annealing temperatures. The forward and reverse boundaries of the CYP1B1 gene were amplified on selected families as previously reported methods (13). Sanger sequencing data were retrieved and analyzed by using Chromas v 2.5 software. Pathogenicity of identified variants was assisted by using different bioinformatics tools to understand the potential impact on health, including Clustal omega W was used to observe amino acid conservation alignment in different homologues species, Phyre2 was used to generate CYP1B1 proteins 3D structures for differentiation between wild and mutant protein, and Ramachandran plots were generated to identify the quality of protein three-dimensional structure by observing torsion angle values.

RESULTS

In this study, eight consanguineous families manifesting the phenotype of primary congenital glaucoma have been enrolled; detailed clinical investigations and history of participants showed no other anomaly with bilateral glaucoma, and pedigree drawing indicates the autosomal mode of penetrance. CYP1B1 gene was sequenced on one family, and a novel homozygous allele c.1187C>T, p.Pro396Leu was found in the SIOVSPCG-06 pedigree (Figure 1C). The novel homozygous allele was segregated into subjects with homozygous and heterozygous status (Figure 1A). The mutation is associated with the onset of glaucoma in one affected individual from the same family tree, manifesting within four to six years of age, and individuals who underwent early surgical procedures preserved vision. On the other hand, those who did not undergo any surgical intervention experienced elevated IOP, severe corneal opacity, and poor visual acuity. The mutation p.Pro396Leu is associated with the development of glaucoma in the SIOVSPCG family, and early surgical intervention appears to have positive effects on managing the condition by maintaining lower IOP and preserving vision. The ClustalW indicates that the Proline at the 396 position is the conserved amino acid of the CYP1B1 gene throughout the ten homologous (Figure 1E). The pathogenicity of novel alleles was predicted using different bioinformatics web tools. The Mutation taster indicates that Pro396Leu change is disease-causing, Provean suggests Pro396Leu change is deleterious with a score of -5.3, Polyphen2 suggest this change is probably damaging with a score of 0.686 score, and the Sift indicates that the change is pathogenic. The DNA of affected and normal participants was subjected to amplification of ArgR390His mutation through ARMS Assay PCR and confirmed by the Sanger sequencing method (Figure 1D). The recurrent mutation c.1169G>A, p.Arg390His of the CYP1B1 gene has been segregated in one pedigree (Figure 1B). The p.Arg390His mutation was found in one family SIOVSPCG-4 in a homozygous state (Figure 1D). The mutation is linked with the onset of glaucoma in two affected individuals from the same pedigree, manifesting within four to five years of age. The individuals who underwent early surgery had low intraocular pressure (IOP) and preserved vision. On the other hand, those who did not undergo any surgical intervention experienced elevated IOP, severe corneal opacity, and poor visual acuity. The mutation p.R390H is associated with the development of glaucoma in the SIOVSPCG-04 family, and early surgical intervention appears to have positive effects on managing the condition by maintaining lower IOP and preserving vision.

Mutation Impact

The Leu396 mutated amino acid is located in a domain essential for binding and connecting other residues; the function is necessary for protein activity (Figure 1E). The mutation in the domain might affect the interaction between the residues and cause disturbances in conveying signals transferring from the binding domain to the activity domain. The wild-type and mutant amino acids vary in structure and size. The mutant residue is larger than the wild-type residue, and due to its larger size, the mutant residue may not fit appropriately in the protein structure; the Phyre2 protein tool was used to assist the wild and mutant protein structure of Pro396Leu (Figure 2A & 2B). The Ramachandran plot was used to analyze how amino acid replacement would affect protein structure by examining the angles of amino acids; it contrasts the stereochemistry and geometry of protein structures that are wild-type and mutant. Torsion angles for the wild-type residue are within the allowed range for Proline, and this position may be necessary for rigidity and stabilization of the protein structure. Mutation into a more flexible residue may destabilize the protein structure. The mutation has the potential to disrupt essential interactions between protein domains, affect signal transfer, and compromise the stability of the protein structure due to changes in size and

rigidity. The range of the mutant and wild-type proteins was not comparable. In favourable and allowed regions, the substitution of Pro396Leu reveals that wild-type protein included 87% and 13% of residues, whereas the mutant structure contained 92% and 8% of residues (Figure 2D).
The metaDome health map shows that the Proline at 396 positions is located in the TCP-1/cpn60 chaperonin family domain and in the neutral region. Metadome protein analysis indicated that Pro396 lies in the intolerant region (Figure 2E). In the recurrent mutation Arg390His, this specific change converts a larger positively charged residue to a smaller neutrally charged residue. The wild-type residue forms hydrogen bonding and a salt bridge between different residues at different codon positions. The phyre2 indicates the wild and mutant positions in the CYP1B1 gene (Figure 2A & 2B); this type of substitution potentially impacts the function and structure of the protein. Mutated residue located in a domain crucial for protein binding and functional activity, the mutation might influence the interaction between these domains and affect signal transduction between the residues. The angles of amino acids to contrast the stereochemistry and geometry of wild-type and mutant protein structures and torsion angles for the wild-type residue (Arg390) are within the allowed range for His390. This specific position of Arg390 in the wild-type protein may be crucial for the rigidity and stabilization of the protein structure (Figure 2A). Mutation into a more flexible residue His390 may lead to destabilization of the protein structure (Figure 2C), and mutation has the potential to disrupt essential interactions between protein domains. It may affect signal transfer and compromise the stability of the protein structure due to changes in size Comparison of Wild-Type and Mutant Structures. The Ramachandran plot indicates that variation of Arg390His shows differences in the distribution of residues in favourable and allowed regions (Figure 2D) in wild and mutant residues, and Metadome protein analysis indicated that Arg390 lies in the intolerant region of CYP1B1 gene (Figure 2E).

DISCUSSION

Primary congenital glaucoma (PCG) is a rare genetic disorder characterized by increased intraocular pressure (IOP) found in early infancy (14). PCG is considered one of the leading causes of blindness in children worldwide. To date, four genetic loci have been reported. Mutations in two genes, CYP1B1 gene, located on loci (GLC3A, 2p22-p21) and LTBP2 located in loci 14q24.2-24.3 have been identified with autosomal recessive in PCG patients(1) and, the genetic basis of PCG is complex, with multiple genes implicated in its pathogenesis (15). A previous study showed that mutations in the CYP1B1 gene caused 87% of reported consanguineous cases with the Phenotype of PCG.

On the other hand, 27% of individuals with sporadic cases affected by PCG are reported to have a mutation in the CYP1B1 gene, where there is no known family history of the condition. Sporadic cases may also involve mutations in the CYP1B1 gene or other genes associated with PCG. It's important to note that while CYP1B1 mutations are associated with a significant proportion of PCG cases, different genes have also been implicated in the disorder. More than 160 pathogenic variations have been reported in CYP1B1gene with the phenotype of PCG disease and distributed a significant amount of the hereditary load in familial and sporadic cases.

In this study, we identified two different allelic variants of the CYP1B1 gene in two isolated families. One novel allele, c.1187C>T, p.Pro396Leu, was found in the SIOVSPCG-06 family, and the allele has not been reported worldwide. Whereas frequently reported allele c.1169G>A, p.Arg390His was segregated in one family. These substitutions of the amino acid were found at the highly conserved domain of the gene and predicted to cause an impact on the normal function of the protein. Importantly, all of these variants were considered to be disease-causing due to their determined functional significance. The clinical investigation revealed that affected individuals with novel homozygous alleles and reported mutation showed varying disease severity between interfamilial and intrafamilial.

According to the worldwide report, research indicated that most pathogenic variations found with disease patterns of PCG were segregated in heterozygous status. A study from Pakistan revealed that 20% of blindness is attributed to pathogenic variation c.1169 G>A, p.Arg390His in the CYP1B1 gene (16). Furthermore, it was identified that patients with the phenotype of PCG exhibit various genetic variations in different populations. The higher association of the CYP1B1 gene is seen in Turkey, Saudi Arabia, India, Brazil, and Morocco. The incidence of PCG varies between 44% and 75% in different populations. The homozygous c.1169 G>A, p.Arg390His allele of the CYP1B1 gene is frequently reported in Pakistani patients exhibiting the phenotype of primary congenital glaucoma. Although Arg390His is noted as the most common pathogenic variation in neighbouring countries, followed by Saudi Arabia, Iran, China and India (3), in the Indian population, the allele Arg390His was also reported with disease phenotype of ectropion uvea by following the frequency 92.3% (12/13) (17).

CONCLUSION

The genetic pattern of PCG is still an active domain of research, and the present study aims to identify additional alleles of genes and co-factors contributing to the cause of this disease. Identifying pathogenic alleles in PCG patients associated with the CYP1B1 gene is used for early disease identification and intervention, which is crucial in managing primary congenital glaucoma and preventing vision impairment. The present study's findings provide valuable insights into the genetic underpinnings of Primary Congenital Glaucoma. The findings of novel and frequently reported mutations significantly advance gene testing protocols, enabling more accurate and targeted diagnoses for PCG in Pakistani families. Additionally, the study's contribution to genetic counselling empowers healthcare professionals to offer informed guidance and support to individuals and families affected by the disease. Overall, the study contributes to the broader understanding of PCG and lays the foundation for improved diagnostic and counselling approaches in the Pakistani population.

ACKNOWLEDGEMENTS

The authors thank all laboratory technicians and relevant staff of SIOVS for identifying patients and supporting laboratory experiments. They also thank the volunteers who participated in this research.

Conflict of Interest: Authors have declared no conflict of interest

Funding agency: Sindh Institute of Ophthalmology & Vision Sciences funded the laboratory experimental expenses from the head of Research and Survey with letter NO.SIOVS/FINANCE/634, Dated 17-10-2022.

AUTHOR CONTRIBUTIONS

Surhio WA: help in drafting and data analysis
Khidiri FF: Critical review
Haroon MI: Identification and clinical investigation
Mehmood S: data analysis
Waryah YM: Study design, collection of data and drafting,

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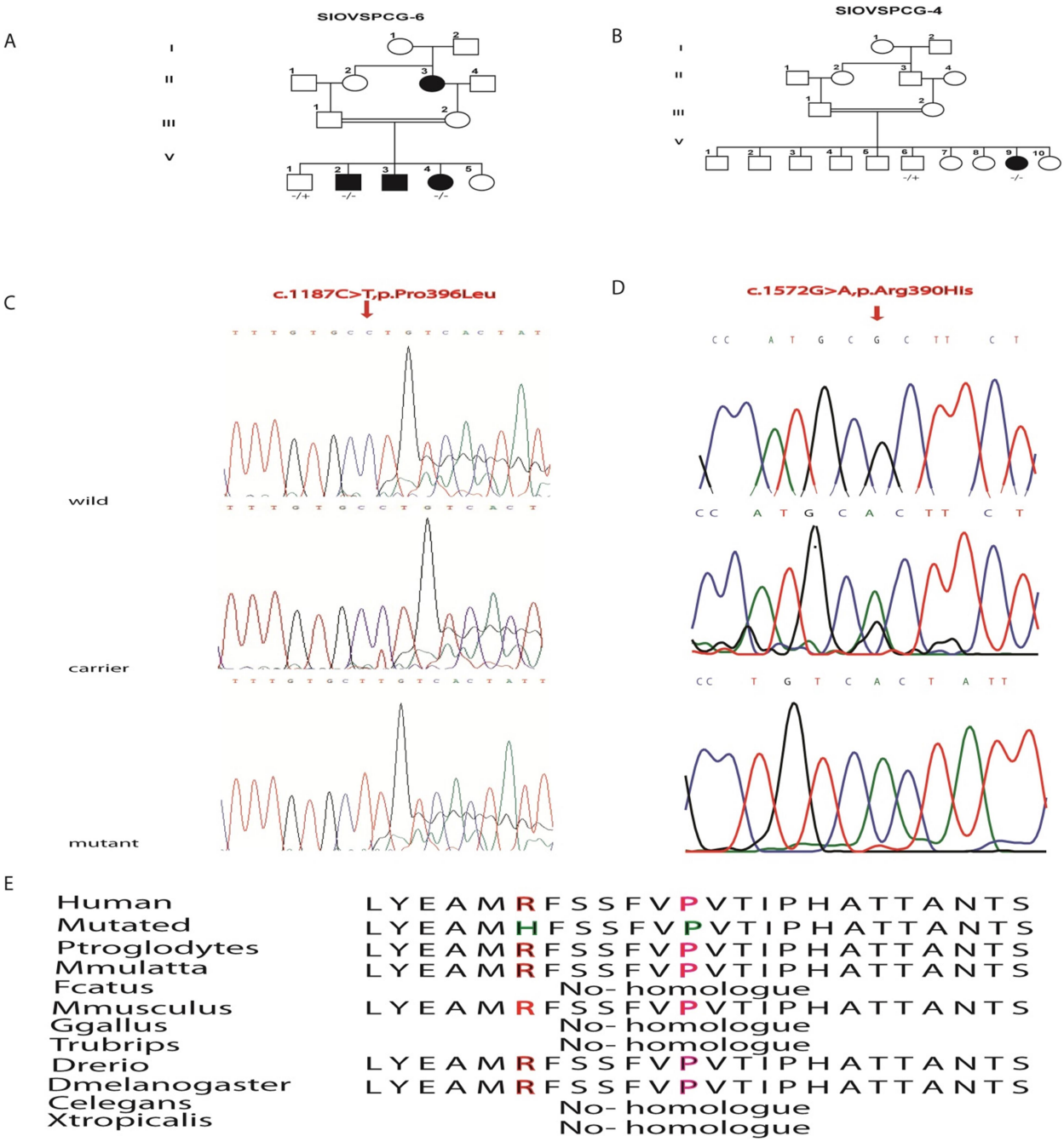

Figure I: (A) shows the pedigree of SIOVSPCG-06; (B) shows the pedigree of SIOVSPCG-04; (C) chromatogram results of c.1187C>T,p.Pro396Leu mutation; (D)chromatogram results of c.1572G>A,p.Arg390His mutation; (E) Clustal-W results of different homologue species.

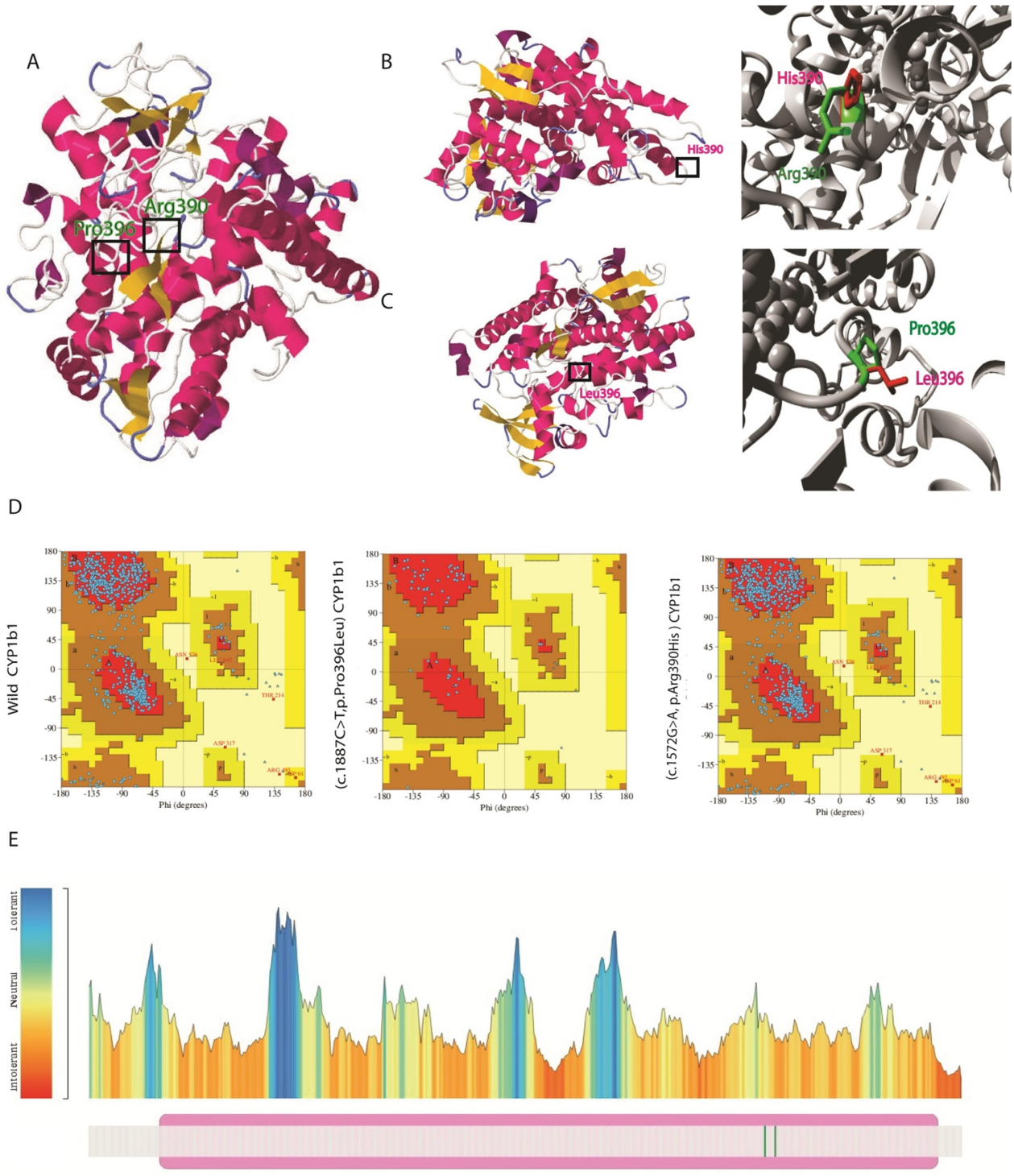

Figure II: (A) Phyre2 protein structure of wild CYP1b1 gene, (B) mutated protein structure of His390, (C) mutated protein structure of Pro396, (D) Ramachandran plot results for wild and mutated CYP1b1 gene, and (E) MetaDome health map shows that the Proline at 396 positions is located in the TCP-1/cpn60 chaperonin family domain and in the neutral region, and Arg390 lies in the intolerant region of the CYP1b1 gene.