Identification of the Single Nucleotide Polymorphisms and their Frequency Present in the Targeted Region of POR Gene in South Indian Population

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**Abstract**

**Background and objectives:** Cytochrome P450 oxidoreductase (POR) gene act as an electron donor to other microsomal cytochrome P450 (CYP) enzymes. This study is attempted to identify the POR genetic variants present in the proximal promoter region, hotspot coding regions that affect the function of POR gene, and the 3′ UTR region encompassing miRNA binding sites among the South Indian population.

**Materials and methods:** Blood samples were collected from 110 south Indian subjects. Genomic DNA was isolated from whole blood and the target regions of the POR gene were amplified using specific primers. The PCR amplicons were sequenced by the Sanger method. The variants present in proximal promoter region, exons (9, 10, 11, and 12) with boundary regions around 100 bp region and 3′ UTR region, were analyzed. The microRNA database (miRDB) prediction tool was used to analyze miRNA binding regions in the 3′ UTR region.

**Results:** The SNPs (rs72553971, rs41301394, rs41301400, rs4732514, rs2286822, rs2286823, rs41301427, and rs17685) were observed in the POR gene with frequency of 2.7, 31.36, 2.27, 57.27, 23.63, 23.63, 14.54, and 35%, respectively. All these SNPs were previously reported in other populations. No novel variation has been observed in the hotspot coding region of the POR gene among South Indian population.

**Conclusion:** The frequency of POR gene polymorphisms is reported for the first time among the South Indian population. This study provides fundamental and useful information on POR pharmacogenetics among this population, which can be utilized for future research.

**Keywords:** Minor allele frequency, Polymorphism, POR gene, Single nucleotide polymorphism, South Indian.

**SBV Journal of Basic, Clinical and Applied Health Science (2020): 10.5005/jp-journals-10082-02269**

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**Introduction**

The human NADPH cytochrome P450 oxidoreductase (POR) gene is present in the chromosome number 7q11.2, which is approximately 72 kb in size and consists of 15 protein-coding exons, with one untranslated exon.¹ The POR enzyme regulates multiple metabolic processing enzymes involved in the drug metabolism, steroidogenesis, and also xenobiotics.² ³ The POR gene is highly polymorphic and exhibits a number of variations among the individuals, which are highly responsible for the altered drug metabolic reactions that are observed in diseases like Antley-Bixler syndrome, polycystic ovarian syndrome, cytochrome P450 oxidoreductase deficiency (PORD), and congenital adrenal hyperplasia.⁴ ⁶ ⁷ The POR protein consists of 680 amino acids with four domains namely, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), hinge domain and nicotinamide adenine dinucleotide domain.⁸ ⁹ Flavin moieties act as a cofactor for the catalytic activity of the protein.¹⁰ ¹¹

The crucial function of the POR protein is to donate electrons through a series of reactions to many other CYP enzymes for their proper biological activity.¹² ¹³ The single nucleotide polymorphisms (SNPs) present in the gene may lead to structural and functional variation in the protein and also alters the drug metabolizing activity among the individuals.¹⁴ Each and every individual shows the differences in the drug response, which can be attributed by the variability in the DNA sequence of the POR gene both in exons as well as introns.¹⁵ Variations present in the intrinsic region have the potential to induce or reduce the splicing mechanism efficiency.¹⁶ The proximal promoter region of the POR gene is present immediate to the upstream of exon 1U, showing various effects of the altering hormone level in case of polymorphisms.¹⁷ ¹⁸ The 3′ UTR region plays an important role for binding the miRNA, to enhance the gene expression and translation process.¹⁹ Some of the SNPs in this region may inhibit the binding specificity of the miRNA.²⁰

The worldwide study reported that the majority of protein-altering variants and disease-causing variants fall in the hotspot coding regions. The risk variants Ala287Pro and Arg457His were
associated with Antley-Bixler syndrome (ABS) and the breast cancer risk was located within the region of exon 9–12 in other population.\textsuperscript{21,22} This study is attempted to assess the frequency of polymorphisms in selected regions (exon 9–12), promoter region, and 3′UTR region of the \textit{POR} gene among healthy individuals of South Indian population.

**Materials and Methods**

The study protocol was approved by the institutional human ethics committee and a written informed consent was obtained from the participants who satisfied the eligibility criteria.

**Inclusion and Exclusion Criteria**

The volunteers were of South Indian origin residing in Tamil Nadu, Kerala, Karnataka, Andhra Pradesh, and Puducherry for at least last three generations. The participants were selected with the result showing normal hematological and biochemical parameters like random blood sugar, hemoglobin, lipid profile, renal function, and routine urine test. Severe and complication illnesses like hypertension, diabetes mellitus, and asthma patients were excluded from the study. Pregnancy and lactating mothers were also omitted. A total of \(n = 110\) unrelated healthy volunteers aged between 18 and 65 years were randomly selected and 2 mL of peripheral venous blood was collected in an EDTA (ethylenediaminetetraacetic acid) anticoagulant tubes. Genomic DNA was isolated from whole blood using commercial kits (Qiagen, Germany) and stored at 4°C. Targeted regions in \textit{POR} gene were amplified through polymerase chain reaction using appropriate forward and reverse primers (Table 1) using the following reaction conditions (Table 2). Each 35 μL reaction consists of 3 μL of genomic DNA (50 ng/μL), 17.5 μL of 10x PCR Top Taq Master Mix, 1 μL of each primer (10 pmol/μL), and 12.5 μL of nuclease free water. Amplification was performed using Veriti 96 well Thermal Cycler (Applied Biosystem, USA). The PCR products were visualized in 1% agarose gel with ethidium bromide staining.

**Sanger Sequencing**

The sequencing of PCR products was performed by Macrogen Inc. (South Korea). The derived electropherogram (Fig. 1) was manually evaluated and confirmed by the sequence with the nucleotide BLAST. The NCBI genomic DNA NC\.000007.14: 75914700–75986855 FASTA sequence was used as a reference to analyze variants. The identified intronic variants are numbered according to the NCBI reference sequence, and exons were identified from the initiation codon ATG (A is considered as +1).\textsuperscript{23} Gene sequence ID 5447 were used to predict the miRNA targeted region in the 3′UTR region. The miRDB (http://mirdb.org) online bioinformatics tool was used to predict the miRNA binding site in the 3′UTR sequences.

Table 1: Primers used to amplify the targeted region of the \textit{POR} gene

| Amplified region | Forward primers 5′–3′ | Reverse primers 5′–3′ | Amplicon size | Ref. |
|------------------|------------------------|------------------------|---------------|-----|
| +36\_as–325\_s   | TCAGGC-CACACCACCT-GAGG | GCCCGAA-GGAGGAG-GCTAGA | 361 bp        | 17  |
| Exon 9–10        | GTAACCG-GTGAGATTTC-CTCAT | ACTATGACAGT-GACGGGGTAGG | 692 bp        | 25  |
| Exon 11–12       | AGGGAG-GCATCAGAGAG-GCATAG | GGCTGGACA-GAAGGGGAGGA | 781 bp        | 25  |
| 3′UTR            | GGGATGTGCA-GAACACCCTC | GTTC-CTGGGGGTCT-GAGTTA | 572 bp        | 34  |

Table 2: PCR conditions for amplifying the \textit{POR} gene

| PCR conditions | Proximal promoter region | Exon 9–10 | Exon 11–12 | 3′ UTR region |
|---------------|--------------------------|-----------|------------|--------------|
| Initial denaturation | 95°C/3 minutes | 95°C/3 minutes | 95°C/3 minutes | 95°C/3 minutes |
| Denaturation   | 95°C/1 minute           | 95°C/1 minute | 95°C/1 minute | 95°C/1 minute |
| Annealing     | 60°C/30 seconds         | 60°C/30 seconds | 59°C/30 seconds | 59°C/30 seconds |
| Extension     | 72°C/2 minutes          | 72°C/2 minutes | 72°C/2 minutes | 72°C/2 minutes |
| Final extension | 72°C/7 minutes         | 72°C/7 minutes | 72°C/7 minutes | 72°C/7 minutes |
| Infinity      | 4°C                     | 4°C        | 4°C        | 4°C          |

Figs 1A to C: Electropherogram shows that the SNP changes in the sequence of the \textit{POR} gene. The black arrow indicates the SNP change: (A) Wildtype alleles CC; (B) Heterozygous alleles CA; (C) Variant alleles
**Statistical Analysis**

The $\chi^2$ test for goodness of fit was used to determine the genotypic distribution of each SNP following Hardy-Weinberg equilibrium (HWE) calculated using Microsoft Excel. Linkage disequilibrium (LD) was calculated using the Haploview Software version 4.1. 24 distribution of each SNP following Hardy-Weinberg equilibrium

Allele frequency of POR (G > A) was found at the 3'UTR region of the POR gene with the frequency of 35%, in the 110 human whole blood samples (Table 3). There were three SNPs found in the ninth intronic region rs13223707 (C > G); rs13240147 (G > A); and rs41301394 (C > T) with the variant allele frequency showing 91.36, 90.45, and 31.36%. One SNP was found in the ninth intronic region rs562843415 (G > A) with the minor allele frequency was less than 1%, but their HWE value showed the significant $p$ value of 0.05. Four SNPs were found in the intronic region of the POR gene, rs41301400 (G > C), with the frequency of variant allele: 2.27; 57.27, 91.81, and 90.45% respectively, whereby three SNPs were consistent with the HWE, except rs4732516. Two SNPs were found in the intronic region rs17685 (G > A), with the minor allele frequency was less than 1%, but their HWE value showed the significant $p$ value of 0.05. According to this study, four SNPs were not in HWE (rs72553972, rs13223707, rs41301400, and rs4732516), showing $p$ value < 0.05. 

The linkage disequilibrium analysis was performed for all 15 SNPs obtained in the South Indian population across the POR gene-targeted regions (Fig. 2). The red color indicates the relatively strong linkage among the SNPs. The $r^2$ value is considered as a guideline for the linkage between a pair of single nucleotide variations and $D'$ indicates the probability for the previous recombinations. According to the $r^2$ value, the perfect linkage was found between the SNPs rs2286822 and rs2286823 ($r^2 = 1$; $D' = 1$). The SNPs between rs4732515 and rs4732516 relatively show strong association by the value $r^2 = 0.8$; $D' = 1$. The value $D' = 1$ indicates that the SNPs were strongly linked between both the pairs rs41301394 and rs2286822; rs41301394 and rs2286823, whereas the $r^2 = 0.1$ exhibited weak linkages.

In this study, we have found SNPs only in the intronic region, hence no SNPs were found in the exonic hotspot/protein-coding regions of the POR gene.

**RESULTS**

The proximal promoter region of the POR gene sequencing showed SNPs rs72553971 and rs72553972 at the position –173 (C > A) and –152 (C > A) with a frequency of 2.7 and 15.45%, respectively, in the South Indian population (Table 3). We have found 12 SNPs in the noncoding intronic region, which were located nearby the boundary of the hotspot exons and also only one variation, rs17685 (G > A), was found at the 3'UTR region of the POR gene with the frequency of 35%, in the 110 human whole blood samples (Table 3). There were three SNPs found in the ninth intronic region rs13223707 (C > G); rs13240147 (G > A); and rs41301394 (C > T) with the variant allele frequency showing 91.36, 90.45, and 31.36%. One SNP was found in the ninth intronic region rs562843415 (G > A) with the minor allele frequency was less than 1%, but their HWE value showed the significant $p$ value of 0.05. Four SNPs were found in the intronic region of the POR gene, rs41301400 (G > C), with the frequency of variant allele: 2.27; 57.27, 91.81, and 90.45% respectively, whereby three SNPs were consistent with the HWE, except rs4732516. Two SNPs were found in the intronic region rs2286822 (C > T) and rs2286823 (G > A). Minor allele frequency of 23.63% was observed for both of the variants, and significant differences in the $p$ value of both HWE were observed. Twelfth intronic region has two SNPs rs41301427 (G > A) and rs377357128 (G > A) with the variant allele frequency: 14.54 and 0.90%, and the $p$ value was significant to the equilibrium. From this study, four SNPs were not in HWE (rs72553972, rs13223707, rs13240147, and rs4732516), showing $p$ value < 0.05. According to the results, POR gene is predicted to be targeted by 14 miRNAs in miRDB, but no variations were found in the miRNA binding site region in South Indian population. The comparison of the worldwide population allele frequency of the POR gene is shown in Table 4.

| S. no. | Chromosomal position | rsID | POR gene region | Variant | Minor allele frequency (%) | Hardy-Weinberg equilibrium ($p$ value) |
|-------|----------------------|------|-----------------|---------|---------------------------|--------------------------------------|
| 1     | 7:75914929           | rs72553971 | –173 proximal promoter | C > A | 2.7 | 0.7687 |
| 2     | 7:75914950           | rs72553972 | –152 proximal promoter | C > A | 15.45 | < 0.05 |
| 3     | 7:75983452           | rs13223707 | 8-intron | C > G | 91.36 | < 0.05 |
| 4     | 7:75983465           | rs13240147 | 8-intron | G > A | 90.45 | < 0.05 |
| 5     | 7:75983485           | rs41301394 | 8-intron | C > T | 31.36 | 0.9366 |
| 6     | 7:75983877           | rs562843415 | 9-intron | G > A | 0.90 | 0.9233 |
| 7     | 7:75983957           | rs41301400 | 10-intron | G > C | 2.27 | 0.8073 |
| 8     | 7:75984680           | rs4732514 | 10-intron | C > T | 57.27 | 0.4548 |
| 9     | 7:75984711           | rs4732515 | 10-intron | T > C | 91.81 | 0.1087 |
| 10    | 7:75984764           | rs4732516 | 10-intron | C > G | 90.45 | < 0.05 |
| 11    | 7:75984970           | rs2286822 | 11-intron | C > T | 23.63 | 0.2570 |
| 12    | 7:75984978           | rs2286823 | 11-intron | G > A | 23.63 | 0.2570 |
| 13    | 7:75985239           | rs41301427 | 12-intron | G > A | 14.54 | 0.6058 |
| 14    | 7:75985331           | rs377357128 | 12-intron | G > A | 0.90 | 0.9618 |
| 15    | 7:75986787           | rs17685 | 3’UTR | G > A | 35 | 0.8422 |

**DISCUSSION**

In recent years, there has been a huge pharmacogenomics interest on POR genetic variants. The POR gene polymorphisms have acquired great attention among researchers, due to their involvement in differential response to cytochrome-mediated drug metabolic activity, steroidogenic metabolism, and they are also indirectly associated with protein expression activity. In this study, we screened the SNPs present in the transcription start site of around 361 bp of untranslated exon (–325/+36). POR –173C > A was found in the basal promoter region at a minor allele frequency of 2.7%, compared to other American populations showing that the Mexican Americans 3.1% is closely related to the South Indian population (Table 4).25 The upstream SNP of POR –173 C > A has been reported to be negatively associated with the warfarin dose and reduces the gene expression in different ethnic groups.26 The functional polymorphism of the POR proximal promoter variant –152 C > A is present at the AP-2 activator protein site, while the effect of the variant remains unclear till today.17 TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) online tool showed that

Table 3: Allele frequency of POR gene in South Indian population

Minor allele frequency of $\chi^2$ test for goodness of fit was used to determine the genotypic distribution of each SNP following Hardy-Weinberg equilibrium (HWE) calculated using Microsoft Excel. Linkage disequilibrium (LD) was calculated using the Haploview Software version 4.1. 24
Table 4: Frequency of POR gene variants in different ethnicities as compared with the South Indian population

| Population     | Allele frequency | Ref |
|----------------|------------------|-----|
|                | rs72553971       |     |
| African American | 0.012 0.026       | 25  |
| Caucasian American | 0.044 0.13       | 25  |
| Chinese American   | 0.015 0.078      | 25  |
| Mexican American   | 0.031 0.077      | 25  |
| Japanese          | 0.019 0.134      | 14  |
| South Indian      | 0.027 0.1545     | PS  |
| Moroccan          | –                 | 14,25 |
| Jewish            | –                 | 14,25 |
| Ashkenazi (T)     | –                 | 14,25 |
| Ashkenazi         | –                 | 14,25 |
| Gomes et al.      | –                 | 3   |
| Saito et al.      | –                 | 14,25 |
| Hart et al.       | –                 | 14,25 |
| 1000 Genomes      | 0.022 0.1040     | 35  |
| TOPMED            | 0.039 0.1122     | 35  |
| HapMap projects   | –                 | 36  |

PS, Present Study
1. Shephard EA, Phillips IR, Santisteban I, West LF, Palmer CNA, Ashworth A, et al. Isolation of a human cytochrome P-450 reductase cDNA clone and localization of the corresponding gene to chromosome 7q11.2. Ann Hum Genet 1989;53(4):291–301. DOI: 10.1111/j.1469-1809.1989.tb01798.x.
2. Lewis DFV. 57 varieties: the human cytochromes P450. Pharmacogenomics 2004;5(3):305–318. DOI: 10.1517/ phgs.5.3.305.29827.
3. Gomes AM, Winter S, Klein K, Turpeinen M, Schaeffeler E, Schwab M, et al. Pharmacogenomics of human liver cytochrome P450
oxidoreductase: multifactorial analysis and impact on microsatellite drug oxidation. Pharmacogenomics 2009;10(4):579–599. DOI: 10.2217/pgs.09.7.

4. Fukami M, Horikawa R, Nagai T, Tanaka T, Naiki Y, Sato N, et al. Cytochrome P450 oxidoreductase gene mutations and Antley-Bixler syndrome with abnormal genitalia and/or impaired steroidogenesis: molecular and clinical studies in 10 patients. J Clin Endocrinol Metab 2005;90(1):414–426. DOI: 10.1210/jc.2004-0810.

5. Huang N, Pandey AV, Agrawal V, Reardon W, Lapunzina PD, Mowat D, et al. Diversity and function of mutations in p450 oxidoreductase in patients with antley-bixler syndrome and disordered steroidogenesis. Am J Hum Genet 2005;76(5):729–749. DOI: 10.1086/429417.

6. Homma K, Hasegawa T, Nagai T, Adachi M, Horikawa R, Fujiwara I, et al. Urine steroid hormone profile analysis in cytochrome P450 oxidoreductase deficiency: implication for the backdoor pathway to dihydrotestosterone. J Clin Endocrinol Metab 2006;91(7):2643–2649. DOI: 10.1210/jc.2005-2460.

7. Arlt W, Walker EA, Draper N, Ivison HE, Ride JP, Hamer F, et al. Congenital adrenal hyperplasia caused by mutant P450 oxidoreductase and human androgen synthesis: analytical study. Lancet 2004;363(9427):2128–2135. DOI: 10.1016/S0140-6736(04)6503-3.

8. Xia C, Panda SP, Marohnic CC, Martásek P, Masters BS, Kim, et al. Structural basis for human NADPH-cytochrome P450 oxidoreductase deficiency. Proc Natl Acad Sci USA 2011;108(33):13486–13491. DOI: 10.1073/pnas.1106623108.

9. Campelo D, Esteves F, Palma BB, Gomes BC, Rueff J, Lautier T, et al. Probing the role of the hinge segment of cytochrome P450 oxidoreductase in the interaction with cytochrome P450. Int J Mol Sci 2018;19(12):3913. DOI: 10.3390/ijms19123914.

10. Wang M, Roberts DL, Paschke R, Shea TM, Masters BS, Kim JJ. Three-dimensional structure of NADPH-cytochrome P450 reductase: prototype for FMN- and FAD-containing enzymes. Proc Natl Acad Sci USA 1997;94(16):8411–8416. DOI: 10.1073/pnas.94.16.8411.

11. Vermilion JL, Ballou DP, Massery V, Coon MJ. Separate roles for FMN and FAD in catalysis by liver microsomal NADPH-cytochrome P-450 reductase. J Biol Chem 1981;256(11):266–277.

12. Ellis J, Gutierrez A, Barsukov IL, Huang W-C, Grossmann JG, Roberts GCK. Domain motion in cytochrome P450 reductase: conformational equilibria revealed by NMR and small-angle x-ray scattering. J Biol Chem 2009;284(52):36628–36637. DOI: 10.1074/jbc.M109.074304.

13. Montellano PROde. Cytochrome P450: structure, mechanism, and biochemistry Springer; 2015. p. 912.

14. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005;21(2):263–265. DOI: 10.1093/bioinformatics/bth457.

15. Huang N, Agrawal V, Giacomini KM, Miller WL. Genetics of P450 oxidoreductase: sequence variation in 842 individuals of four ethnicities and activities of 15 missense mutations. Proc Natl Acad Sci USA 2008;105(15):1733–1738. DOI: 10.1073/pnas.0711621015.

16. Zhang X, Li L, Ding X, Kaminsky LS. Identification of cytochrome P450 oxidoreductase gene variants that are significantly associated with the interindividual variations in warfarin maintenance dose. Drug Metab Dispos 2011;39(8):1433–1439. DOI: 10.1124/dmd.111.038836.

17. Mirunalini R, Pavithra G, Dhas DBB, Adithan C. Genotype and allele frequency of P450 oxidoreductase *28* gene polymorphism in South Indian population. J Pharmacol Pharmacother 2019;10(1):7–10. DOI: 10.4103/jpp.JPP_55_18.

18. Liu S, Chen R, Li J, Zhang Y, Wang X, Fu Q, et al. The POR rs1057868-rs2868177 GC-GT diplotype is associated with high tacrolimus concentrations in early post-renal transplant recipients. Acta Pharmacol Sin 2016;37(9):12518. DOI: 10.1038/aps.2016.77.

19. Hart SN, Wang S, Nakamoto K, Wesselman C, Li Y, Zhong X. Genetic polymorphisms in cytochrome P450 oxidoreductase influence microsatopol 450-catalyzed drug metabolism. Pharmacogenom Genomics 2008;18(11):11–24. DOI: 10.1097/FPC.0b013e3282f2f212.

20. Nakamoto K, Wang S, Jenison RD, Guo GL, Klaassen CD, Wan YJ, et al. Linkage disequilibrium blocks, haplotype structure, and hSNPs of human CYP1A1 gene. BMC Genet 2006;7(12):9. DOI: 10.1186/1471-2156-7-29.

21. Zhang H-F, Li Z-H, Liu J-Y, Liu T-T, Wang P, Fang Y, et al. Correlation of cytochrome P450 oxidoreductase expression with the expression of 10 isoforms of cytochrome P450 in human liver. Drug Metab Dispos 2016;44(8):1193–1200. DOI: 10.1124/dmd.116.069849.

22. Pecorini GF, Verna F, Sesta A, Messina M, Menegatti E, Einanudi S, et al. SAT-346 POR rs2286822 polymorphism is associated with clinical features in patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency. J Endocr Soc 2019;3(Suppl. 1);SAT-346. DOI: 10.1210/js.2019-SAT-346.

23. Gong L, Zhang CM, Lv JF, Zhou HH, Fan L. Polymorphisms in cytochrome P450 oxidoreductase and its effect on drug metabolism and efficacy. Pharmacogenet Genomics 2017;27(9):337–346. DOI: 10.1097/PFC.0000000000000297.

24. Hart SN, Li Y, Nakamoto K, Wesselman C, Zhong X. Novel SNPs in cytochrome P450 oxidoreductase. Drug Metab Pharmacokinet 2007;22(4):391–402. DOI: 10.2133/dmpk.dmpk-07-111.

25. Ensembl genome browser 100 [Internet]. [cited 2020 May 28]. Available from: https://www.ncbi.nlm.nih.gov/gnp/.