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Splice, Insertion-Deletion and Nonsense Mutations that Perturb the Phenylalanine Hydroxylase Transcript Cause Phenylketonuria in India

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ABSTRACT

Phenylketonuria (PKU) is an autosomal recessive metabolic disorder caused by mutational inactivation of the *phenylalanine hydroxylase* (*PAH*) gene. Missense mutations are the most common *PAH* mutation type detected in PKU patients worldwide. We performed *PAH* mutation analysis in 27 suspected Indian PKU families (including 7 from our previous study) followed by structure and function analysis of specific missense and splice/insertion–deletion/nonsense mutations, respectively. Of the 27 families, disease-causing mutations were detected in 25. A total of 20 different mutations were identified of which 7 "unique" mutations accounted for 13 of 25 mutation positive families. The unique mutations detected exclusively in Indian PKU patients included three recurrent mutations detected in three families each. The 20 mutations included only 5 missense mutations in addition to 5 splice, 4 each nonsense and insertion–deletion mutations, a silent variant in coding region and a 3′UTR mutation. One deletion and two nonsense mutations were characterized to confirm significant reduction in mutant transcript levels possibly through activation of nonsense mediated decay. All missense mutations affected conserved amino acid residues and sequence and structure analysis suggested significant perturbations in the enzyme activity of respective mutant proteins. This is probably the first report of identification of a significantly low proportion of missense *PAH* mutations from PKU families and together with the presence of a high proportion of splice, insertion–deletion, and nonsense mutations, points to a unique *PAH* mutation profile in Indian PKU patients. J. Cell. Biochem. 115: 566–574, 2014.

KEY WORDS: PHENYLKETONURIA; *PHENYLALANINE HYDROXYLASE*; SPLICE SITE MUTATION; *PAH* TRANSCRIPT; NONSENSE MEDIATED DECAY

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Phenylketonuria (PKU; OMIM:261600) is an inborn error of amino acid metabolism inherited as an autosomal recessive trait and is caused by mutations in the gene coding for the hepatic enzyme phenylalanine hydroxylase (PAH). PAH is responsible for hydroxylation of Phenylalanine to Tyrosine in the presence of Oxygen and the cofactor Tetrahydrobiopterin (BH4). Insufficient/absent PAH enzyme activity results in mental retardation in affected children due to accumulation of phenylalanine in the brain where it acts as a neurotoxin. A phenylalanine restricted diet prevents brain damage [Blau et al., 2011] and therefore early detection of affected individuals through mutation screening assumes significance. In addition, mutation analysis facilitates genetic counseling of affected families.

More than 560 PKU causing mutations have been identified in the *PAH* gene from various populations (*PAH* mutation database [PAHDB]; www.pahdb.mcgill.ca). Majority of mutations identified worldwide are rare or "private"; very few recurrent mutations have been reported [Zschocke, 2003]. In our previous study [Bashyam et al., 2010], we identified four novel mutations in seven suspected PKU families. In the current study, we have extended the analysis to an additional 20 families. Our results suggest the occurrence of a unique *PAH* mutation spectrum in Indian PKU patients.

MATERIALS AND METHODS

PATIENTS, SAMPLES, AND MUTATION SCREENING

The study was approved by the institute ethics committee and included 20 PKU families (in addition to 7 families analyzed in our previous study [Bashyam et al., 2010]). Eighteen of 27 families were from the South Indian State of Andhra Pradesh while the rest belonged to different geographical regions within India. Samples were collected from probands for 18 families (families 1–18; Table I) and from the parents for two families (families 19–20; Table I). Families 19 and 20 had a previous history of PKU and were therefore referred for carrier testing; the proband samples were unfortunately unavailable. All patients exhibited classical PKU symptoms which are described in Table I and Document S1. Establishment of disease, sample collection, and the mutation screening procedure has been described earlier [Bashyam et al., 2010]. Each mutation was confirmed through bi-directional DNA sequencing. Interestingly, the proband from family 2 exhibited features of Metachromatic

TABLE I. Clinical and Molecular Genetic Analyses of Indian PKU Families

Family	Patient	Gender/age	Phe/Tyr levels (μmol/L)[a]	Mutation[b]	Homozygous/heterozygous	Consanguinity	Family status	
							Mother	Father
01	01	M/1 year, 8 months	1,260/45	c.168-2A>G (IVS2-2A>G)	Homozygous	Present	Carrier	Carrier
02[c]	02	M/1 year	300/24	c.168-2A>G (IVS2-2A>G)	Homozygous	Present	NA	NA
03	03	F/7 years	1,200/70	c.358delT	Homozygous	Present	Carrier	Carrier
04	04	M/2 years	1,177/25	c.472C>T (p.R158W)	Homozygous	Absent	Carrier	NA
05	05	F/1 year, 2 months	1,240/31	c.558_559delAT	Homozygous	Present	Carrier	Carrier
06	06	F/6 months	700/40	c.612T>C (p.Y204Y)	Heterozygous	Present	Normal	Carrier
07	07	M/2 years, 6 months	440/122	c.618C>A (p.Y206X)	Homozygous	Absent	Carrier	Carrier
08	08	F/2 years, 6 months	450/70	c.727C>T (p.R243X)	Homozygous	Present	Carrier	Carrier
08	09 (younger sibling)	F/8 months	NA/NA	c.727C>T (p.R243X)	Homozygous	Present	Carrier	Carrier
09	10	M/2 years, 6 months	1,519/42	c.755G>A (p.R252Q)	Heterozygous	Absent	Normal	Carrier
				c.1066-11G>A (IVS10-11G>A)	Heterozygous		Carrier	Normal
10	11	F/1 year, 4 months	1,100/27	c.781C>T (p.R261X)	Homozygous	Present	Carrier	Carrier
11	12	F/10 months	1,900/70	c.781C>T (p.R261X)	Homozygous	Present	NA	NA
12	13	M/11 months	1,243/34	c.842C>T (p.P281L)	Homozygous	Present	NA	NA
13	14	F/22 days	2,129/66	c.913-7A>G (IVS8-7A>G)	Heterozygous	Present	Normal	Carrier
				c.934G>C (p.G312R)	Heterozygous		Carrier	Normal
14	15	M/10 months	506/32	c.1177insT	Homozygous	Present	NA	NA
15	16	M/2 years	1,405/38	c.1177insT	Homozygous	Present	NA	NA
16	17	M/10 months	NA/NA	c.1503A>G	Heterozygous	Present	NA	NA
17	18	M/6 years, 6 months	1,117/32	Mutation not detected	–	Present	–	–
18	19	M/10 months	1,400/63	Mutation not detected	–	Present	–	–
19	Carrier parents	Father	–	**c.1103A>G (p.E368G)**	Heterozygous	–	–	–
		Mother	–	**c.1103A>G (p.E368G)**	Heterozygous		–	–
20	Carrier parent	Father	–	c.1503A>G	Heterozygous	–	–	–

Reference sequences: *PAH*–GenBank accession no. NM_000277.1, cDNA, and amino acid nomenclature considers "A" of translation initiation codon (ATG) as the first nucleotide and ATG/methionine as the first codon/amino acid, respectively.

[a]Levels were measured from blood samples using high performance liquid chromatography.

[b]Novel mutation is shown in bold.

[c]Proband harbors *Arylsulfatase A* gene mutation in addition to the *PAH* mutation.

IVS, InterVening Sequence (intron); M, male; F, female; Phe, phenylalanine; Tyr, tyrosine; NA, not available.

leucodystrophy (in addition to classical PKU symptoms) and a mutation in the *Arylsulfatase A* gene (*ARSA*) was also identified (Ranganath et al., personal communication).

QUANTITATION OF *PAH* TRANSCRIPT

Two micrograms of total RNA obtained from EBV transformed lymphoblasts was reverse transcribed using oligo dT primer (GE Healthcare) to generate cDNA as previously described [Bashyam et al., 2010]. Appropriate dilutions of the cDNA were used for quantitative PCR performed on the SDS 7500 (ABI, Foster City, CA) using the SYBR Premix Ex Taq (Perfect Real Time kit; Takara Bio, Inc., Japan) in the presence of primers PAH-RTF and PAH-RTR [Bashyam et al., 2010]. PCR and quantitation of relative *PAH* levels with respect to *GAPDH* were performed as described before [Bashyam et al., 2010].

PROTEIN SEQUENCE AND STRUCTURE ANALYSIS

Sequence and structure analysis of effect of missense mutations was performed as described earlier [Bashyam et al., 2010, 2012a,b,c]; details are given in Methods S1.

RESULTS

SPLICE, INSERTION–DELETION, AND NONSENSE PAH MUTATIONS APPEAR TO BE THE MAJOR CAUSE OF PKU IN INDIA

The study was carried out on 28 patients belonging to 27 families (inclusive of our previous study [Bashyam et al., 2010]). Mutations were detected in all except two families (Table I and Fig. 1 and Fig. S1). Of the total 20 mutations identified in our current and previous studies, only 5 were missense including 2 novel mutations (Fig. 1 and Table I). The novel missense mutations viz. p.G312R (c.934G>C) and p.E368G (c.1103A>G) affected amino acid residues that exhibited significant conservation (Fig. 1). Absence of the novel mutations in 100 (200 chromosomes for c.1103A>G) and in 50 (100 chromosomes for c.934G>C) normal individuals from the same local population was independently confirmed using DNA sequencing (data not shown). The c.612T>C silent variant is reported as an SNP in NCBI database (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=rs62514928) with unknown clinical relevance and frequency, though it was not detected in 69 (138 chromosomes) normal individuals in this study (data not shown).

An analysis of PAHDB reveals missense *PAH* mutations to be the most common form (341/567; 60%); therefore, the low frequency detected in the current study (5/20; 25%; Table II) is highly significant. None of the common missense mutations listed in PAHDB including p.R408W (6.6%) and p.I65T (4%) were detected. On the other hand, the frequency of splice (5/20; 25%), insertion/deletion (4/20; 20%), and nonsense (4/20; 20%) mutations detected in Indian PKU patients (Fig. 2A and Table II) was significantly higher than the corresponding frequencies reported in the PAHDB (11.5%, 15%, and 5% for splice, insertion–deletion, and nonsense mutations, respectively). More importantly, 20 of 25 (88%) mutation positive families harbored potential *PAH* transcript perturbing mutations including splice, nonsense, insertion–deletion, and UTR mutations (Table II). In addition, 13 of the 25 families harbored mutations which were exclusive for Indian PKU patients and were not detected in patients from other countries (Table II), suggesting thereby the possible existence of a unique *PAH* mutation spectrum among Indian PKU patients, as already suggested for other Mendelian disorders (hypohidrotic ectodermal dysplasia [Bashyam et al., 2012a] and familial hypertrophic cardiomyopathy [Bashyam et al., 2012c]). Among the unique mutations, c.1503A>G, c.1177insT, and c.168-2A>G were detected in three families each (Table II).

As expected, the carrier parents in families 19 and 20 harbored heterozygous mutations (Table I). Of the 16 probands in whom mutations were identified, homozygous mutations were detected in 12, heterozygous mutations in 2 (families 6 and 16; Table I) and compound heterozygous in 2 (families 9 and 13; Table I). Frequent occurrence of consanguineous marriages is one reason for high frequency of homozygous mutations in Indian PKU patients. It is possible that the second mutation in probands of families 6 and 16 could be located in regions which were not screened (such as deep within introns). Similarly, mutations not detected in families 17 and 18, could also be located in intronic regions not covered in the mutation screening strategy. Exonic copy number gains and/or deletions, not screened in this study, could be the other cause of disease in these affected families. All parent samples were confirmed to harbor the respective mutation in heterozygous condition (Table I).

FUNCTIONAL CHARACTERIZATION OF SELECT MUTATIONS REVEALS POSSIBLE ROLE OF NONSENSE MEDIATED DECAY (NMD) INDUCED DEGRADATION OF MUTANT PAH TRANSCRIPT

Three nonsense (p.Y206X, p.R243X, and p.R261X) and two deletion (c.358delT and c.558_559delAT) mutations identified in this study are expected to result in the generation of premature termination codon (PTC) situated 90, 117, 60, 123, and 114 bases upstream of the subsequent exon–exon junctions, respectively. Therefore, the five mutations may trigger NMD-based degradation of the respective mutant transcript [Bashyam, 2009]. In order to test this possibility, we isolated total RNA from EBV transformed lymphoblasts generated separately from three probands (harboring c.358delT, p.Y206X, and p.R243X mutations [Fig. 3A]; samples from other two probands were unavailable for transcript analysis) and quantified the respective *PAH* transcript levels with respect to RNA isolated from normal lymphoblasts. All three mutations resulted in significant reduction in the respective mutant transcript levels (Fig. 3B) indicating transcript degradation perhaps through nonsense mediated decay (NMD). The mutations however did not result in an obvious change in transcript structure (such as exon skipping due to the PTC through nonsense-associated altered splicing or "NAS" [Wang et al., 2002]), as confirmed by reverse transcription polymerase chain reaction analyses (data not shown). In addition, the c.1177insT and c.976delT mutations (Table I) may not trigger NMD as already described in our previous study [Bashyam et al., 2010].

PROTEIN SEQUENCE AND STRUCTURE ANALYSIS REVEALS POSSIBLE PERTURBATIONS DUE TO MISSENSE MUTATIONS

Multiple sequence alignment (MSA) of PAH with its homologues revealed significant conservation of the wild-type amino acid residues at the mutation sites (Fig. 1 and Fig. S2) and therefore, their substitutions by dissimilar residues are expected to hinder

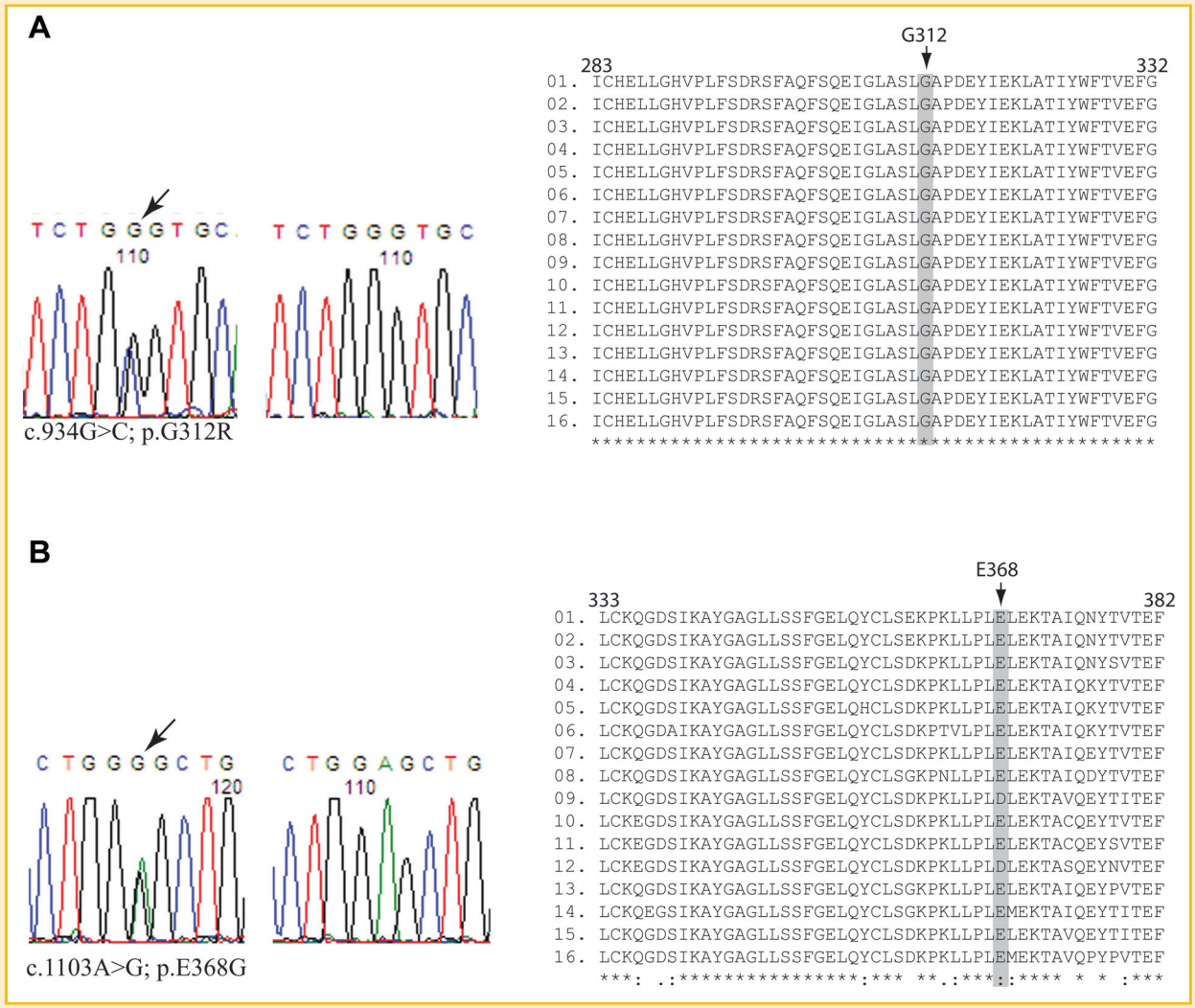

Fig. 1. Identification and analysis of two novel *PAH* missense mutations (c.934G>C [p.G312R]; Panel A and c.1103A>G [p.E368G]; Panel B). For each mutation, electropherogram for the mutant sequence is on the left and for the normal sequence is on the right; each mutation is indicated by an arrow in the electropherogram for the mutant sequence. Multiple sequence alignment with *PAH* homologues from other species for the region surrounding the affected amino acid residue for each mutation is also shown. The homologues are as follows: 1. gi|4557819|ref|NP_000268.1| *Homo sapiens*; 2. gi|114646575|ref|XP_001156919.1| *Pan troglodytes*; 3. gi|332241630|ref|XP_003269981.1| *Nomascus leucogenys*; 4. gi|355786459|gb|EHH66642.1| *Macaca fascicularis*; 5. gi|109098481|ref|XP_001094859.1| *Macaca mulatta*; 6. gi|296212713|ref|XP_002752958.1| *Callithrix jacchus*; 7. gi|345781140|ref|XP_532671.3| *Canis lupis familiaris*; 8. gi|301759315|ref|XP_002915501.1| *Ailuropoda melanoleuca*; 9. gi|149742972|ref|XP_001497778.1| *Equus caballus*; 10. gi|171543886|ref|NP_032803.2| *Mus musculus*; 11. gi|129975|sp|P04176.3| *Rattus norvegicus*; 12. gi|354475067|ref|XP_003499751.1| *Cricetulus griseus*; 13. gi|291389824|ref|XP_002711342.1| *Oryctolagus cunicu*; 14. gi|344267654|ref|XP_003405681.1| *Loxodonta africana*; 15. gi|114051455|ref|NP_001039523.1| *Bos taurus*; 16. gi|348580699|ref|XP_003476116.1| *Cavia porcellus*.

protein function. The position-specific profile (Gribskov's scores) at the mutation sites (Table III) confirmed this inference. In addition, all five missense mutations were identified as "Disease" causing mutations by the web-server "Hansa" (hansa.cdfd.org.in:8080) [Acharya and Nagarajaram, 2012; Bashyam et al., 2012a,b,c] (Table III).

Each missense mutation affected an amino acid located in the PAH catalytic domain (Fig. 2A,B). Arg158 appears to form a salt bridge with Glu280 which may be important for maintaining the PAH active site structure (Fig. 2C). Hence, substitution of Arg158 by the larger aromatic residue Trp is expected to preclude the aforementioned interaction which in turn may eventually affect enzymatic activity [Kobe et al., 1999]. Pro281 has been thought to provide conformational stability to the co-factor-binding motif and is a part of the active site [Kobe et al., 1999]. In addition, Pro281 occurs very close to the Fe residue (Fig. 2C) and may help in defining the architecture of the active site. Therefore, any substitution is expected to affect conformational stability of the active site and may disrupt co-factor binding. Arg252 forms a salt bridge with Asp315 besides forming hydrogen bond with the carbonyl Oxygen of Ala313 (Fig. 2C). Any substitution at Arg252 is expected to result in disruption of these aforementioned stabilizing interactions in the catalytic domain. Gly at position 312 is located in the loop connecting the two helices (Asp296–Ser310 and Asp315–Trp326) and is buried. Gly312 is

TABLE II. List of *PAH* Mutations Identified in Indian PKU Families

Mutation	Location	Mutation type	No. of families	Source
c.60+5G>A (IVS1+5G>A)	Intron 1	Splice	1	Previous study[b]
c.168-2A>G (IVS2-2A>G)[a]	Intron 2	Splice	3	Previous[b] and current study
c.358delT	Exon 4	Deletion	1	Current study
c.472C>T (p.R158W)	Exon 5	Missense	1	Current study
c.526C>T (p.R176X)	Exon 6	Nonsense	1	Previous study[b]
c.558_559delAT	Exon 6	Deletion	1	Current study
c.612T>C (p.Y204Y)[a]	Exon 6	Silent variant	1	Current study
c.618C>A (p.Y206X)	Exon 6	Nonsense	1	Current study
c.727C>T (p.R243X)	Exon 7	Nonsense	1	Current study
c.755G>A (p.R252Q)	Exon 7	Missense	1	Current study
c.781C>T (p.R261X)	Exon 7	Nonsense	2	Current study
c.842C>T (p.P281L)	Exon 7	Missense	1	Current study
c.842+1G>A (IVS7+1G>A)	Intron 7	Splice	1	Previous study[b]
c.913-7A>G (IVS8-7A>G)	Intron 8	Splice	1	Current study
c.934G>C (p.G312R)[a]	Exon 9	Missense	1	Current study
c.976delT[a]	Exon 10	Deletion	1	Previous study[b]
c.1066-11G>A (IVS10-11G>A)	Intron 10	Splice	1	Current study
c.1103A>G (p.E368G)[a]	Exon 11	Missense	1	Current study
c.1177insT[a]	Exon 11	Insertion	3	Previous[b] and current study
c.1503A>G[a]	3′UTR	3′UTR	3	Previous[b] and current study

[a]Mutations detected exclusively in Indian families.
[b]Bashyam et al. [2010].
IVS, InterVening Sequence (intron); UTR, untranslated region.

present in left handed helical conformation that is mostly favored by itself and to some extent by Asn [Nagarajaram et al., 1993]. More importantly, position 312 is in close proximity to the tetramerization and regulatory domains of the protein and makes stearic clashes with surrounding residues (Fig. 2D). Therefore, mutation to Arg at this position is expected to disturb the local backbone conformation and hence may affect the protein fold. In addition, Arg, being bulkier and positively charged, is expected to affect the protein structure. The Glu368 residue is involved in formation of main-chain hydrogen bond with Lys371, Thr372, and Pro366. Furthermore, analysis of the PAH tetrameric structure (2PAH [Fusetti et al., 1998]) revealed that Glu368 is in close proximity with its counterpart from the other subunit (in the dimer/tetramer; Fig. 3E). From our structure analysis, we conclude that mutation to Glycine at this position may result in increased conformational freedom and a substantial change in side chain volume in addition to the loss of Glu–Glu contact at the dimer interface (Fig. 3E), which may in turn destabilize the dimer/tetramer structure.

DISCUSSION

In the current study, we have performed mutation analysis of 27 PKU families from India including 7 from our previous study [Bashyam et al., 2010]. A total of 20 different mutations were detected in 25 families; no mutation was detected in 2. Therefore, the mutation detection frequency was 25/27 (93%), similar to other reports [Stojiljkovic et al., 2006; Groselj et al., 2012]. The c.168-2A>G, c.1177insT and c.1503A>G mutations appear to be the most common *PAH* mutations causing PKU in the Indian population. This result assumes significance, given the paucity of recurrent mutations detected from PKU patients worldwide.

In the previous study [Bashyam et al., 2010], we had provided the first evidence for a possible role of NMD in PKU, based on analysis of the novel c.168-2A>G mutation. In the current study, we have characterized two nonsense (p.Y206X and p.R243X) and one deletion (c.358delT) *PAH* mutations (Fig. 3A) and detected a significant reduction in the levels of the respective mutant transcripts suggesting possible NMD-induced transcript degradation in all three (Fig. 3B). Two additional mutations detected in this study and one in our previous study (p.R176X) are also expected to trigger NMD though we were unable to test the same due to lack of sample availability. Our analyses therefore constitute the only evidence for role of NMD-based *PAH* transcript degradation in PKU. In addition, this is probably the first report of characterization of *PAH* nonsense mutations at the transcript level in PKU patients. A few earlier studies have however reported alterations of the *PAH* transcript due to specific mutations [Dobrowolski et al., 2010; Stojiljkovic et al., 2010; Heintz et al., 2012]. Given the low proportion of missense mutations and the corresponding high frequency of splice, insertion–deletion, and nonsense mutations, alterations of the *PAH* transcript may be a common cause of PKU in the Indian population, unlike other populations. Our study has underscored the importance of transcript analysis for all truncation mutations for the presence of possible transcript perturbations rather than restricting the analyses to the truncated protein alone. The other significant feature of our study is the demonstration of the importance of identification of disease causing mutations in carriers, especially in families where PKU is established based on past history but proband sample is unavailable. Knowledge of mutation in such families facilitates genetic counseling.

The nonsense and missense mutations detected in the present study (with the exception of the novel p.E368G and p.G312R mutations) have been reported earlier from other populations. The p.R261X

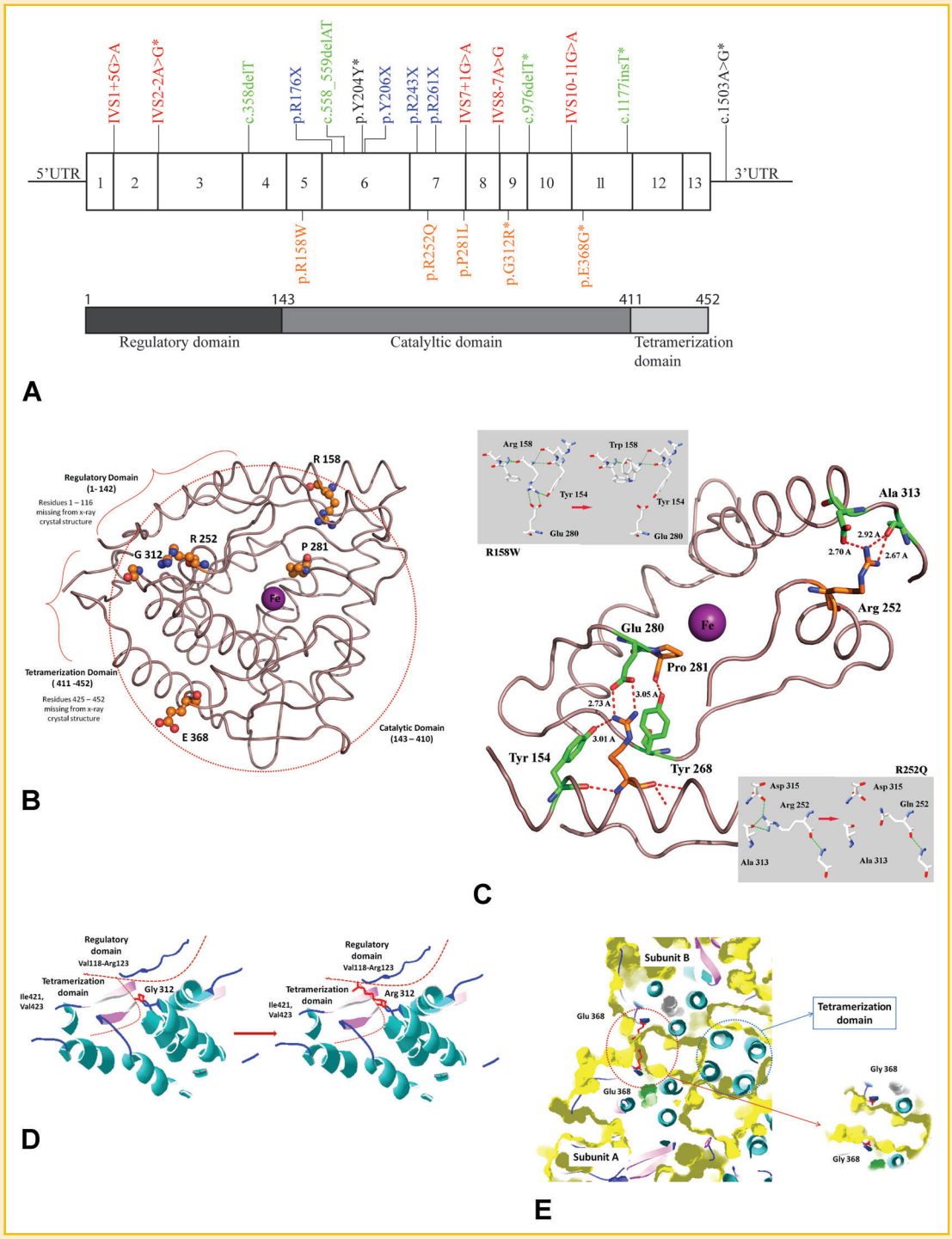

Fig. 2. Analyses of PAH mutations identified from Indian PKU patients. A (top): Location of the mutations in the *PAH* gene. Mutations are color coded: Red, splice; blue, nonsense; green, in/del; orange, missense; black, silent and 3′ UTR. Mutations identified exclusively in Indian patients are denoted by "*." A bar diagram (bottom) depicting three important PAH domains is also shown. B: Positions of all five PKU associated missense mutations in the human PAH protein structure in orange ball and stick model; Fe is depicted in magenta sphere. C: Structure context of the mutated R158, R252, and P281 residues. Hydrogen bond is represented by dashed line. D: Position of G312 in close proximity of the tetramerization and regulatory domains. Only neighboring residues within 10 A^{O} of G312 are shown. Arg at 312 shows stearic clash with neighboring atoms. E: Structure context of the E368G missense mutation; PAH tetramer is shown in surface model with Glu368 (from two chains) in red involved in dimer contacts. The surface of the molecule is shown in yellow, helices are shown in blue, and beta sheets in pink. Inset shows surface changes when Glu368 mutates to Gly.

Fig. 3. Characterization of nonsense and deletion mutations identified in this study. A: Position of each mutation with respect to the PAH amino acid sequence; the premature termination codon generated due to each mutation is denoted in bold italic font. For the c.358delT mutation both normal (top) and mutant (bottom) sequence is shown. The deleted "T" nucleotide is indicated by horizontal line. For each mutation the complete nucleotide sequence of the corresponding exon (c.358delT, exon 4; p.Y206X, exon 6; p.R243X, exon 7) is shown. B: Result of reverse transcription quantitative PCR carried out on RNA isolated from each proband and from a normal sample.

TABLE III. Evaluation of Missense Mutations Identified in PKU Patients

Mutation[a]	Domain	Structural explanation	Gribskov's score	Predicted mutation status by Hansa
p.R158W	Biopterin_H[b]	Disruption of the shape of the active site	+5.00 to 0.00	Disease
p.R252Q	Biopterin_H[b]	Destabilization of interactions in the catalytic domain	+5.00 to +1.00	Disease
p.P281L	Biopterin_H[b]	Conformational destabilization in the active site	+7.00 to −3.00	Disease
p.G312R	Biopterin_H[b]	Unfavorable backbone conformation leading to destabilization of protein structure	+6.00 to −2.00	Disease
p.E368G	Biopterin_H[b]	Loss of dimer contacts and destabilization of tetramer	+4.62 to −1.88	Disease

[a]Novel mutations are shown in bold.
[b]Biopterin-dependent aromatic amino acid hydroxylase.

mutation was identified in PKU patients from Brazil [Santana da Silva et al., 2003] and Iran [Zare-Karizi et al., 2011]. The p.R243X mutation was detected in PKU patients from Morocco and several European countries [Dahri et al., 2010] and was reported to be mildly responsive to BH4 therapy [Perez-Duenas et al., 2004]. The p.Y206X mutation was reported earlier from China [Song et al., 2005] and from other populations [Erlandsen and Stevens, 1999]. The p.R158W mutation is also reported from China [Takarada et al., 1993b] and Korea [Takarada et al., 1993a]; similarly the p.R252Q has been reported from Taiwan [Chien et al., 2004]. The p.P281L mutation is reported to be a common mutation in the Mediterranean population [Perez et al., 1992] and has been suggested to induce skipping of exon 8, though the mutation itself is located in exon 7. However, the exon skipping effect is partial since both full and partial length transcripts are detected in the affected lymphoblasts [Ellingsen et al., 1999]; the disease phenotype associated with this mutation therefore could be a net result of altered protein sequence due to exon skipping and altered protein active site due to the amino acid substitution (Fig. 2C). We detected a novel silent mutation c.612T>C in family 6. Though it is reported as a variant with unknown clinical significance in the NCBI SNP database, it was not detected in the normal population in our study. It is possible that this mutation may have an effect on the *PAH* transcript. Of note, two mutations affecting the same codon viz. c.612T>G and c.611A>G were reported earlier. The c.612T>G generates a PTC [Guldberg et al., 1993] whereas the c.611A>G mutation was shown to generate a cryptic 5′ splice site leading to defective splicing [Ellingsen et al., 1997].

Each of the five missense mutations detected in the present study affected conserved amino acid residues (Fig. 1 and Fig. S2 and Table III) and the perturbations are expected to destabilize enzyme activity. Erlandsen and Stevens [1999] using a PAH model (constructed by superimposing the human PAH catalytic and tetramerization domains on the rat PAH regulatory domain), reported Arg252 to form a salt bridge with Asp315 and a hydrogen bond with Asp27side-chain. However, these interactions are not seen in the human PAH crystal structure [Fusetti et al., 1998] which is used in the present study and hence there is no perceivable evidence that substitution of R252 by Gln would result in disruption of the above-mentioned stabilizing interactions.

Several observations made in the current study point to the possible existence of a unique *PAH* mutation profile among Indian PKU patients. Firstly, a total of 7/20 (35%) mutations are exclusive to the Indian population (Table II). Secondly, missense mutations occur at a significantly lower frequency compared to other populations. In contrast, the frequencies of splice, insertion–deletion, and nonsense mutation types likely to perturb *PAH* transcript, are significantly higher than that reported in the PAHDB. Therefore, mutations affecting *PAH* transcript rather than PAH protein/enzyme activity appear to be the major cause of PKU in India unlike other populations. Of note, the low missense mutation frequency reported in the current study is similar to that reported from Iran [Zare-Karizi et al., 2011] but not from China [Song et al., 2005] and Korea [Lee et al., 2004]. However, recurrent mutations reported from Indian PKU patients are distinct from those identified from other Asian countries.

A few important clinical and socio-economic observations in the current study are worth mentioning. Firstly, most patients presented with mental retardation and/or developmental delay. One reason could be non-availability of special (phenylalanine-restricted) diet for the patient. More importantly, we observed a significantly high frequency of consanguineous marriages among PKU families. Fifteen of 18 probands were associated with consanguinity resulting in high frequency of homozygous mutations (Table I) as opposed to compound heterozygosity. In fact, consanguinity appears to be a common factor in several autosomal recessive genetic disorders in India [Bashyam et al., 2004; Kumar et al., 2013]. Therefore, it is important to educate the patients and affected families (through genetic counseling) and the population in general (through Government sponsored mass education programs) about this social problem.

In conclusion, the current study has revealed a significantly low frequency of missense mutations causing PKU in the Indian population. In contrast, mutations affecting the *PAH* transcript appear to be a common cause of PKU, as against other populations. The current study therefore has important implications for PKU screening, patient management, and genetic counseling in India and underscores the importance of analyzing the *PAH* transcript.

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