Analysis of common genetic mutations in a cohort of Sri Lankan children with salt wasting form of Congenital Adrenal Hyperplasia

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Abstract

Introduction Steroid hydroxylase deficiency due to *CYP21A2* gene mutation is the most common cause of Congenital Adrenal Hyperplasia (CAH). Mutation spectrum in Sri Lankan CAH patients has not been investigated adequately.

Objectives This study attempted to study the spectrum of mutations in *CYP21A2* gene in 30 patients with salt wasting form of CAH in Sri Lanka.

Methods Allele specific polymerase chain reaction was carried out using mutation site specific primers for eight mutations (P30L, I2G, 8bp deletion, I172N, E6 cluster, V281L, Q318X and R356W) reported as frequently occurring in other populations.

Results Fourteen patients had homozygous mutations; six patients were compound heterozygotes as determined by investigating parents of the patients, one patient had a large gene deletion which was previously reported and the remaining patients had at least one heterozygous mutation. The following allele frequencies were observed for each mutation P30L - 10%, I2G - 40%, 8 bp-18.33%, I172N - 3.33%, E6 cluster - 5%, Q318X - 40% and R356W - 3.33%. V281L mutation was not observed in the study cohort. DNA sequencing revealed a novel mutation G292S in one patient.

Conclusion This is the first report describing a broad spectrum of mutations in *CYP21A2* gene in Sri Lankan patients with CAH. Mutation frequencies did not vary from other ethnic groups reported around the world.

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Introduction

Congenital Adrenal Hyperplasia (CAH), an inherited condition disrupting synthesis of adrenal cortical hormones [1] affects one in 15,000-20,000 children worldwide [2]. Among the five enzymes required for cortisol synthesis, 21-hydroxylase (21-OH) is the most commonly affected in CAH [1]. *CYP21A2* gene coding for 21-hydroxylase is located on chromosome 6 and has 10 exons. It has a pseudogene *(CYP21A1P)* which is 98% homolo-gous to the normal gene located in close proximity [3]. Recombination events that occur between the normal gene and the pseudogene during meiosis, in view of high sequence homology, leads to a variety of mutations including point mutations, deletions, frameshift and intronic mutations [4]. Deficiency of 21-OH results in a wide range of clinical presentations, the classic salt wasting form which can sometimes be fatal, classic simple virilizing form which leads to genital ambiguity and non-classical form which is a milder form of the disease [5]. Salt wasting CAH is observed in approximately 75% of the classic CAH patients, making it the most frequent severe form of the disease [6]. Approximately 11 deleterious mutations have been reported in the coding region of the *CYP21A1P* gene [7]. Most of them arise due to either gene conversion from the pseudogene [1] or large deletions. Approximately 25-30 % of disease causing genes are chimeric [8]. One of the most frequent mutations which cause splicing defects in *CYP21A2* gene is I2G [9]. An 8bp deletion in exon 3 results in an inactive enzyme while cluster of 3 point mutations in exon 6 abolishes the enzyme activity [10]. Q318X and R356W mutations in exon 8 results in a truncated protein [11, 12] and affect the substrate binding site [10] respectively. I172N, a mutation in exon 4 may alter the hydrophobic region of the enzyme to a polar region weakening the interaction of the enzyme with endoplasmic reticulum [10]. P30L and V281L mutations in exons 1 and 7 respectively only alter the secondary structure of the protein with no severe impact on the protein [10]. Mutation spectrum of CAH patients is reported to sometimes vary depending on the ethnic group. We do not have sufficient data for Sri Lanka as only large gene deletions have been studied previously [13]. Therefore in this study several point mutations and the 8bp deletion were analysed among a cohort of CAH patients in Sri Lanka.

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Methods

Patients

Thirty patients from 28 families were recruited from the Lady Ridgeway Children's Hospital (LRH), Colombo, Sri Lanka. They had been admitted to the University Paediatric ward with dehydration, ambiguous genitalia (in female patients) and progressive pigmentation. Biochemical assays showed hyponatremia, hyperkalaemia, elevated levels of serum 17-hydroxyprogesterone and higher amounts of dehydro-epiandrosterone sulphate which confirmed the salt wasting form of CAH [14]. After obtaining informed consent from the parents, 2.5mL of venous blood was collected from the patients into 5 mL EDTA coated tubes. Once the genetic analysis of the patients was completed, blood samples (5 mL) were also collected from the parents of the patients suspected to be compound heterozygotes. This study received ethical approval (EC-15-050) from the Ethics Review Committee, Faculty of Medicine, University of Colombo Sri Lanka.

Molecular analysis

DNA was extracted from whole blood and *CYP21A2* gene was analyzed for eight mutations namely P30L, I2G, 8bp deletion, I172N, E6 Cluster, V281L, Q318X and R356W using Allele Specific PCR (ASPCR). Each chromosome was checked for all eight mutations. Representative samples were sequenced for the confirmation of ASPCR results. When patients were suspected to be compound heterozygotes their parents' DNA was also analysed.

Allele Specific PCR

Two PCR reactions were performed for each mutation site. One reaction with the normal primer to detect the normal allele and the second reaction with the mutation specific primer to detect the mutated allele. A common primer was used in the reverse or forward orientation with the normal and mutated type primer. The common primer amplified only the normal gene, not the pseudogene. Normal and mutated primers differed only by their 3' terminal which correspond to the specific allele. But the primers for 8bp deletion and E6 cluster mutations had high dissimilarity with their normal primers (Supplementary Table 1, Figure 1).

Polymerase Chain Reaction (PCR) amplification was performed using respective primers (IDT Integrated DNA technologies, Coravallee, Iowa, USA) in a final volume of 25 µl containing 100 ng genomic DNA and Green GoTaq® reaction buffer, $MgCl_2$, dNTPs and GoTaq® Flexi DNA Polymerase (Promega™ M8295 Corporation, Madison, WI, USA). Amounts of each component used varied for different primer sets. PCR reactions were performed in five steps which includes initial denaturation at 94ºC for 5 minutes, followed by 30 cycles containing denaturation at 94º C for 1 minute, annealing at 60-70ºC (depending on the primer) for 30 seconds, extension at 72ºC for 1 minute and a final extension at 72ºC for 5 minutes. Then the products were visualized through ethidium bromide stained agarose gel electrophoresis.

DNA sequencing

To confirm the reliability of the ASPCR results representative samples were subjected to sequencing of the specific allelic regions. The whole gene was sequenced to detect possible novel mutations in the patients who did not have any of the eight mutations tested. PCR reactions were performed and the products were purified using Wizard® SV Gel and PCR clean-up system (Promega). Sequencing was performed in Applied Biosystems™ 3500 Dx series Genetic Analyzer using Big Dye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA). Along with some allele specific primers sequencing primers were used for sequencing depending on the exon/intron sequence of interest (Supplementary Table 2).

In silico analyses

Reference DNA sequence was downloaded from ensemble (accession number- ENSG00000231852) at https://asia.ensembl.org/Homo_sapiens/Gene/Sequence?db=core;g=ENSG00000231852;r=6:32038265-32041670. Novel variants were identified by aligning the patient's gene sequence with the reference sequence in Bioedit software. To predict the effect of novel variants in the protein YASARA version 18.4.24 software foldx plugin was used. The structure of 21-Hydroxyalse enzyme was obtained from https://www.modelarchive.org/doi/10.5452/ma-anifj [15]. Protein structure which will be generated due to the variations p.G292S and p.R103K were constructed using foldx command. Constructed protein structure and the reference protein structure were superimposed to identify the energy difference between them ($\Delta\Delta G$ value). If $\Delta\Delta G > 0$ then the mutation is destabilizing the protein and if $\Delta\Delta G < 0$ then the mutation is stabilizing the protein. (Energy differences such as 0.5kcal/mol are negligible)

Results

Thirty patients from 28 families comprised of 20 females and 10 males. Majority (22) were Sinhalese in ethnicity. There were 3 and 5 patients of Tamil and Moor ethnicity respectively. Seven patients were off springs of consanguineous unions. Homozygous mutations were observed in thirteen patients (43.3%), fourteen patients (46.6%) had at least one heterozygous mutations (Table 1) and among them 10 had more than one heterozygous mutation suggesting compound heterozygosity.

Table 1. Mutations detected in the patients tested

Patient	*P30L*	*I2G*	*8bp*	*I172N*	*E6 cluster*	*V281L*	*Q318X*	*R356W*
C001		Het					Het	
C002		Het						
C003		Het						Het
C004		Het						Het
C005**	Hom	Hom	Hom					
C006		Hom	Hom					
C007		Het					Het	
C008								
C009**								
C010								
C011							Hom	
C012	Het	Het	Het				Het	
C013			Het				Het	
C014**							Hom	
C015							Het	
C016							Het	
C017		Het					Het	
C018	Het	Het					Hom	
C019		Het					Het	
C020		Het					Het	
C021				Hom			Het	
C022		Het	Het				Het	
C023		Hom			Het			
C024		Hom	Hom				Het	
C025**		Hom					Het	
C026**		Hom					Het	
C027	Hom						Het	
C028**			Hom				Het	
C029**					Hom			
C030							Het	

Het-Heterozygous; Hom-Homozygous mutated, **Off springs of consanguineous unions

Allele frequencies observed is given in Table 2. Sequencing results of the selected samples corroborated the ASPCR results as given in Figure 1.

Table 2. Allele frequencies of CAH patients

Mutation	*P30L*	*I2G*	*8bp del*	*I172N*	*E6 cl*	*Q318X*	*R356W*
Frequency	8.33%	40%	18.33%	3.3%	5%	40%	3.3%

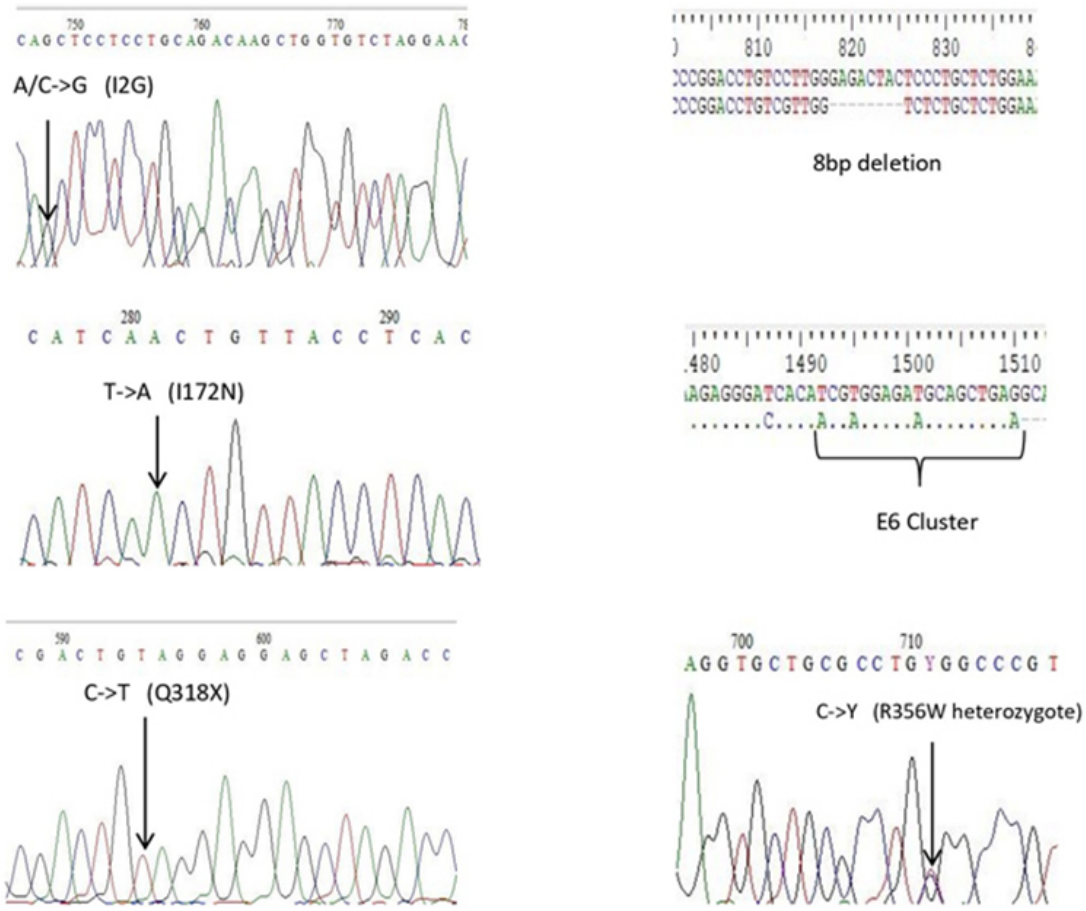


Figure 1. **Electropherogram representing each mutation observed.**

One of the remaining three patients had been previously detected to have a large gene deletion. Sequencing of the complete gene in the remaining two patients revealed two variants. One variant NC_000006. 12:g.32039109G>A in exon 3, which leads to amino acid substitution of p.R103K was observed patients C008 and C010. Second variant observed in patient C008 in exon 7 - NC_000006.12:g. 32040140G>A leads to amino acid substitution of p.G292S. When energy difference was calculated using foldX, ΔΔG values of 0.274kcal/mol and 2.290kcal/mol were observed for R103K and G292S respectively suggesting that G292S will destabilize the 21 hydroxylase enzyme.

DNA sequencing also revealed the presence of chimeric gene. Patient C005 had pseudogene like sequence up to exon 3 and the remaining exons/introns had wild type *CYP21A2* gene. Patient 29 had wild type gene sequence up to exon 4 and the subsequent introns and exons had pseudogene like sequence.

One or both parents were available for mutation testing in eight out of ten patients suspected to be compound heterozygotes. Table 3 shows results obtained. Figure 2 shows the results observed in family C012.

Table 3. Analysis of suspected compound heterozygous patients: heterozygous mutations observed in the patients and their parents, and predicted from parental analysis

	Mutations observed			*Mutations Predicted*	*Compound heterozygosity*
	Patient	Mother	Father		
C001	Q318X and I2G	Q318X	I2G	Q318X/I2G	Yes
C003`	R356W and I3G	No mutations	No sample	Unpredictable	Unpredictable
C004					
C007	Q318X and I2G	I2G	Q318X	I2G/Q318X	Yes
C012	8bp,Q318X, I2G, P30L	Q318X	8bp	Q318X/8bp	Yes
C013	Q318X and 8bp	8bp	Q318X	8bp/Q318X	Yes
C017	Q318X and I2G	I2G	No sample	I2G/Q318X*	Yes*
C019	Q318X and I2G	No sample	Q318X	I2G*/Q318X	Yes*

*Most likely to be heterozygous as the parent available for analysis carried one mutation, the other mutated allele is most likely to be inherited from the parent who was not tested.

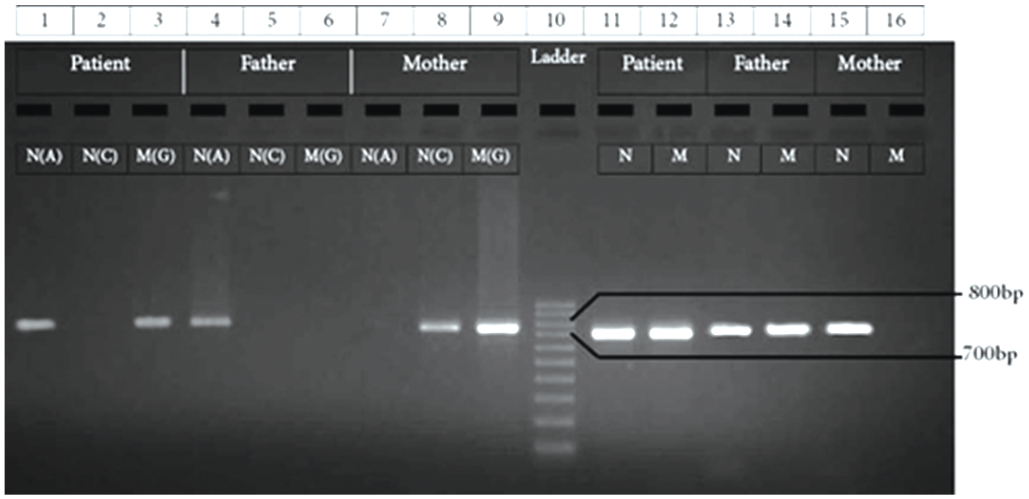


Figure 2. **Allele Specific PCR results of family of patient C012.**

Affected child was heterozygous for I2G and 8bp deletion. Mother was heterozygous for I2G and the father was heterozygous for 8bp deletion resulting in compound heterozygosity in the affected child.

Discussion

Thirty patients with salt wasting form of CAH in a Sri Lankan cohort were genotyped using allele specific PCR to find out the presence of eight common mutations in the *CYP21A2* gene. When patients are suspected as compound heterozygotes their parent's samples were also analyzed by ASPCR. DNA sequencing was performed for the confirmation of ASPCR results and to investigate for possible novel mutations in two patients who did not carry any of the common mutations. This is the first study to report the spectrum of *CYP21A2* mutations in CAH patients in Sri Lanka.

One of the most frequent mutations in our cohort was I2G with 40% of alleles having this aberration. Similar frequencies are reported from Hungary [16] and Japan [17]. Highest frequency (around 48%) was observed in Mexico [18] and the lowest frequency (around 10%) is in Argentina [19]. I2G mutation is usually associated with salt wasting form of CAH.

We observed an allele frequency of 40% for Q318X. But only three patients (10%) were homozygous. Six patients were compound heterozygotes. A much higher allele frequency (53%) of Q318X mutation has been reported in Tabriz population [20] and a lower frequency of 35.5% and 25.7% from Tunisia and Brazil respectively. Only a few ethnic groups have higher frequency of Q318X allele, the reason for higher frequency in our cohort is unknown, may be it is due to a founder effect [21]. Further evidences are needed to prove this hypothesis.

Eleven alleles had 8bp deletion in exon 3 giving a prevalence of 18.33%. 8bp deletion frequency was 13% in Iranian population [22], 12.7% in an Indian cohort [23] and 12% in Romania [24]. Lowest allele frequencies were observed for the E6 cluster (5%) and R356W (3.3%) which are associated only with the salt wasting phenotype.

Only one patient had I172N mutation (homozygous mutation), but this mutation results in the simple virilizing form of CAH [25]. However this patient was also heterozygous for Q318X mutation which may have led to salt wasting phenotype. Patient C009 who was reported to carry a large genomic rearrangement in the *CYP21A2* gene previously [13] was also included in the present study and PCR amplification failed with all the primers in this patient further confirming the large gene deletion.

Frequently 21 OH deficiency occurs due to compound heterozygote genotype of the patient [2]. In our study ten patients were suspected as compound heterozygous. Analyses of their parents' DNA samples confirmed six patients to be compound heterozygous and for two patients parents' samples were not available. Among the confirmed compound heterozygous patients four patients had I2G/Q318X alleles, one patient had I2G/8bp alleles and one patient had 8bp/Q318X alleles.

Patients C003 and C004 were siblings and they both were heterozygous for the I2G and R356W mutations. Their mother did not carry either of these two alleles. Unfortunately father was not available for testing. Thus whether one or both mutations are of paternal or de novo in origin cannot be ascertained. Patients C025 and C026 were also siblings; and both were homozygous for I2G mutation and heterozygous for the Q318X mutation.

Consanguineous marriages increase the risk of genetic disorders because they share the common ancestors. In the present study the patients who were

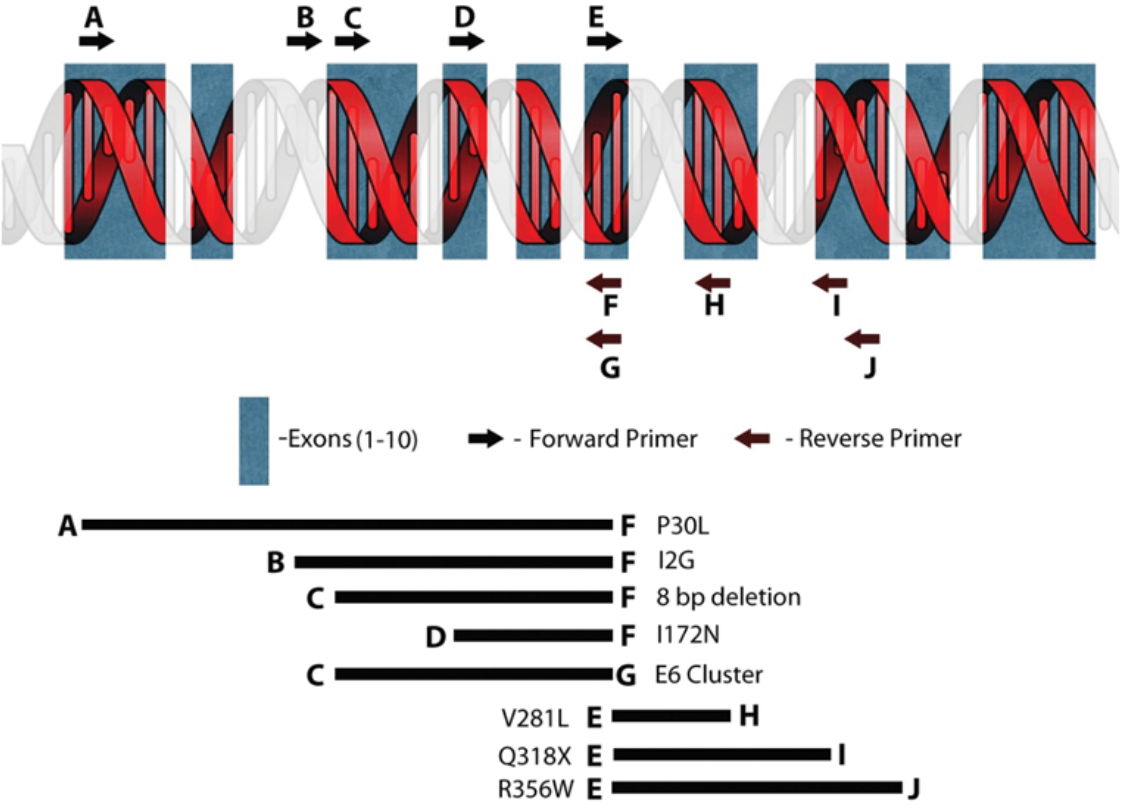
offspring of consanguineous marriages C005, C009 and C014 showed severe mutations. C005 had three homozygous mutations (I2G, 8bp deletion, P30L), C009 had a large gene deletion as previously reported [13] and C014 was homozygous for Q318X mutation. All these mutations will give rise to severe salt wasting form of CAH.

Sequencing of the *CYP21A2* gene showed the presence of chimeric genes in two patients. In patient C005 psuedogene sequences were observed up to exon 4 and after exon 4 normal gene sequences were observed, the pseudogene normal gene junction site was between end of intron 3 and exon 4. This patient had 3 mutations (P30L, I2G & 8b) as detected by ASPCR. In patient C029 normal gene sequence was observed up to exon 4 and pseudogene sequences thereafter with the junction within exon 4. In this patient PCR amplification failed. The common primers used in ASPCR were specific to the normal gene. But the ASPCR result was positive for primers of E6 cluster in which forward primer was normal gene specific while the reverse primers were allele specific. Here only the mutated allele specific primer produced a band which confirms the pseudogene sequences in this patient.

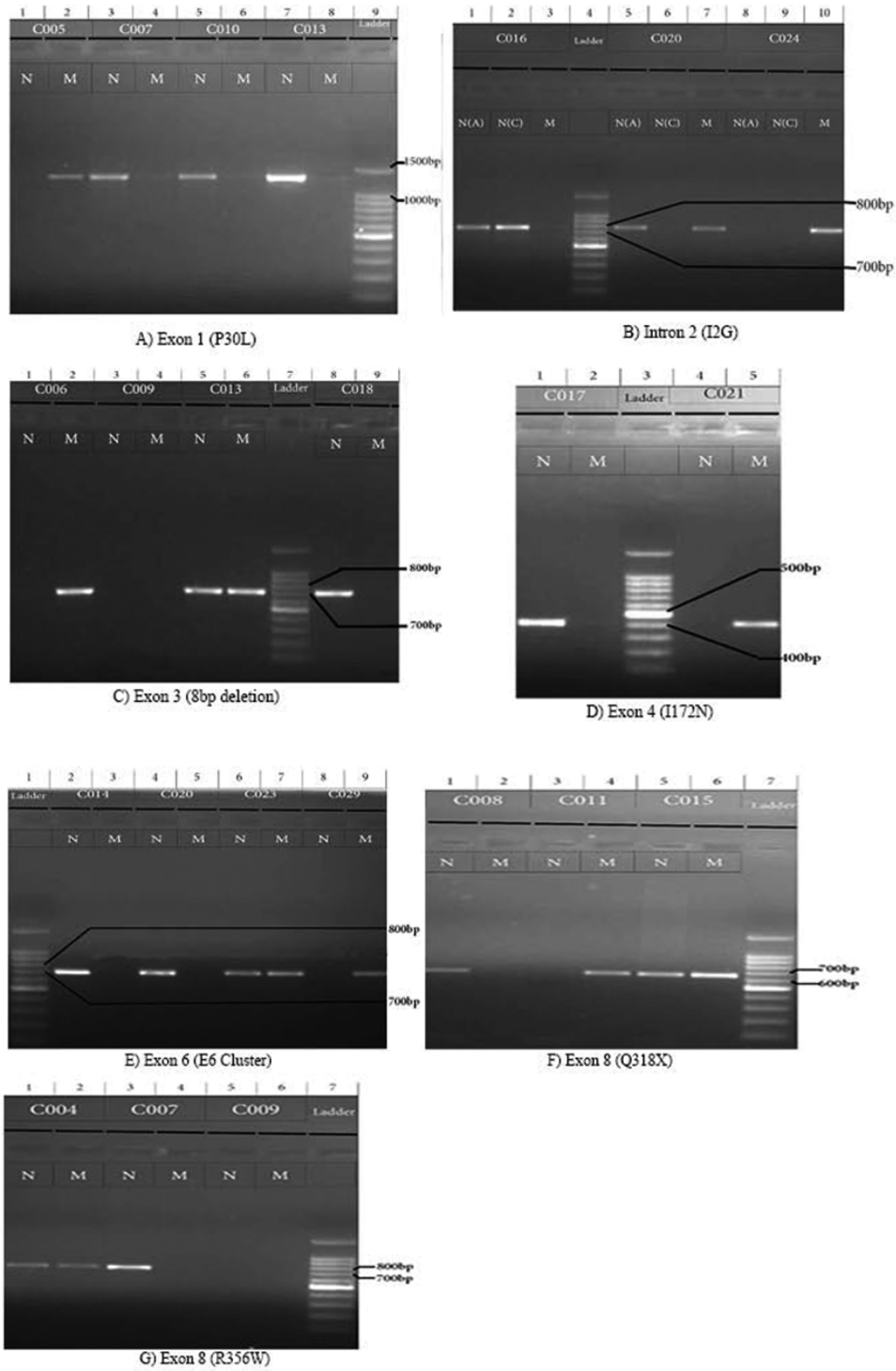
Sequencing results also revealed two novel variants; R103K and G292S. Patient C008 had both R103K and G292S which gives an energy difference of 2.290kcal/mol suggesting that the mutations are destabilizing the protein. But patient C010 carried only R103K which gives an energy difference of 0.274kcal/mol which will not have any impact on the protein.

In conclusion this is the first study which has provided the spectrum of genetic mutations in Sri Lankan CAH patients. The mutation spectrum was similar to what is reported for other ethnic groups around the world. Furthermore one novel pathogenic mutation was identified. The presence of two chimeric genes was a very unique finding as these have been reported only in a few ethnic groups. This study will lay the foundation for genetic testing and genetic counseling for CAH in Sri Lanka.

Supplementary materials



Supplementary Figure 1. **Location of the primers used for allele specific PCR**



Supplementary Figure 2. **Representative allele specific PCR results for all 7 mutations observed in different samples. For each sample two reactions were performed one with the normal and the other with the mutated primer (Designated as N and M). Depending on the product formation normal, heterozygous and homozygous mutations were determined. No bands were observed in sample C009 (shown in panel C and G) perhaps due to the large gene deletion which was previously reported for the patient.**

In Figure 2, panel A shows P30L mutation results of samples C005,C007,C010 and C013. Since the product was formed only in N lane sample C010, C007 and C013 were wild type, sample C005 had product in M lane hence it is homozyogus mutated. Panel B depicts I2G mutation results of sample C016, C020 and C024. Since C016 has products only in N lanes it is wild type, C020 has products in both N and M lanes it is heterozygous mutated and C024 has product only in M lane it is homozygous mutated. Panel C shows the result of 8bp deletion results of sample C006,C009,C013 and C018. Sample C006 is homozygous mutated, C013 is heterozgous mutated and C018 is wild type. Panel D shows the I172N mutation results of sample C017 and C021. Sample C017 is wild type and C021 is homozyogus mutated. Panel E shows the E6 cluster mutation results of C014,C020,C023 and C029. Sample C014 and C020 are wild type, sample C023 is heterozygous mutated and sample C029 is homozygous mutated. Panel F shows the Q318X mutation results of C008,C011 and C015. Sample C008 is wildtype, sample C011 is homozyogus mutated and sample C015 is heterozyogus mutated. Panel G shows the R356W mutation results of sample C004,C007 and C009. Sample C004 is heterozygous mutated and sample C007 is wildtype.

Supplementary Table 1. Primer sequences used in ASPCR

Mutation	*Forward primer*		*Reverse primer*	*Size*	*Annealing temperature*
	N 5'TCCGGAGCCTCCACCTCCC				
P30L				1326bp	65.5°C
	M 5'TCCGGAGCCTCCACCTCC**T**				
	N 5'TTCCCACCCTCCAGCCCCCAA				
I2G	N 5'TTCCCACCCTCCAGCCCCCAC	N	5'AGCTGCATCTCCACGATGTGA	761bp	66.7°C
	M 5'TTCCCACCCTCCAGCCCCCA**G**				
8bp	N 5'CGGACCTGTCCTTGGGAGACTAC			704bp	62.9°C
deletion	M 5'ACTACCCGGACCTGTCCTTGGTC				
	N 5'TCCTCACCTGCAGCATCAT			417bp	61.1°C
I172N	M 5'CTCTCCTCACCTGCAGCATCA**A**				
E6 cluster	N 5'CGGACCTGTCCTTGGGAGACTA			706bp	65.7°C
		M	5'TCAGCTGC**T**TCTCC**T**CG**T**TGTG		
		N	5'TCCACTGCAGCCATGTGCAC		
				333bp	64.3°C
V281L		M	5'TCCACTGCAGCCATGTGCA**A**		
	N 5'GAGGGATCACATCGTGGAGATGCA	N	5'TTCGTGGTCTAGCTCCTCCTG		
Q318X		M	5'AGTTCGTGGTCTAGCTCCTCCT**A**	647bp	63.8°C
		N	5'CTAAGGGCACAACGGGCCG		
R356W		M	5'CTAAGGGCACAACGGGCC**A**	757bp	67.1°C

Site of point mutations are indicated in Boldface. Underlined sequence in EX3NS shows the deleted 8bp sequence of *CYP21A2* gene. N= Normal and M= Mutant.

Supplementary Table 2. Primers used for sequencing

Primer	*Sequence*	*Position*
1F	5'-TCGGTGGGAGGGTACCTGAA-3'	nt -122 to -103
2R	5'-GGGAACTGATGAAGGCAGCT-3'	nt 753-734
3R	5'-TCCAGAGCAGGGAGTAGTCTC-3'	nt 840-821

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Conflicts of interest

The authors declare no conflicts of interest.

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