SUMMARY & CONCLUSIONS
6. SUMMARY AND CONCLUSIONS

The present study entitled “IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF MUTATIONS AND POLYMORPHISM IN UGT1A1, OATP AND G6PD GENES IN TERM AND LATE-PRETERM NEONATES WITH SEVERE HYPERBILIRUBINEMIA” was performed to investigate the spectrum of polymorphisms and mutations in UGT1A1, OATP 2 and G6PD genes in hyperbilirubinemia neonates.

The findings of this study are summarized as follows:

6.1 Study design and population: The subjects of the study were babies born at Nehru hospital and those referred because of jaundice to the neonatal unit of Post Graduate Institute of Medical Education and Research, Chandigarh. Neonates with gestational age ≥34 weeks with severe unconjugated hyperbilirubinemia defined as serum total bilirubin (STB) of ≥20 mg/dl with in the first 14 days of life were included as cases. Those with no clinical jaundice or having the peak STB < 15 mg/dl were enrolled as controls. Babies with evidence of isoimmunization, elevated conjugated bilirubin fraction (≥ 1.5 mg/dl) or who had received blood transfusion were excluded. This was a nested case-control study.

Among 107 patients, fifty-seven hyperbilirubinemic and 50 control neonates were included in the study from which 64.5% were male and 35.5% were female. Mean birth weight for controls was 2375 grams and for subjects were 2593 grams. Median TSB for peak STB for controls was 9.3 mg/dL and for subjects were 23 mg/dL. Phototherapy was given to all subjects.

6.2 UGT1A1 gene:

1. (TA)n promoter was detected by SSCP and the most common polymorphism of UGT1A1 gene viz. (211)G→A/(G71R), (686)C→A/(P229Q), (1091)C→T/(P364L) and (1456)T→G/(Y486D) were detected by PCR based RFLP technique.

2. Significantly more number of babies in the study group had variation of the (TA)n promoter compared to control group (OR 8.63, 95% CI 3.2 – 24.1; p =0.000). Even
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the frequency of heterozygous genotype (TA)6/(TA)7 was significantly more in the hyperbilirubinemic group compared to control group (OR 2.42, 95% CI 1.1 – 5.36; p = 0.016). The allele frequency of (TA)7 in hyperbilirubinemic neonates was 0.487 compared to 0.29 in the controls (OR 2.32, 95% CI 1.32 – 4.12; p = 0.0018). The rare (TA)8 allele was found only in hyperbilirubinemic subjects with the allele frequency of 0.058.

3. We did not find any Gly71Arg mutation either in hyperbilirubinemics or controls. We found Ala72Pro polymorphism at the codon position 72 of the exon 1 of bilirubin UGT1A1 gene. Ala72Pro of exon 1 was found to be the most common mutation of UGT1A1 gene from our population. 24 patients were heterozygous and 2 were homozygous for A72P. There was a significant difference in the genotype frequency of the three variants of Ala72Pro and Pro364Leu polymorphism in control and hyperbilirubinemic patients ($\chi^2 = 11.95, 2.55, P=0.0005^*, 0.11$) and ($\chi^2 = 15.96, P=0.001^*$). There was no significant difference in the genotype frequency of the three variants of Pro229Glu and Tyr486Val polymorphism in control and hyperbilirubinemic patients.

4. SSCP analysis of all the 5 exons of UGT1A1 gene demonstrated either mobility shift or deletion or insertion of bands in 57 DNA samples. Subsequent automated DNA sequencing identified 17 mutations-1 already reported (D359N) and 16 novel mutations. These 16 novel mutations included nine missense mutations (A64S, D70H, G71E, S143N, D146Y, S191P, R257K, W335R and L489V), five silent mutations (I571, K114K, S334S, T349T and T482T), one frame shift mutation (1363 ins C) and one nonsense mutation (E534X).

5. 8 novel mutations were found from exon 1 of UGT1A1, 4 novel mutations were found in exon 3 of UGT1A1 gene and 4 novel mutations were found from exon 5. Exon 1,3 and 5 are the hotspots of mutations in Indian hyperbilirubinemia patients.

6. All the novel mutations identified and characterized in UGT1A1 gene have been registered with International Database, GenBank, maintained by National Centre for Biotechnology Information (NCBI), USA. The mutations have been assigned specific accession numbers and are freely available at http://www.ncbi.nlm.nih.gov/GenBank.
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7. Pathological prediction using SIFT and PolyPhen programs revealed that 9 novel substitution mutations (D146Y, S191P, P229Q, R257K, W335R, D359N, P364L, Y486D and L489D) identified in our population were predicted to be damaging whereas 5 mutations viz (A64S, D70H, G71E, A72P AND S143N) were predicted to be tolerated mutations. Threshold value for pathological mutations was ≤ 0.5. None of the mutations were found to have any splicing defects.

6.3 OATP2 gene:

1. Identification of the most common 388 (G→A)/ Asn130Asp, 463 (C→A)/ Pro155Thr, 521(T→C)/ Val174Ala, and 1463 (G→C)/Cys485Ser polymorphism of OATP 2 gene by Polymerase Chain Reaction based Restriction Fragment Length Polymorphism Analysis and by sequencing.

2. There was a significant difference in the genotype frequency of the three variants of 388(G→A) polymorphism in control and hyperbilirubinemic patients. Statistical analysis revealed that homozygotes and heterozygotes frequencies were significantly higher in hyperbilirubinemic group to that of control (p <0.01, $\chi^2$ = 7.04). There was no significant difference in the genotype frequency of the three variants of Pro229Glu polymorphism in control and hyperbilirubinemic patients. We did not find 521(T→C) and 1463 (G→C) in our population.

3. 388G>A polymorphism was found to be present in 10 patients in homozygous state and 39 patients in heterozygous state on the other hand this polymorphism was also found in control group also viz 26 patients in homozygous state and 5 patients in heterozygous state. It is the most common polymorphism in our population which is associated with hyperbilirubinemia.

4. Subsequent automated DNA sequencing identified 4 novel mutations viz. 369(T→A), 370(T→A) and 411(G→T) in exon 4 of OATP 2 gene and 523(T→C) in exon 5 of OATP 2 gene. Exon 4 and 5 of OATP 2 gene are the hotspots of mutations in Indian hyperbilirubinemia patients. All four infants had serum bilirubin more than 27mg/dL.

5. 523T>C variant (51% in homozygous) in hyperbilirubinemia group was significantly higher to that of control group (24% in homozygous). Allele
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frequency of c.523T>C was also significantly higher in hyperbilirubinemia group as compared to control (24%).

6. Pathological prediction using SIFT and PolyPhen programs revealed that 4 novel substitution 370(T→A) and 523(T→C) mutations were predicted to be tolerated mutations. Threshold value for pathological mutations was ≤ 0.5. None of the mutations were found to have any splicing defects.

8. There was no significant change among the different individual variants of OATP2 gene in gestation age, birth weight and ABE as compared to wild type. Notably acute bilirubin encephalopathy (ABE) was found to be significantly higher in compound variants as compared to wild type. However, the serum total bilirubin was significantly higher in all variants as compared to wild type.

**6.4 G6PD Gene:**

1. Identification of the most common Mediterranean mutation and Orissa mutations of G6PD gene by Polymerase Chain Reaction (PCR) based Restriction Fragment Length Polymorphism (RFLP) Analysis.

2. We did not find any significant difference in various parameters in G6PD Mediterranean and Orrisa mutation.

3. There was a trend for earlier day of peak STB as the TA repeat number increased (p=0.068). We did not find any significant interaction of TA repeat numbers and G6PD deficiency and their effects on jaundice parameters.

4. Presence of variant (TA)n promoter (OR 11.54, 95% CI 3.3 – 40.1) and G6PD deficiency (OR 41.7, 95% CI 4.6 – 378.1) were independent risk factors associated with significant hyperbilirubinemia.

We did not find any significant difference in birth weight, Phototherapy, ET and bilirubin encephalopathy in the interaction of UGT1A1, OATP2 and G6PD gene mutations/polymorphisms. The peak STB of OATP2 and UGT1A1/OATP2 group were >26 mg/dL as compare to other groups. The variation in the OATP2 and UGT1A1 genes are also at high risk to develop severe hyperbilirubinemia.
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Conclusion: In conclusion, clinical and genetic data demonstrates the prevalence of UGT1A1, OATP2 and G6PD gene mutations in hyperbilirubinemic neonates in India. In bilirubin metabolism, increased destruction of erythrocytes, defect in the function of organic anion transporter polypeptide 2 (OATP2) or UDP-glucuronosyltransferase 1A1 (UGT1A1) may result in unconjugated hyperbilirubinemia. It was observed that G6PD deficiency, polymorphisms/mutations in the UGT1A1 as well as OATP2 gene were associated with severe hyperbilirubinemia in neonates. The (TA)n promoter polymorphism of bilirubin UGT1A1 gene was significantly associated with hyperbilirubinemia in term and late-term neonates. The Gly71Arg mutation of bilirubin UGT1A1 was not found in either neonates with hyperbilirubinemia or controls. Ala72Pro polymorphism was found to be the most common mutation at the codon position 72 of the exon 1 of bilirubin UGT1A1 gene. 1091C>T was second most common variation with homozygous form with allele frequency 32% in our population. 16 novels mutations and one already known mutation were found using SSCP and subsequent DNA sequencing in UGT1A1 gene. The allele frequency of variant (TA)7 of UGT1A1 gene in our study population and controls was 48.7% and 29%, respectively. We did not find 521(T—>C) and 1463 (G—>C) in our population in OATP2 gene. 4 novel mutations were found in OATP2 gene by DNA sequencing. Exon 1, 3, 5 of UGT1A1 and exon 4 and 5 of OATP2 gene are the hotspots of mutations in Indian hyperbilirubinemia patients. The new knowledge on the association of UGT1A1, OATP2 and G6PD genes variants with neonatal hyperbilirubinemia warrants further study to elucidate their precise role and mechanism.