The present study was performed to investigate the spectrum of polymorphisms and mutations in UGT1A1, OATP 2 and G6PD genes in hyperbilirubinemic neonates.

Hyperbilirubinemia is one of the most common problems encountered in term newborns. Historically, management guidelines were derived from studies on bilirubin toxicity in infants with hemolytic disease. More recent recommendations support the use of less intensive therapy in healthy term newborns with jaundice. Although up to 60% of term newborns have clinical jaundice in the first week of life, few have significant underlying disease (Behrman et al., 2000). However, hyperbilirubinemia in the newborn period can be associated with severe illnesses such as hemolytic disease, metabolic and endocrine disorders, anatomic abnormalities of the liver, and infections. Jaundice typically results from the deposition of unconjugated bilirubin pigment in the skin and mucus membranes. Depending on the underlying etiology, this condition may present throughout the neonatal period.

Infants without identified risk factors rarely have total serum bilirubin levels above 15 mg per dL. As the number of risk factors increases, the potential to develop markedly elevated bilirubin levels also increases (Behrman RE et al., 2000). G6PD deficiency, ABO incompatibility, Rh incompatibility, sepsis, bleeding into tissues like cephalhematoma, intrauterine infections, use of oxytocin in the mother, polycythemia, galactosemia, hypothyroidism, etc are the major causes of pathological jaundice (Narang et al., 1997). Inspite of the usual investigation 34% of cases are labelled as ‘Idiopathic’ as no cause is found. In recent years, many of these etiologies have been found to have a genetic origin in various parts of the world (Najati et al., 2010).

The serum total bilirubin (STB) concentration at any point represents the contributing force of bilirubin production, primarily from the catabolism of heme, on the one hand and the elimination of bilirubin from the body, by hepatic conjugation and subsequent excretion, on the other (Dennery et al., 2001). As long as there is equilibrium between these processes, the STB should remain within the physiologic range and does not pose a danger (Kaplan et al., 2002). However, bilirubin production-elimination imbalance may lead to excessive accumulation of bilirubin with resultant hyperbilirubinemia.
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The bilirubin conjugation process is governed in the hepatocyte by the conjugating enzyme UGT1A1 (Kaplan et al., 2003). For neonates, hyperbilirubinemia is mainly caused by abnormal liver function, haemolysis and/or genetic defect (Clarke et al., 1997; Beutler, 1994; Huang et al., 2002a; Huang et al., 2005). Unconjugated bilirubin is produced mainly by the turnover of erythrocytes. It may be transported by organic anion transporter polypeptide 2 (OATP 2) to the liver (Cui et al., 2001; Huang et al., 2005), it then is conjugated by glucuronosyltransferase 1A1 (UGT1A1) before being excreted into the bile (Clarke et al., 1997; Huang et al., 2005). Another genetic defect, glucose-6-phosphate dehydrogenase (G6PD) deficiency, an X-linked abnormality, is most-commonly seen genetic defect, affecting over 400 million individuals worldwide (Beutler, 1994; Huang et al., 2005). As a consequence of G6PD deficiency amongst neonates, the mean life span of erythrocytes may be shortened somewhat, and/or lowgrade haemolysis may occur, and both situations may lead to the increased production of bilirubin (Beutler, 1994). Chromosomal mapping studies revealed that UGT1A1 and OATP2 genes are located at chromosomes 2q37 and 12p12, respectively (Clarke et al., 1997; Tamai et al., 2000), whereas the G6PD gene is located at Xq28 (Beutler et al., 1994).

The results of our study demonstrated that variation of the UGT1A1, OATP2 and G6PD genes are the important risk factors for unconjugated hyperbilirubinemia amongst North Indian neonates. Detailed analyses revealed that variation in the UGT1A1 gene is the major risk factor for unconjugated hyperbilirubinemia, whereas G6PD deficiency and a variant OATP2 gene are additive risk factors.

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.
5.1 Identification and Molecular Characterization of Mutations/polymorphisms in UGT1A1 gene:

5.1.1 Analysis of most common/known polymorphisms of UGT1A1 gene in neonatal Hyperbilirubinemia:

Genetically determined unconjugated hyperbilirubinemia constitutes a spectrum of clinical entities characterized by augmentation of serum bilirubin values related to graded reduction of UGT1A1 enzyme activity. In this study, we have characterized the molecular defects in UGT1A1 gene by analysing the whole coding sequence of the 5 exons. First of all, we established allele frequency of four most common polymorphisms (211G>A, 686C>A, 1091C>T and 1456T>G) in Indian hyperbilirubinemic neonates. PCR-RFLP method was based on the fact that all mutations create a different restriction site in different exons G71R creates a new AvaiI site in exon 1 of UGT1A1 gene, 686C—>A creates a new Bsr I site in exon 1 of UGT1A1 gene, 1091C—>T creates a new Bsl I site in exon 3 of UGT1A1 gene and 1456T—>G create a new AvaiI site in exon 5 of UGT1A1.

We did not find any Gly71Arg (211G>A) mutation either in hyperbilirubinemics or controls in our population. We found Ala72Pro (214G>C) polymorphism at the codon position 72 of the exon 1 of bilirubin UGT1A1 gene. 214G>C of exon 1 was found to be the most common polymorphism of UGT1A1 gene from our population. In present study, the neonates who carry variation 214G>C in the UGT1A1 gene are at high risk for experiencing severe hyperbilirubinemia. In this study genotype distribution of these variation revealed that A72P was predominantly present in heterozygous state. It has been seen that polymorphisms found in exon 1 might reduce the affinity of the ligand, bilirubin, for the catalytic site in the amino- terminal half of the molecule, which is encoded by exon 1 of the UGT1A1 gene (Agrawal et al., 2009). The G211A missense UGT1A1 coding sequence mutation that underlies Gilbert syndrome in Asian populations (Huang et al., 2004; Huang et al., 2002a; Kamisako, 2004; Takeuchi et al., 2004; Yamamoto et al., 2002) was not observed in our population. Yamamoto et al. (1998) reported that UGT1A1 enzyme activities of the G71R variations in the heterozygous or homozygous state were decreased to 60.2 and 32% of normal respectively (Yamamoto et al., 1998). These decreased enzyme activities are thought to cause delayed elimination of bilirubin (Clarke et al., 1997) and ultimately occurrence of
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hyperbilirubinemia. The conversion of G→A at nucleotide 211 (G71R) is the predominant variation and is highly associated with neonatal hyperbilirubinemia and adult hyperbilirubinemia in Taiwanese population (Huang et al., 2002; Huang et al., 2002; Huang et al., 2000; Huang et al., 2005).

In our study, P229Q (686C>A) was found to be present in three hyperbilirubinemic neonates in heterozygous state. However 686C>A is in linkage disequilibrium with variation in the promoter area in Taiwanese population (Huang et al., 2002a; Huang et al., 2000; Huang et al., 2005; Huang et al., 2002b). 1091C>T was second most common variation with homozygous form with allele frequency 32% in our population in 18 subjects and 1 control (Table 4.4). Pro364Leu (1091C>T) was mostly reported in Chinese (Huang et al., 2002, Huang et al., 2002, Huang et al., Huang et al., 2002). Homozygous variant of nucleotide 1091C>T polymorphisms of UGT1A1 gene was a significant risk factor associated with severe hyperbilirubinemia among Indian, Malaysian and Chinese newborns (Boo et al., 2009). Allele frequency of other two variants i.e. 686C>A and 1456T>G were not significantly different among controls and subjects. However, 1456T>G variation was found to be present in four hyperbilirubinemic neonates in heterozygous state and STB of all four neonates were >25mg/dL and 2 neonates had kernicterus. 1456T>G may be the risk factor of neonatal hyperbilirubinemia Two polymorphisms viz 214G>C and 1091C>T were most common polymorphisms with the frequency of 26.3% and 16.7% respectively indicating exon 1 and exon 5 are hot spots of the UGT1A1 gene polymorphisms.

We also attempted to establish correlation between genotype and phenotype. Four subjects who had 214G>C and 1091C>T polymorphism as compound homozygous variation had serum bilirubin level >24 mg/dL. Notably, other two cases having 1091C>T variant had significant hyperbilirubinemia (>25mg/dL) and showed kernicterus. Our study showed that the subjects who carrying 214G>C and 1091C>T polymorphism of UGT1A1 gene were at high risk for significant hyperbilirubinemia.

5.1.2 Identification and characterization of unknown mutation in UGT1A1 gene in neonatal Hyperbilirubinemia:

For unknown mutation analysis of all five exons two sets of primers were used. Various Conditions were established for ideal SSCP viz. running temperature, concentration of acrylamide, concentration of running buffer, concentration of glycerol and time required
for denaturation of the PCR products. SSCP of the amplified product was run at 100 volts for 18 hours at 10°C. Subsequent automated DNA sequencing identified 17 mutations in which 1 was already reported (D359N) and 16 were novel mutations. These 16 novel mutations included nine missense mutations (A64S, D70H, G71E, S134N, D146Y, S191P, R257K, W335R and L489V), five silent mutations (I57I, K114K, S334S, T349T and T482T), one frame shift mutation (1363 ins C) and one nonsense mutation (E534X). Even though, only one subject developed kernicterus in R257K mutation who had peak bilirubin level of 25.6mg/dL. In two cases 214G>C alone and along with the presence of one mutation D70H showed kernicterus and serum bilirubin levels were more than 21mg/dL in both subjects. D70H alone was present in 3 hyperbilirubinemic neonates which resulted in aspartic acid to histidine change at nucleotide 208. It is noteworthy here that two cases of kernicterus were associated with homozygous status of variant. UGT1A1 polymorphism 1941C>G (Saeki et al., 2007) have been showed to be related to TSB levels. The high incidence of hyperbilirubinemia may be attributable to the high frequency of missense mutations (Maruo et al., 1999). A study by a Japanese group showed that silent mutations were able to change the rate of protein folding, and thus affect the affinity of the protein toward substrate binding (Kimchi et al., 2007).

The effect of genetic mutations on phenotype is of significant interest in genetics. Output prediction scores were assessed for all the mutations identified and characterized in this study using computer based SIFT (Sorting Intolerant from Tolerant) programme. The threshold value for pathological mutations was ≤0.5 (http://blocks.fhrc.org/sift/SIFT.html). The amino acid substitution was predicted to be damaging if the score is ≤ 0.5 and tolerated if the score is > 0.5. Pathological predictions were further confirmed by another computer algorithm (http://genetics.bwh.harvard.edu/pph). Human Splicing Finder (HSF) matrix (http://www.umd.be/HSF/HSF.html) was used to predict any difference in splicing between the novel mutants and their respective wild-type reference sequences. By using sequence homology, these programs predicted the effect of all possible substitutions at each position. Nine 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 (Table 4.13). The software’s did not predict the affect
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of nonsense mutation and silent mutation since they are designed for predicting the effects of missense mutations only. The UGT1A1 polymorphisms have recently acquired significance because they predispose individuals to altered metabolism (Ostrow and Tiribelli, 2001; Esteban et al., 1999; Le Guellec et al., 1995; Ando et al., 2000). Exons 2-5 are shared by other UGT1A1 transcripts and isoenzymes that mediate metabolism of xenobiotics involved in bilirubin glucuronidation (Zucker et al., 2001). The identification of these novel mutations in the UGT1A1 gene, increasing the mutational spectrum of UGT1A1 allelic variants, contributes to a better understanding of the molecular pathology of disorders characterized by unconjugated hyperbilirubinemia.

5.1.3 Frequency of (TA)n promoter polymorphism:

The most common genetic polymorphism encountered with Gilbert’s syndrome in Caucasians is an additional TA insertion in the TATAA box of the UGT1A1 gene promoter. The usual sequence is (TA)6; insertion of additional (TA) sequences, resulting in a (TA)7 or occasionally (TA)8 causes progressively diminished expression of the UGT1A1 enzyme. The importance of this polymorphism varies with different ethnic populations.

In the present study, we found a higher frequency of (TA)n promoter polymorphism in hyperbilirubinemic subjects compared with controls. This observation is in accordance with the findings of Laforgia et al. who found significantly higher frequency for the variant (TA)7/(TA)7 genotype in Italian neonates with STB concentrations ≥13 mg/dL compared with controls whose STB values did not exceed that concentration (26.8% vs. 12.2%, p <0.05) (Laforgia et al., 2002). However, in case-control studies done on Turkish neonates, no significant difference in the frequency of variant promoter between jaundiced babies and controls were found (Babaoglu et al., 2006; Muslu et al., 2007).

The allele frequency of variant (TA)7 in our study population and controls was 48.7% and 29%, respectively. The allele frequency of (TA)7 reported in healthy adult population of Indian origin was 35% in a study done on migrated Indians in Singapore (Balram et al., 2002), 38% in a report from Eastern India (Farheen et al., 2006) and 41% in a study from Northern India (Premawardhena et al., 2003). The variant (TA)8 allele has been reported earlier in hyperbilirubinemic subjects, with the allele frequency being 5.8% in Indian population (Agrawal et al., 2009). This allele was first described by
Beutler et al. (Beutler et al., 1998) in 6.9% of healthy individuals of North and Central America with varying degrees of African ancestry. Moreover, the UGT1A1-enzyme activity was observed to have decreased progressively as the number of TA repeats in the promoter area of the UGT1A1 gene increased from five to eight (Bosma et al., 1995; Beutler et al., 1998). The variations in the promoter area of the UGT1A1 [A(TA)yTAA] instead of [A(TA)xTAA] is reportedly related to Gilbert’s syndrome for Caucasian individual (Bosma et al., 1995; Monaghan et al., 1996), whilst variants in the promoter and within the coding region of this gene are involved in Gilbert’s syndrome for Asian population (Huang et al., 2005; Hsieh et al., 2001; Sato et al., 1996).

5.1.4 Effect of TA repeats number on jaundice parameters:

The serum bilirubin levels of individuals homozygous or even heterozygous for 7 TA repeats have been found to be higher than those with wild type 6 TA repeats in adult patients of Gilbert syndrome (Bosma et al., 1995; Kadakol et al., 2001). Supportive of the concept that the mechanism for this hyperbilirubunemia is diminished bilirubin conjugation includes the demonstration that UGT1A1 enzyme activity was lowest in hepatic tissue from homozygotes for the variant 7/7 (749 nmol/g liver/h; p<0.005) and intermediate in 6/7 heterozygotes (985 nmol/g liver/h; p<0.05), compared with normal 6/6 homozygotes (1565 nmol/g liver/h) (Raijmakers et al., 2000). Similarly Bancroft et al (measured transcutaneous bilirubin index in first 2 days of life) and Roy-Chowdhury et al (STB values at 96 hours of life) found higher values in those homozygous for the variant A(TA)7TAA promoter compared to homozygous normal A(TA)6TAA controls (Bancraft et al., 1998; Roy Chowdhury et al., 2002). However in these studies they were recording STBs at fixed intervals, while we have recorded only peak STBs. In addition, majority of babies in our study received phototherapy and/or exchange transfusion which would have altered the natural peak STB values. The duration of phototherapy could be a surrogate marker for severity of jaundice but again that will be modified with exchange transfusions as 70% of our babies underwent exchange transfusion. Similar to our findings, the studies done by Ulgenalp A and Muslu N (both case-control study) did not find any significant difference in the mean peak STB values between different genotype groups (Ulgenalp et al., 2003; Muslu et al., 2007). In the study by Muslu et al., onset of jaundice and numbers requiring phototherapy and exchange transfusion were also not significantly different between 6/6 groups compared to 6/7 + 7/7 group.
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In their study, using a reporter gene, they showed that there is an inverse relationship between number of TA repeats and the activity of promoter through the range of 5 to 8 TA repeats. (TA)8 allele is very rare in Caucasians; despite many published studies, only a few cases have been described (Coelho et al., 2004; Ostanek et al., 2007). Bancroft et al., (Bancroft et al., 1998) who measured transcutaneous bilirubin index in first 2 days of life and Roy-Chowdhury et al. (Roy-Chowdhury et al., 2002) who measured STB values at 96 hours of life found higher values of STB in neonates homozygous for the variant (TA)7 promoter compared with homozygous normal (TA)6 controls. The onset of jaundice and peak STB values in the various genotype groups in our study was not different statistically. However, there was a trend of earlier attainment of peak STB and increasing duration of phototherapy as TA repeat number increased. This may be because the majority of babies in our study received phototherapy and/or exchange transfusion, which may have altered the peak STB. The duration of phototherapy can be a surrogate marker for severity of jaundice but again that could have been modified with exchange transfusions, as 70% of the jaundiced babies underwent exchange transfusion.

Our findings reveal that it is crucial to carry out detailed surveys on genetic variations in and around the UGT1A1 gene and functional studies on these variants for a deeper understanding of quantitative anomalies of bilirubin. Further, the study also demands investigations into the functional aspects of novel variants in cell based system to predict their pathological significance.

**5.2 Identification and Molecular Characterization of Mutations/polymorphisms in OATP 2 gene:**

**5.2.1 Analysis of most common mutations of OATP 2 gene in neonatal Hyperbilirubinemia:**

The locus of the OATP 2 gene is at chromosome 12p12 (Tamai et al., 2000). There are 2073 nucleotides and 14 exons in this gene (Tamai et al., 2000). It has been reported that OATP 2 is involved in the transportation of both conjugated and unconjugated bilirubin (Cui et al., 2001). A variant of Organic anion transporter polypeptide 2 gene has been incriminated in unconjugated hyperbilirubinemia in Taiwanese population (Huang MJ et al., 2004). To date very little information is available on the prevalence of genetic polymorphisms of OATP 2 gene among the Indian population despite increasing
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evidence supporting a role of these transporters in the hepatocellular uptake of endogenous compounds and drugs. Genetic analysis for the presence of most common polymorphisms in OATP 2 gene was carried out by using PCR based RFLP. The method was based on the fact that all mutations create a different restriction site in different exons: 388G>A creates a new Taq I site in exon 4 of OATP 2 gene, 463C>A creates a new Hpa II site in exon 4 of OATP 2 gene, 521T>C creates a new Hha I site in exon 5 of OATP 2 gene and 1463G>C create a new Alu I site in exon 10 of OATP 2 gene. It was demonstrated recently that OATP 2 is responsible for the transportation of organic anions into hepatocytes (Cui et al., 2001). This mechanism may also be involved in the transportation of unconjugated bilirubin (Cui et al., 2001). Variations at nucleotides 388, 463, 521, and 1463 in the OATP 2 gene have been reported (Tirona et al., 2001; Nozawa et al., 2002). In previous studies, only the G→A base variation (D130N) at nucleotide 388 was shown to significantly increase the risk of nonphysiological unconjugated hyperbilirubinemia (Huang et al., 2004; Tirona et al., 2001; Tamai et al., 2000; Nozawa et al., 2002; Nishizato et al., 2003). High affinity uptake of unconjugated bilirubin by OATP2 occurred in the presence of albumin (Silverman, 2000; Fromm, 2000). In vitro, OATP2 has been shown to transport both unconjugated and conjugated bilirubin (Kalliokoski and Niemi, 2009; Cui et al., 2001; Niemi et al., 2009). Also Cui et al showed that bilirubin bound to albumin is taken up across the basolateral membrane by OATP2. The differentiation between carrier-mediated and diffusional bilirubin uptake into the liver is supported by the identification of mutations in the OATP2 (SLC21A6) gene leading to the loss or functional impairment of OATP2 in the basolateral membrane of hepatocytes (Kalliokoski and Niemi, 2009; Cui et al., 2001).

In this study, we have characterized previous reported polymorphisms (Table 4.20) and novel polymorphisms/mutations in OATP 2 gene (Table 4.21). Allele frequency of 388G→A (Asn130 Asp) variance was significantly higher in hyperbilirubinemic group (51.8%) which is consistent with the findings of Huang et al who reported that 388G>A variant of OATP2 gene as a significant risk factor associated with hyperbilirubinemia (Huang et al., 2004; Wong et al., 2009). The change of amino acid (aspartic acid to asparagine, encoded by nucleotides 388–390) at codon 130 of OATP 2 may reduce the function in unconjugated bilirubin uptake (Huang et al., 2004). 388A>G variant of OATP 2 gene was highly polymorphic in Malaysian population, Chinese population, Taiwan and Japanese populations (Wong et al., 2009; Huang et al., 2004; Zhang et al., 2001).
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2010). In our study, neonates who had the variants 388 in the OATP2 gene were at high risk to develop severe hyperbilirubinemia. In our study, 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. Buyukkale et al., found that in males with the 388 A>G mutation of OATP-2, unconjugated bilirubin in plasma was significantly increased compared with females who have the same mutation. (Buyukkale et al., 2011). Other studies also suggested that c.388G>A of OATP2 was the most common and highly polymorphic in Asian Population with special reference to Malaysian, Chinese and Taiwanese population (Xu et al., 2007; Jada et al., 2007; Wang et al., 2003). The frequency of c.521T>C variant was found to be 13% in Chinese, Taiwanese and Japanese population (Wong et al., 2009; Jada et al., 2007; Nozawa et al., 2002; Yamada et al., 1992). A388G and T521C polymorphisms have been previously reported in association with unconjugated hyperbilirubinemia (Huang et al., 2005; Huang et al., 2004; Ieiri et al., 2004) and at allele frequencies intermediate of European American and African American descent (Ho et al., 2007). Expression of OATP 2 gene variants resulting in putative OATP 2 loss of function haplotypes (Ho et al., 2006) was observed but, infrequently. Although more recent observations called into question a direct role of OATP 2 in hepatic bilirubin uptake, (Ho et al., 2007; Wang et al., 2003), Huang et al., (Huang et al., 2004) reported a significantly increased risk for bilirubinemia ≥20 mg/dL (≥342μmol/L) in Taiwanese newborns who were homozygous for the A388G OATP 2 variant. Taiwanese carry the variations only at nucleotide 388 and 521. Variation at nucleotide 521T>C reduces the transportation function for estrone-3-sulphate to <50% of normal in a experiment using HeLa cells (Tirona et al., 2001). In our study, genotype and allele frequencies of c.463C>A variant were not different among hyperbilirubinemic and control groups. The genotype frequency of c.521T>C was found to be 14% in European-Americans and c.1463G>C was observed to be 9% in African-Americans (Tirona et al., 2001). In our study we did not find 521T>C variant. Instead, we found 523T>C novel variant by DNA sequencing. Genetic analysis revealed that c.523T>C variant (51% in homozygous) in hyperbilirubinemia group was significantly higher to that of control group (24% in homozygous). Allele frequency of c.523T>C was also significantly higher in hyperbilirubinemia group as compared to control (24%).
The results of our study suggest that variations of G to A at nucleotide 388 and T to C at nucleotide 521 are more important variants of OATP2 gene regarding disease susceptibility which is consistent with the reports of others (Wong et al., 2009; Michalski et al., 2002; Tirona et al., 2001). Recently, Huang et al (2005) demonstrated that change of amino acid at either codon 130 or codon 174 of OATP 2 may reduce the normal functional level of unconjugated-bilirubin eliminated in Taiwanese adults (Michalski et al., 2002). Huang and colleagues demonstrated 3-fold increase risk of marked hyperbilirubinemia in infants with an OATP2 gene polymorphisms at position 388 (Wong et al., 2009). These results inferred that the variants nucleotide 388 and nucleotide 523 in the OATP2 gene may alter certain functions of the transporter OTAP2. Similarly, in an experiment using He La cells, the variant nucleotide 521 reduced the transportation for function estrone 3 sulfate to less than 50% of normal (Tirona et al., 2001).

**5.2.2 Identification and characterization of unknown mutations of OATP 2 gene in neonatal hyperbilirubinemia:**

Furthermore, three novel polymorphism viz. c.370T>A (Ser124Thr), C.411G>T (Ser137Ser) in exon 4 and c.523T>C (Phe175 Leu) in exon 5 were identified in OATP2 gene from Indian population by DNA sequencing. Genetics analysis revealed that genotype frequency was significantly higher in hyperbilirubinemic group as compared to controls (Table 4.19). Most of the genotypes of these novel polymorphisms in hyperbilirubinemic infants were in homozygous form. Importantly, a SNP c.369T>A in exon 4 of OATP2 gene was detected in four hyperbilirubinemia infants which generate a stop codon at 123 position (Figure 4.43). This nonsense mutation in mRNA transcript will translate a truncated non-functional OATP transporter. Interestingly, as per the nature of this mutation, all four infants had serum bilirubin more than 27mg/dL. This mutation in all the four infants was found in homozygous form in this study. Hence, these findings suggest that variants of OATP2 gene are significant risk factors as observed in case of UGT1A1 gene (Michalski et al., 2002).

Several variants were found at a relatively high incidence (A388G> C463A>T521C >A2000G> G1463C), and their genotypic frequencies appear to be dependent on race (36). Interestingly, Tirona et al., reported that Gly488Ala (OATP-C protein) amino acid substitution takes place in an area of the protein that could be considered a signature
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motif for all members of OATP transporter family. However, these SNPs were found to have modest changes in OATP transporter activity (Tirona *et al.*, 2001). We also checked the effect of our novel polymorphisms at the website SNP structure, function, disease. A primary purpose of the SNPs3D resource is to provide a means of selecting candidate genes likely to influence disease susceptibility, and to further select the most relevant non-synonymous SNPs within those genes. Pathological predictions were further confirmed by another computer algorithm (http://genetics.bwh.harvard.edu/pph). Human Splicing Finder (HSF) matrix (http://www.umd.be/HSF/HSF.html) was used to predict any difference in splicing between the novel mutants and their respective wild-type reference sequences. The underlying genetic change was 370T>A and 523T>C mutation was predicted to be a tolerable mutation by polyphen with an output prediction score of 0.00 and 0.005 (threshold ≤ 0.5) (Table 4.17).

5.2.3 Jaundice Parameter in the study group according to polymorphisms status in OATP 2 gene:

In our study there was no significant difference among (370(T→A), 388(G→A), 411(G→T) and 523 (T→C) variants in gestation age, birth weight and ABE as compared to wild type (Table 4.21). Notably acute bilirubin encephalopathy was found significantly higher in compound variants (370(T→A)+ 411(G→T), 370(T→A)+ 523 (T→C), 411(G→T)+523 (T→C), 370(T→A)+ 411(G→T)+ 523 (T→C) and 388(G→A) +523 (T→C)) as compared to wild type (Table 4.22) whereas it was not significantly higher in individual variants (Table 4.21). However, the serum total bilirubin was significantly higher in all variants as compared to wild type (Table 4.21). There was slightly elevated level of STB in 388(G→A) and 370(T→A) variants as compared to 411(G→T) and 523 (T→C) variants (Table 4.21). The mean serum bilirubin levels in the compound variants with three variants (370(T→A) + 411(G→T)+523 (T→C), STB was 27.6 mg/dL and in two compound variants 370(T→A) and 411(G→T), STB was 28.1mg/dL. There were significantly higher as compared to individual variants (Table 4.22). Moreover, the need of phototherapy was also significantly higher in individual variants infants as compared to compound variants (Table 4.21 and Table 4.22). Therefore 370(T→A), 411(G→T) and 523 (T→C) variation could be the genetic marker of OATP 2 gene in hyperbilirubenic neonates.
5.3 Identification and Molecular Characterization of Mutations in G6PD gene:

The etiological relationship between G6PD deficiency and neonatal hyperbilirubinemia has been confirmed by several studies. G6PD-deficient babies are 3-fold more prone to neonatal jaundice than G6PD-deficient infants (Al-Naama et al., 1987; Dawodu et al., 1998). Most G6PD-deficient individuals are entirely asymptomatic and develop symptoms only in response to oxidant stress. G6PD deficiency does not affect the life expectancy of affected individuals. G6PD deficiency is the most common red cell enzymopathy to cause neonatal hemolysis and jaundice. Good population data are available from West Africa (Bienzle, 1981), the Mediterranean (Milbauer et al., 1973) and the Far East (Tan, 1981) and it is clear that perhaps as many as one third of all males with neonatal jaundice have G6PD deficiency, a similar proportion of male children with G6PD deficiency developing neonatal jaundice (NNJ). Early observations suggested that the incidence of NNJ was significantly lower among subjects of African Ancestry in the USA than in Africa. It is, however, clear that all subjects with G6PD are at increased risk of NNJ (Bienzle U et al., 1976) and kernicterus has been reported in all at-risk population groups. Public health programs have significantly reduced the incidence of kernicterus in some parts of the world viz Singapore (Tay, 1995) but not others viz Pakistan (Rehman et al., 1995). It has long been assumed that these factors combined with an increased susceptibility of neonatal erythrocytes to hemolysis, give rise to an increased incidence and extent of jaundice.

However, recent evidence suggests that hemolysis is only partly responsible; decreased bilirubin conjugation and elimination playing a major role in the pathogenesis of NNJ (Kaplan et al., 1996). The presence of an additional hemolytic process such as ABO incompatibility was found to have little impact on the degree of hemolysis and hyperbilirubinemia (Kaplan et al., 1998). These observations confirm those first made in Sardinia, where the severity of NNJ does not correlate with red cell G6PD activity (Meloni et al., 1987), and hyperbilirubinemia is largely the result of an impairment of liver function caused by G6PD deficiency in the liver (Oluboyede et al., 1979).

The prevalence of G6PD deficiency is more in severe hyperbilirubinemia as compared to moderate hyperbilirubinemia (Behjati-Ardakani et al., 2007). In our study out of 107 we
found 19 babies with the Mediterranean mutation (12 were hemizygous males, 6 were heterozygous female and 1 was homozygous female) and 3 babies with Orissa mutation (2 were hemizygous male and 1 was heterozygous female). The mean enzyme activity for the G6PD normal males (n = 24) was 6.45 ± 2.12 U/g Hb. Hence a G6PD activity of less than 2.21 U/g Hb (mean – 2SD of normal value) was taken as being indicative of deficiency in males. The mean enzyme activity for the G6PD normal females (n = 21) was 6.05 ± 1.61 U/g Hb. Hence a G6PD activity of less than 2.83 U/g Hb (mean – 2SD of normal value) was taken as being indicative of deficiency in females. We did not find any significant difference in various parameters in G6PD Mediterranean and Orissa mutation but there were only 3 infants with G6PD Orissa mutation.

5.4 Interaction of TA repeats and G6PD deficiency and their effects on jaundice Parameters:

There was no significant interaction between G6PD deficiency and TA promoter polymorphism in our study. Two Italian case-control studies also reported no significant interaction between G6PD deficiency and (TA)n promoter polymorphism among those who developed hyperbilirubinemia (Galanello et al., 1999; Iolascon et al., 1999). However, Kaplan and Hammerman (Kaplan and Hammerman, 2005b) described that in G6PD deficient Israeli neonates, superimposition of the variant UGT1A1 promoter resulted in a significant increase in the incidence of hyperbilirubinemia in an allele dose dependent response. However, their study design was different in that, they took G6PD deficient and G6PD normal neonates with no other risk factors for neonatal hyperbilirubinemia (except G6PD deficiency) and reported the percentage developing hyperbilirubinemia with respect to their UGT1A1 variant genotype status. When Kaplan et al. (Kaplan et al., 2001b) analyzed their data in the manner we have done, they did not find any significant interaction between G6PD deficiency and TA promoter polymorphism. Therefore, the differences in the findings among various studies may be related to study design or the interactions may actually be population specific. Similarly, Huang et al have shown the additive effect of G6PD deficiency in neonates carrying the variant G71R in the exon 1 of UGT1A1 in the genesis of hyperbilirubinemia (Huang et al., 2002b). G6PD deficiency plays a major role in the pathogenesis of severe neonatal hyperbilirubinemia in Nigeria. Slusher et al., (Slusher et al., 1995) found that 31% of 45 clinically jaundiced infants were G6PD deficient of whom 36% died of presumed...
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kemicterus. Similarly, Owa (Owa, 1989) reported that 62.3% of 106 severely jaundiced Nigerian neonates were G-6-PD deficient compared with 13.3% of 128 in non-jaundiced controls. Boo et al 2009, showed that it was G6PD level rather than the actual level of G6PD enzyme that was significantly associated with the development of severe hyperbilirubinemia (Boo et al., 2009). Hence it is possible that there might be an interaction between both in producing hyperbilirubinemia but because of different study design, we were unable to pick it up.

5.5 Interaction of UGT1A1, OATP 2 and G6PD and their effects on jaundice Parameters:

Severe neonatal hyperbilirubinemia is a prototypic pediatric complex trait or disorder that is both prevalent (≥1%) in the newborn population (total serum bilirubin ≥20 mg/dL) and the product of multiple gene loci each with relatively weak effects interacting with other genes and environmental contributors (Mathew, 2001; Badano et al, 2002). The gene variants that underlie complex disorders are characteristically common in the population, often at allele frequencies of 20% or higher, (Eichner et al., 2002) carried by affected and unaffected individuals alike. Such nonsynonymous polymorphisms are individually of little overt functional impact (ie, not physiologically disruptive but when coexpressed can collectively play an important modulatory role in defining phenotype and risk). It is the contribution of multiple different coexpressed susceptibility genes, individually conferring a small increase in risk, that is required coupled with environmental factors to generate complex disorder phenotypes. Severe neonatal hyperbilirubinemia is no exception, as evidenced by the reported allele frequencies of UGT1A1 and OATP 2 polymorphisms and their modulatory role in the genesis of marked neonatal hyperbilirubinemia when coexpressed with each other and/or other icterogenic conditions (Kaplan et al, 1997; Huang et al., 2004; Huang et al., 2002c; Maruo et al., 2000; Yamamoto et al., 2002).

Table 4.28 showed the interaction of UGT1A1, OATP 2 and G6PD gene mutations/polymorphisms on jaundice parameters. There was not significant difference between the above genotypes for birth weight, Phototherapy, ET and bilirubin encephalopathy. The peak STB of OATP2 and UGT1A1+OATP2 group were >26 mg/dL as compare to other groups. After Post doc analysis there was a significant
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difference in TSB between (UGT1A1 + OATP2) and OATP2 group with UGT1A1, G6PD, (UGT1A1+OATP2+G6PD) and wild type groups (Table 4.28).

5.6 Future research:

In all likelihood, additional genes are involved beyond the 3 studied herein, and their identification is warranted. Knowledge of each susceptibility gene polymorphism is essential to understanding more fully the molecular pathogenesis of neonatal hyperbilirubinemia, providing genetic markers for clinical risk assessment, and characterizing potential novel therapeutic targets, all meritorious lines of future investigation. The degree of genetic heterogeneity and variant coexpression across UGT1A1, OATP 2 and G6PD genes observed in this cohort underscore the likely complex polygenic nature of neonatal hyperbilirubinemia. A more comprehensive study is warranted. However, due to strong evidence in the literature, genetic polymorphism still should be considered in clinical management, especially in those neonates who have existing risk factors, in order for early intervention to take place and to prevent neurotoxicity. Denaturing high performance liquid chromatography (DHPLC) serves as a rapid, accurate and inexpensive method for genetic diagnosis. Awareness of bilirubin conjugation genetics and the modern-day ability to identify genetic mutations of bilirubin conjugation may be useful adjuncts to the American Academy of Pediatrics (AAP) parameters for identifying neonates. DHPLC using ion-pairing reverse-phase chromatography columns is inexpensive, fast and powerful for analyzing the entire human genome, and allows automated detection of single-base substitutions, small insertions and/or deletions. Sensitivity and specificity ranges from 96% to 100%, compared with direct sequencing, and its clinical use in diagnosis of several genetic disorders has been reported (O’Donovan et al., 1998; Xiao and Oefner, 2001; Su et al., 2003).