Correlation analysis of UGT1A1 gene and neonatal hyperbilirubinemia based on MLPA-NGS technique

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Research Article

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Abstract

Objective: This study aims to explore whether there was a correlation between unexplained severe *neonatal hyperbilirubinemia* and the following six gene pathogenic variants: c.211G>A of UGT1A1 gene, c.388A>G, c.597C>T, c.521T>C of SLCO1B1 gene, c.175T>C, and c.7G>A of BLVRA gene.

Methods: This was a *case-control study.* The hyperbilirubinemia group, including 65 neonates with unexplained severe hyperbilirubinemia (serum total bilirubin level ≥342 μmol·L^{-1}), was hospitalized in the neonatal intensive care unit of Shanghai Children's Hospital from January 2019 to September 2020. The control group included 52 neonates with birth defects and serum total bilirubin level £221 μmol·L^{-1}. The six gene pathogenic variants mentioned above were investigated using MLPA-NGS technique. Pathogenic variants and *allele* frequencies were compared between the two groups.

Results: Compared *with* previous sequencing results, the concordance *rate of* c.211G>A was 98.3% (115/117), while all other pathogenic variants had a concordance *rate of 100%.* The pathogenic variants frequencies were as follows: c.388A>G of SLCO1B1 gene (93.84%)>c.7G>A of BLVRA gene (89.23%)> c.597C>T of SLCO1B1 gene (81.54%)> c.211G>A of UGT1A1 gene (69.23%)> c.521T>C of SLCO1B1 gene (26.15%)> c.175T>C of BLVRA gene (18.46%). *A allele* frequency of c.211G>A of hyperbilirubinemia group was 45.38%, *while A allele* frequency of control group was 21.15%. The differences were statistically significant, with c^2=14.988, P<0.001. No significant difference was observed in allele frequency of other gene pathogenic variants between the two groups, with P>0.05. Binary *logistic* regression results revealed that c.211G>A of UGT1A1 gene was a high-risk factor for unexplained severe *neonatal hyperbilirubinemia,* with OR=4.233, 95% CI: 1.896-9.451. No significant difference was observed in serum total bilirubin levels between mutation types.

Conclusions: c.211G>A of UGT1A1 gene was significantly correlated with unexplained severe *neonatal hyperbilirubinemia* and was a high-risk factor for the disease. MLPA-NGS technique was a highly accurate, rapid, and cost-effective method in identifying common and known gene loci of severe *neonatal hyperbilirubinemia. It can be employed as a point* mutation screening approach for detecting hyperbilirubinemia-related pathogenic variants.

Introduction

Hyperbilinemia occurs in 60% of newborns.The pathogenic causes of neonatal hyperbilirubinemia include hemolysis, various infective pathogens, and other causes. Neonatal hyperbilirubinemia varies in severity due to different etiologies and scavenging ability of bilirubin. Severe neonatal hyperbilirubinemia was defined as a TSB concentration above 20 mg/dL (342μmol/L)[1].Some children develop bilirubin encephalopathy due to high serum unconjugated bilirubin concentration as a result of an imbalance between increased bilirubin production and decreased conjugation rates. *Uridine diphosphate-glucuronosyl-transferase 1A1* (*UGT1A1*), the key enzyme in bilirubin metabolic pathway, is mainly distributed in the liver. *UGT1A1* gene polymorphisms reduce the activity of *UGT1A1*, eventually leading to elevated levels of unconjugated bilirubin[2]. Previous research revealed that 211G > A and (TA)7 polymorphisms of *UGT1A1* gene are linked to neonatal hyperbilirubinemia[3]. Indeed, 130 pathogenic variants have been discovered to date in *UGT1A1* gene[4]. However, our investigation of neonates with severe unexplained hyperbilirubinemia discovered that 14% of children do not have *UGT1A1* gene mutations. Several genes are involved in bilirubin metabolism; are there other genetic variants in these children? We reviewed the literature and found that *organic anion transporting polypeptide 2*(*OATP2*), *biliverdin reductase A* (*BLVRA*), and *sodium taurocholate co-transporting polypeptide* (*NTCP*), encoded by *SLCO1B1* and *BLVRA* genes, respectively, are involved in bilirubin metabolism, the pathogenic variants in these genes may play a key role in neonatal hyperbilirubinemia [5–7]. The study included six

polymorphisms: 211G > A polymorphism of *UGT1A1* gene, c.175T > C and c.7G > A polymorphisms of *BLVRA* gene, c.388A > G c.521T > C and c.597C > T polymorphisms of *SLCO1B1* gene. The reason for choosing these loci is that in previous studies on these genes, the pathogenic variants of these gene is a popular research topic, but the research conclusions are not completely consistent, so this study aimed to further investigate whether these pathogenic variants are correlated with neonatal hyperbilirubinemia. MLPA(Multiplex Ligation-dependent probe amplification)was first reported by Schouten et al in 2002. It is a new technique developed in recent years for qualitative and semi-quantitative analysis of DNA sequences to be examined. However, it has numerous disadvantages, including the fact that it can only detect 60 genic sites at a time. In terms of this study, relying on MLPA technology is time-consuming and costly, given the vast number of genic sites. MLPA-NGS technology combines MLPA technology with NGS (Next Generation Sequencing) technology, and changes fragments by length to distinguish fragments by sequence, and the detection site is much larger than that of MLPA technology. As a result, we have used MLPA-NGS technique, which is inexpensive, highly accurate, and specific to detect these above SNPs.

Methods

Subjects and Clinical Data

This was a case-control study. From January 2019 to September 2020, the hyperbilirubinemia group, including 65 neonates with unexplained severe hyperbilirubinemia, was hospitalized in the neonatal intensive care unit of Shanghai Children's Hospital. The control group included 52 neonates with birth defects. Inclusion criteria for hyperbilirubinemia group were as follows: 37 w≤ gestational age≤42 w, birth weight≥2500 g, postnatal age≤14 d, all subjects were Han Chinese, and serum total bilirubin level≥20mg/dL(342μmol/L)[1],at the same time, the direct bilirubin level < 2mg/dL(34.2μmol/L). The control group met the same inclusion criteria as the hyperbilirubinemia group, except that the serum total bilirubin level ≤12mg/dL(205μmol/L) and whole-exome sequencing was performed. Exclusion criteria included the following: asphyxia, hypoalbuminemia, hemolysis, G6PD deficiency, infection, and liver disease. The above clinical information was obtained by reviewing medical records. The Ethics Committee of Shanghai Children’s Hospital approved the study and written informed parental consent was obtained before patient enrollment.

Probe Design

The probe name,abbreviation and sequence:

probe name	abbreviation	Sequence(5’-3’)
UGT1A1-c.211G > A		
UGT1A1-c.211G > A-L	L03	CCTACACGACGCTCTTCCGATCTGTCCTAGCACCTGACGCCTCGTTG
UGT1A1-c.211G > A-M	M03	TACATCAGAGACRGAGCATTTTACA
UGT1A1-c.211G > A-R	R03	CCTTGAAGACGTACCCTGTGCCATTCCAACCCTTAGGGAACCCCGATC
SLCO1B1-388A > G		
SLCO1B1-388A > G-L	L11	CCTACACGACGCTCTTCCGATCTACAGTTACAGGTATTCTAAAGAAACTAATAGCR
SLCO1B1-388A > G-R	R11	ATTCATCAGAAAATTCAACATCGACCTTATTCCAACCCTTAGGGAACCCCGATC
SLCO1B1-c.521T > C		
SLCO1B1-c.521T > C-L	L15	CCTACACGACGCTCTTCCGATCTTCTGGGTCATACATGTGGATATATGY
SLCO1B1-c.521T > C-R	R15	GTTCATGGGTAATATGCTTCGTGTCCAACCCTTAGGGAACCCCGATC
BLVRA-c.7G > A		
BLVRA-c.7G > A-L	L24	CCTACACGACGCTCTTCCGATCTGTGACCGAAGGAAGAGACCAAGATGATTR
BLVRA-c.7G > A-R	R24	CAGAGGTGAGTTCTTTACAAAGACCAGTTTTCCAACCCTTAGGGAACCCCGATC
BLVRA-c.175T > C		
BLVRA-c.175T > C-L	L25	CCTACACGACGCTCTTCCGATCTGCTCGGGAGCATTGATGGAGTCCA
BLVRA-c.175T > C-M	M20	GCAGATTTCTYTGGAGGATGC
BLVRA-c.175T > C-R	R25	TCTTTCCAGCCAAGAGGTGGAGGTTCCAACCCTTAGGGAACCCCGATC
SLCO1B1-c.521T > C		
SLCO1B1-c.521T > C-L	L15	CCTACACGACGCTCTTCCGATCTTCTGGGTCATACATGTGGATATATGY
SLCO1B1-c.521T > C-R	R15	GTTCATGGGTAATATGCTTCGTGTCCAACCCTTAGGGAACCCCGATC

Probe Configuration

A phosphate group was introduced at the 5'-end of right probe and middle probe, and mixed with an equal volue of 0.1xAE then diluted to obtain a concentration of 2 fmol/μL and named hyperbilirubinemia probe working fluid.

DNA Collection and Analysis

Genomic DNA was extracted using a blood DNA extraction kit (Tiangen Biotech) and preserved at − 20°C in the refrigerator. 5 μl of DNA sample (50–250 ng) was denatured at 98°C for 5 min and gradually cooled to 25°C. The MLPA buffer and hyperbilirubinemia probe working fluid were mixed homogeneously. The thermocycler program was continued at 95°C for 2 min, 65°C for 1 h, 64°C for 1 h, 63°C for 1 h, 62°C for 1 h, 61°C for 1 h, 60°C for 1 h, 59°C for 1 h, 58°C for 1 h, 57°C for 1 h, 56°C for 1 h, 55°C for 1 h, and then 54°C for 3–10 h. Each reaction consisted of 25 μl DH2O, 3uL ligase buffer A, 3uL ligase buffer B and 1uL ligase 65 then incubated for 25min at 54°C, followed by deactivation for 5 minutes at 98°C and cooled to 20°C. The PCR products sent to the NuoHeZhiYuan company (Nanjing of Jiangsu, China) for high-throughput sequencing on the Illumina MiSeq platform.

Statistical Analyses

Statistical analyses were performed with SPSS 23.0. Accord with normal distribution of continuous variables with $\bar{x}$ ±⍰ said, between groups comparing with independent samples t test, two sets of the above comparison, using the single factor analysis of variance; Continuous variables that do not conform to the normal distribution are represented by M (Q1, Q3), and the Mann-Whitney U test is used for comparison between groups, and the Kruskall-Wallis test is used for comparison between more than two groups. Count data expressed in cases (%), group comparison between the χ^2test. $P < 0.05$ was considered statistically significant.

Results

Verification of MLPA-NGS Results

Sanger sequencing or next-generation sequencing (NGS) were employed to verify the results. The coincidence rate of 211G > A polymorphism was 98.3%, while all other pathogenic variants had a 100% coincidence rate (Table 1).

Table 1
Verification of MLPA-NGS results

Pathogenic variants	Genotype	MLPA-NGS		Sanger/NGS		Coincidence rate
		Cases	Controls	Cases	Controls	
UGT1A1-c.211G > A	A/A	14	3	14	3	98.3%
	G/A	31	16	31	16	
	G/G	20	33	20	33	
SLCO1B1-c.388A > G	G/G	28	27	28	27	100%
	G/A	33	19	33	19	
	A/A	4	6	4	6	
SLCO1B1 -c.597C > T	T/T	13	15	13	15	100%
	C/T	40	25	40	25	
	C/C	12	12	12	12	
SLCO1B1-c.521T > C	C/C	0	0	0	0	100%
	T/C	17	11	17	11	
	T/T	48	41	48	41	
BLVRA-c.175T > C	C/C	1	0	1	0	100%
	T/C	11	12	11	12	
	T/T	53	40	53	40	
BLVRA-c.7G > A	A/A	27	20	27	20	100%
	G/A	31	22	31	22	
	G/G	7	10	7	10	

There were no significant differences in gender gestational age, birth weight, and delivery mode between case and control groups ($P > 0.05$); however, there were significant differences in total bilirubin levels between groups ($t = 24.164$, $P < 0.001$) (Table 2).

Table 2
Demographic characteristics of newborns enrolled in the study

	Cases	Controls	χ^2/t/Z	*P* value
Bilirubin level (μmol/L,mg/dL)	444.78 ± 75.29 (26.01 ± 4.40)	175.52 ± 31.21 (10.26 ± 0.26)	24.164	0.001
Gender (male/female)	32/33	32/20	1.766	0.184
Gestational age (weeks)	38.52 ± 1.23	38.53 ± 0.89	-0.032	0.975
Birth weight (g)	3273 ± 344	3266 ± 324	0.110	0.912
Age (days)	6(5,8)	7(6,8)	-1.024	0.306
Mode of delivery(cesarean delivery/natural delivery)	38/27	31/21	0.016	0.900
Feeding modalities (Breast and formula/ Formula fed)	36/29	24/28	0.985	0.321

Allele Frequency

There were 65 patients in the hyperbilirubinemia group and 52 in control group. The frequency of A allele was higher in the hyperbilirubinemia group than in the control group (45.38% vs. 21.15%, P < 0.001). Other alleles did not differ significantly between hyperbilirubinemia and control groups (Table 3). Figure 1 displays the results of bivariate logistic regression analysis. 211G > A polymorphism was linked to an increased risk of developing severe hyperbilirubinemia (OR = 4.233, 95% CI: 1.896–9.451).

Table 3
Allele frequencies of polymorphisms

Pathogenic variants	Allele	Case group n(%)	Control group n(%)	χ2	*P* value
SLCO1B1-c.388A > G	G	89(68.46%)	73(70.19%)	0.281	0.776
	A	41(31.54%)	31(29.81%)	-	-
SLCO1B1 -c.597C > T	T	66(50.77%)	55(52.88%)	0.104	0.748
	C	64(49.23%)	49(47.12%)	-	-
SLCO1B1-c.521T > C	C	17(13.08%)	11(10.58%)	0.343	0.558
	T	113(86.92%)	93(89.42%)	-	-
UGT1A1-c.211G > A	A	59(45.38%)	22(21.15%)	14.988	< 0.001
	G	71(54.62%)	82(78.85%)	-	-
BLVRA-c.175T > C	C	13 (10.00%)	12(11.54%)	0.143	0.705
	T	117(90.00%)	92(88.46%)	-	-
BLVRA-c.7G > A	G	45(34.62%)	42(40.38%)	0.823	0.364
	A	85(65.38%)	62(59.61%)	-	-

Bilirubin Level

No statistically significant differences were observed in bilirubin levels between different genotypes (Table 4), $P > 0.05$.

Table 4
Bilirubin level in different genotypes

Genotype	n	Bilirubin level (μmol/L,mg/dL)	F	*P* value
211G/G	20	444.75 ± 66.11 (26.00 ± 3.86)	0.005	0.995
211G/A	31	444.04 ± 90.77 (25.97 ± 5.31)		
211A/A	14	446.47 ± 50.01 (26.11 ± 2.92)		
521T/T	48	443.11 ± 70.38 (25.91 ± 4.12)	-0.299	0.766
521T/C	17	449.51 ± 89.96 (26.29 ± 5.26)		
597C/C	12	451.85 ± 68.11 (26.42 ± 3.98)	0.381	0.685
597C/T	40	438.44 ± 69.99 (25.64 ± 4.09)		
597T/T	13	457.78 ± 98.45 (26.77 ± 5.76)		
388A/A	3	525.34 ± 158.99 (30.72 ± 9.30)	2.559	0.086
388G/A	39	439.12 ± 55.19 (25.68 ± 3.23)		
388G/G	23	439.95 ± 77.42 (25.73 ± 4.53)		
7G/G	7	479.89 ± 118.30 (28.06 ± 6.92)	1.004	0.372
7G/A	31	435.47 ± 79.80 (25.47 ± 4.67)		
7A/A	27	446.37 ± 54.21 (26.10 ± 3.17)		
175T/T	52	449.19 ± 79.72 (26.27 ± 4.66)	0.943	0.349

Genotype	n	Bilirubin level (μmol/L,mg/dL)	F	*P* value
175T/C	13	427.16 ± 52.93 (24.98 ± 3.10)		

Discussion

This study examined six gene loci using MLPA-NGS technique. The mutation frequencies were as follows: c.388A > G (93.84%) > c.7G > A (89.23%) > c.597C > T (81.54%) > c.211G > A(69.23%) > c.521T > C(26.15%) > c.175T > C(18.46%). The c.211G > A polymorphism was correlated with severe hyperbilirubinemia, increasing the risk of its development. The results are consistent with previous findings[8–11]. In the present study, the mutation frequency of c.211G > A was 69.23% and allele frequency was 45.38%, which were higher than in Japan (34%) and Vietnam (27.3%)[12]. A study from Vietnam has suggested that total bilirubin levels were higher in c.211G > A homozygous pathogenic variants than in wild type[13]. However, in our study, there was no statistically significant difference between c.211G > A homozygous neonates, heterozygous neonates, and wild-type neonates. This is most likely linked to the fact that this study included only neonates with severe hyperbilirubinemia and not all neonates with hyperbilirubinemia. Unconjugated bilirubin is transported specifically into liver cells using *organic anion transporter 2* (*OATP2*) on liver membrane. *OATP2* is encoded by *SLCO1B1* gene. Prior research indicated that mutations in this gene cause reduced transport function of the transporter[14]. The c.521T > C has been extensively researched, and the reduced transport function caused by this mutation may be associated with a miscarriage of proteins to the cell membrane[15]. A study from northern China indicated that c.388A > G is linked to severe hyperbilirubinemia occurrence[16]. While Liu J et al. demonstrated that c.388A > G is associated with a higher risk of incident hyperbilirubinemia, there is no significant correlation between C.521T > C and hyperbilirubinemia[17]. Another study revealed that c.388A > G, c.597C > T, and c.521T > C had no link to the incidence of neonatal hyperbilirubinemia in Guangxi[18], consistent with our findings. A study in 2005 discovered that while c.388A > G and G6PD deficiency both contribute to hyperbilirubinemia occurrence, the serum bilirubin concentration is unaffected by c.388A > G mutation only[19]. These findings appear to be contradictory. As a result, a multicenter study with large sample size is warranted. During bilirubin metabolism, hemoglobin is released from human red blood cells and degraded into heme. Heme oxygenase (HO) catabolizes the heme group into CO, biliverdin, and iron; biliverdin is further reduced to bilirubin by *BLVRA*[20]. Once the genes encoding *BLVRA* are mutated, the activity of this enzyme is altered, thus affecting bilirubin levels. The results of this study indicate that *BLVRA* c.7G > A had the highest rate of mutations (89.23%), followed by c.175T > C (18.46%) in the hyperbilirubinemia group, there was no significant correlation between the pathogenic variant and hyperbilirubinemia. There are relatively few studies on this at home and abroad. A 2016 study indicated that c.175T > C were linked to hyperbilirubinemia[21]. Yang et al.'s study on genetic factors of neonatal hyperbilirubinemia revealed that *BLVRA* gene c.7G > A was not significantly associated with disease's occurrence[22]. Lin et al. investigated the general population of Kazak, Uyghur, and Han Chinese and discovered that C.7G > A mutation was insufficient to alter bilirubin levels[23]. At present, the mechanism of *BLVRA* gene mutation affecting bilirubin level is incompletely clear and basic experiments must be designed for further study.

In this study, MLPA-NGS technique was employed for the first time to detect genes in neonates with hyperbilirubinemia. The neonates in the hyperbilirubin and control groups were independently sequenced using Sanger sequencing and next-generation sequencing. We compared MLPA-NGS sequencing results with previous sequencing results and discovered that the coincidence rate of UGT1A1c.211G > A was 98.3% (115/117), and all other pathogenic variants had a coincidence rate of 100%. In addition to genetic disease detection, some scholars have

recently applied MLPA-NGS technique to high-throughput gene amplification detection of tumor samples, achieving 100% sensitivity and 96% specificity[24, 25]. In this study, we first utilized MLPA-NGS technique to identify neonatal hyperbilirubinemia gene mutation sites. The sequencing results were basically consistent with Sanger sequencing or next-generation sequencing results. An advantage of MLPA-NGS technique is its significantly lower economic cost. It could be promoted as a screening method for mutations at known common loci of neonatal hyperbilirubinemia.

Our study has some limitations. First, the sample size is small. Second, our study was limited to children with severe hyperbilirubinemia, not including children with mild hyperbilirubinemia. I think we can group the children with hyperbilirubinemia according to serum bilirubin levels in future studies to clarify the relationship between bilirubinemia and the above gene mutations.

Conclusions

In this study,c.211G > A of *UGT1A1* gene was significantly linked to unexplained severe neonatal hyperbilirubinemia and was a high-risk factor for the disease. MLPA-NGS technique was a highly accurate, rapid, and cost-effective method in identifying common and known gene loci of severe neonatal hyperbilirubinemia. It can be employed as a point mutation screening approach for detecting hyperbilirubinemia-related pathogenic variants.

Declarations

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics Statement

The studies involving human participants were reviewed and approved by Ethics Committee of Shanghai Children's Hospital (2021R027-E01). Written informed consent to participate in this study was provided by the participants' legal guardian.

Author Contributions

DY completed the data processing and manuscript writing.DY and YY was responsible for sample testing.YH was responsible for the revision and review of the paper.XG,HZ and SW provided the ideas of the manuscript.HZ,XW and KG were responsible for collecting data. All authors contributed to the article and approved the submitted version.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures

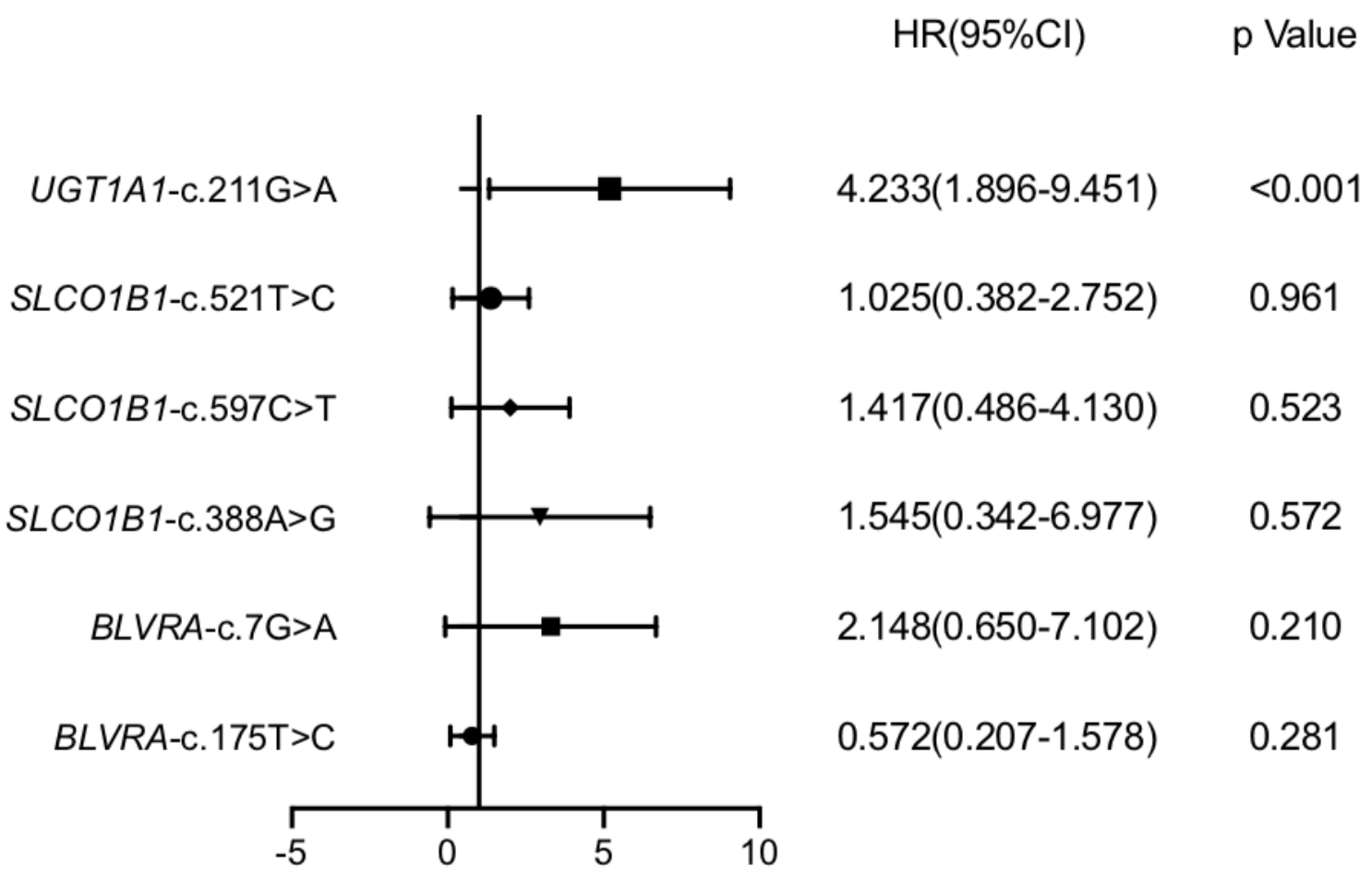

Figure 1

Correlation between pathogenic variants and severe neonatal hyperbilirubinemia