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Organic anion transporter protein (OATP1B1) encoded by *SLCO1B1* gene polymorphism (388A>G) & susceptibility in gallstone disease

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***Background & objectives*: Organic anion transport protein 1B1 (OATP1B1) is a major transporter protein for bile salt uptake in the enterohepatic circulation of bile salts. As the role of *SLCO1B1* gene (encodes OATP1B1 or liver specific transporter-1) 388 A>G polymorphism in susceptibility towards gallstone disease is unclear the prevalence of this polymorphism in healthy north Indian population was investigated.**

***Methods*: Peripheral venous blood of 270 unrelated northern Indian patients with symptomatic gallstone disease and 270 unrelated healthy control subjects was screened for *SLCO1B1* gene 388 A>G polymorphism by PCR-RFLP method and genotyping was done on 12 per cent polyacrylamide gel. The cross-sectional data on accrual of cases and controls were collected and odds ratio with 95 per cent CI calculated as for case-control design.**

***Results*: Allele frequencies of 388 G were 45 per cent in gallstone cases and 44 per cent in controls with no statistical significance. Genotype frequencies in gallstone cases and controls for, genotype AA were 30 and 32 per cent; AG: 51 and 47 per cent and GG: 16 and 21 per cent respectively. No significant association of any allele or genotype with gallstone disease was found.**

***Interpretation & conclusions*: Although the prevalence of *SLCO1B1* gene 388A>G polymorphism in north Indian population in high, yet this polymorphism does not appear to play a significant role in susceptibility to gallstone formation.**

Key words Gallstone - LST-1 - OATP-C - OATP1B1 - polymorphism - *SLCO1B1*

Gallstone is a multifactorial disease with several environmental and genetic risk factors. Although, gallstone disease has a significant genetic component[1], very little is known about the genetic factors that contribute to the development of this disease. A prerequisite for gallstone formation is the supersaturation of bile with cholesterol in gallbladder[2]. Major components of bile are bile acids (80%), lecithin and other phospholipids (16%) and unesterified cholesterol (4%)[3]. Bile acids play essential role in the absorption of fat and contribute to the regulation of cholesterol synthesis[4].

The main source of bile salts in gallbladder is the enterohepatic circulation. If there is any disturbance

in the enterohepatic circulation of bile salts, it can decrease the relative concentration of bile salts in gallbladder and can lead to the formation of cholesterol stones[5,6].

At physiological *p*H, the hepatic uptake of bile salts is a rate limiting step in the entero-hepatic circulation[7]. In sodium independent pathway of hepatic uptake of bile salts, major transporter protein is organic anion transporter protein1B1 (OATP1B1) (especially for chenodeoxycholic acid)[8]. This is an important transporter protein of super family of organic anion transporter proteins (OATPs)[9]. This protein is highly expressed in liver and supports the membrane translocation of a broad range of compounds such as bile acids, sulphate and glucuronide conjugates[10], thyroid hormones[11], peptides[12] and drugs such as pravastatin[13], methotrexate[14,15] and rosuvastatin[16]. From mouse model studies it is confirmed that Lith6 (loci involved in gallstone disease) represents a complex locus containg genes for OATPs and Pparg[18]. *SLCO1B1* gene expressing OATP1B1 is highly polymorphic. A 388A>G polymorphism of this gene has been reported to considerably reduce the transporting property of drugs like pravastatin[17], and may be involved in the Na^+ independent pathway of bile salt uptake in enterohepatic circulation. Therefore, we undertook this study to estimate the prevalence of *SLCO1B1* 388A>G polymorphism in north Indian population and to evaluate its association with gallstone disease.

Material & Methods

Subjects: A total of 270 patients with gallstone disease were recruited consecutively from the Departments of Gastroenterology, Medicine and Surgery, Sanjay Gandhi Post Graduate Institute of Medical Sciences, and Department of Surgery, CSM Medical University, Lucknow, India, from August 2005 to May 2007. Patients were admitted either for endoscopic retrograde cholangiopancreatography (ERCP) or for cholecystectomy. All patients were ultrasonographically confirmed for gallstone disease and consented to participate in the study. The study was duly approved by the local ethical committees of the respective Institutes. Age and sex matched control subjects comprised of 270 healthy, unrelated, gallstone free individuals (ultrasonographically confirmed), recruited from screening clinics at the same institutions. All individuals were personally interviewed using pre-designed and validated questionnaire through which information about individual's demographic data, types of substance abuse and parity (for female participants) were recorded. Radiological and clinical data were collected from the hospital record section. Subjects suffering from any chronic disorder like diabetes and cancer were excluded from the study. The sample size was calculated using QUANTO 1.1 program (http://hydra.usc.edu/gxe, accessed on July 11, 2005) with input of following variables; design of study = case-control, ratio of case and control: 1:1, significance level = 0.05 (2 sided), power = 80 per cent, model of inheritance = log additive (for polygenic disease), allele frequency = 0.30 for 388 site of *SLCO1B1* gene, genetic effect (odds ratio) = 2.5. Calculated sample size by software was 97. However, for genetic polymorphism study, it was feasible to collect 2.5 times (n = 270) cases and equal number of control subjects.

Five ml venous blood samples in EDTA vials were taken from each subject and stored at -70°C. Leukocyte DNA was extracted using salting out method[19].

Genotyping: The *SLCO1B1* 388A>G polymorphism was analysed by genomic DNA amplification using PCR and followed by digestion with restriction enzyme *TaqI* (Banglore Genei, India) at 65^0C for 6 h. PCR amplification was conducted in a total volume of 20 µl with 20 pmol of each primer[20] (custom synthesized from IDT, USA); 150-200 ng genomic DNA, 10 mM dNTPs (Bangalore Genei, India); 3 units of *Taq* DNA polymerase (Bangalore Genei, India) in the buffer provided by the manufacturer. The digested products were separated on 12 per cent polyacrylamide gel and genotypes were based on band sizes: 151 and 63 bps for AA, 128, 63 and 23 bps for GG, 151, 128, 63, 23 bps for GA (Fig.). The PCR were carried out in a DNA thermal cycler (DNA Engine PTC-200, MJ

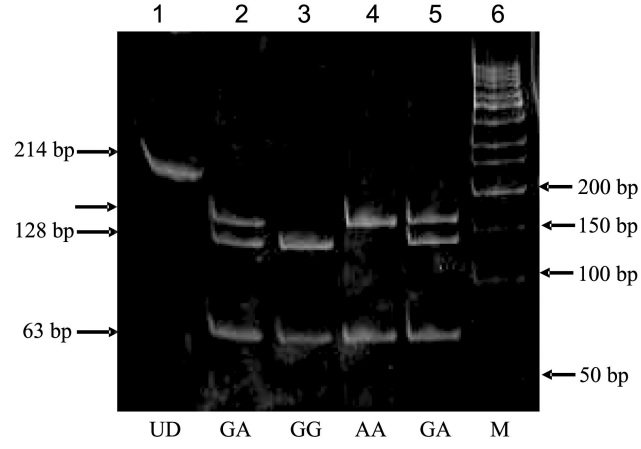

Fig. Representative polyacrylamide gel picture (15%) for 388A>G gene polymorphism. Lane 1: undigested PCR(UD) product, lanes 2 & 5: heterozygote GA genotype, lane 3: homozygous GG genotype, lane 4: homozygous AA genotype, lane 6: 50 bp ladder.

Research, Inc, USA). PCR products and molecular weight markers were visualized after staining with ethidium bromide. Gel documentation was done by Alphaimager™ 1220, (Alpha Innotech Corporation, USA). Every precaution was taken to prevent cross contamination between samples during DNA extraction and PCR assays. About 20 per cent of samples (105) from both patients and controls were re-genotyped by an independent person. Approximately 10 per cent of all samples were sequenced and negligible discrepancy in genotyping was noticed.

Statistical analysis: Continuous variables were presented as mean ± SD and discrete variables by frequencies and percentages. Allele and genotype frequencies were tested for Hardy-Weinberg equilibrium in control group and deviation was tested using chi-square test. For estimating genotyping and allelic frequencies difference between gallstone cases and controls, Pearson's chi-square test was used. The genotyping and allelic risk as odds ratios with 95 per cent confidence intervals (95% CI) were measured. Statistical analyses were performed using SPSS software version 15.0 (*www.spss.com*). Tests of statistical significance were two-sided and differences were taken as significant when *P* value was <0.05.

Results

We recruited total 270 patients (age range 18-82, 48.41 ± 13.23) and 270 control subjects (age range 18-80, 46.12 ± 13.97). Out of 270 patients, there are 163 females (age range 18-82, 47.85 ± 13.31) and 107 male (age range 19-72, 48.41 ± 13.23) subjects while in control group there are 145 females (age range 20-72, 46.70 ± 12.08) and 125 male (age range 18-80, 47.98 ± 13.86) subjects.

Allele frequencies of the *SLCO1B1* 388A>G polymorphism for gallstone cases and controls were 56 and 55 per cent for A; 44 and 45 per cent for G respectively showing no significant difference (OR=0.97; 95% CI 0.76-1.24) (Table I). Genotype frequencies in gallstone cases and controls for AA

Table I. Distribution of genotype and allele frequencies of *SLCO1B1* 388 A>G polymorphism between patients and controls

Genotype	Gallstone cases #n (%)	Controls n (%)	OR (95%CI)	*P*
AA	82 (30.37)	86 (31.85)	1 (Reference)	-
AG	138 (51.11)	126 (46.67)	1.19 (0.84-1.70)	0.30
GG	50 (15.52)	58 (21.48)	0.83 (0.53-1.30)	0.38
Allele				
A	302 (56)	298 (55)	1 (Reference)	
G	238 (44)	242 (45)	0.97 (0.76-1.24)	0.80

OR, Odds ratio

Total number of subjects 270 (for genotypes) and total number of chromosomes 540 (for alleles) in patients and controls

Table II. Distribution of genotype and allele frequencies of *SLCO1B1* 388 A>G polymorphism between patients and controls in female and male groups separately

Genotype	Female				Male			
	Patients *n (163) (%)	Controls **n (145) (%)	OR (95%CI)	*P*	Patients +n (107) (%)	Controls ++n (125)(%)	OR (95%CI)	*P*
AA	47 (28.83)	48 (33.10)	1 (Reference)		35 (32.71)	38 (30.40)	1 (Reference)	
AG	81 (49.69)	64 (44.14)	1.29 (0.74-2.25)	0.33	57 (53.27)	62 (49.60)	1.00 (0.53-1.87)	0.99
GG	35 (21.47)	33 (22.76)	1.08 (0.55-2.12)	0.80	15 (14.02)	25 (20)	0.65 (0.27-1.54)	0.28
Allele								
A	175 (53.68)	160 (55.17)	1 (Reference)		127 (59.35)	138 (55.20)	1 (Reference)	
G	151 (46.42)	130 (44.83)	1.06 (0.76-1.48)	0.71	87 (40.65)	112 (44.80)	0.84 (0.57-1.2)	0.36

*Total number of patients 163 (for genotypes) and chromosomes 326 (for alleles) in females; **Total number controls 145 (for genotypes) and chromosomes 290 (for alleles) in females; +Total number of patients 107 (for genotypes) and chromosomes 214 (for alleles) in males; ++Total number controls 125 (for genotypes) and chromosomes 250 (for alleles) in males

were 30 and 32 per cent; for AG were 51 and 47 per cent (OR=1.19; 95% CI=0.84-1.70); for GG were 16 and 21 per cent (OR=0.83; 95% CI= 0.53-1.30), respectively. Allele frequencies in control population were in Hardy-Weinberg equilibrium. There were no significant differences between patients and controls in terms of frequencies of alleles and genotypes (Table I).

On stratified analysis of data between females and males, there were no significant differences between patients and controls in the frequency of the above alleles or genotypes. In females group, genotype frequencies in gallstone cases and controls for AA were 29 and 33 per cent; for AG 50 and 44 per cent (OR=1.29; 95% CI=0.74-2.25) and for GG were 21 and 23 per cent (OR=1.08; 95% CI= 0.55-2.12) and at allelic level frequencies were 46.42 and 44.83 per cent respectively, again with no statistically significant difference (OR=1.06; 95% CI=0.76-1.48). In male group genotype frequencies in gallstone cases and controls for AA were 33 and 30 per cent; for AG 53 and 50 per cent (OR=1.00; 95% CI = 0.53-1.87) and for GG were 14 and 20 per cent (OR=0.65; 95% CI= 0.27-1.54), and at allelic level frequencies were 40.65 and 44.80 per cent respectively, with no statistically significant differences (OR=0.84; 95% CI = 0.57-1.2) (Table II).

Discussion

The hitherto undescribed role of *SLCO1B1* gene polymorphism in the pathogenesis of gallstone disease was studied. It was done to test a hypothesis that gallstone formation is more likely to occur in individuals who exhibit greater association with *SLCO1B1* gene G allele at 388 position as it may be involved in the Na^+ independent pathway of bile salt uptake in enterohepatic circulation. Several polymorphisms of this gene are reported in literature, which alter the transportation activity of this protein[15]. 388A>G polymorphism reduces the transportation activity of OATP1B1 up to 50 per cent for several drugs like pravastatin[13]. However, the results of our study showed that there may not be any significant role of this polymorphism in gallstone disease. The prevalence of gallstone disease is different in female and male being in the ratio of 3:1[21]. A stratified analysis was also undertaken between genders but it also did not show any significant difference in the frequency of any alleles and genotypes between genders (in this study female male ratio is 1.52).

OATP1B1 is an important transporter protein in the basolateral membrane of hepatocytes. It mediates the transportation of several important drugs and their metabolites. It is also an important transporter in the elimination pathway of several toxic substances from the body through liver. All OATP proteins have twelve trans-membrane domains[9] and 388A>G polymorphism is present in one of the extracellular loops[22]. This polymorphism changes the amino acid asparagine to aspartic acid, which in turn increases the overall negative charge of the protein. Substrate recognition or binding is the first step in the transportation of any molecule through a membrane. Specific substrate recognition by all OATPs is usually high molecular weight molecules with steroid nucleus[9]. It may be possible that this particular loop with aspargine is important for efficient drug molecule binding but not for bile salts although we have no specific proof for the proposed hypothesis. Lyons *et al*[17] showed that this polymorphism does not affect the OATP1B1 m-RNA levels.

World wide frequency of *SLCO1B1* 388G allele is in large range which can be explained on the basis of inter-ethnic differences among different population groups[23]. The frequency of this allele in north Indian population was similar to that reported for Singapore based Indian population[24] (Table III). No apparent association of this polymorphism with frequency of gallstone disease

Table III. Ethnic distribution of *SLCO1B1* 388G allele frequency

Ethnic group	No. of subjects studied	*SLCO1B1* 388G allele (%)
Finnish[25]	468	46
Caucasian[26]	423	37
European-American[27]	49	30
African-American[15]	22	75
Japanese[28]	267	64
Japanese[29]	120	63
Korean[30]	22	75
Chinese[24]	100	20
Malay[24]	100	13
Asian-Indian[24]	100	43
North India (present study)	270	44.81

Highest frequency was reported in African-Americans and lowest in Malay population

Superscript numerals denote reference numbers

was observed, suggesting that the polymorphism may not significantly influence the gallstone formation. Based on mouse model studies, *SLCO1B1* was found to be a candidate gene for gallstone disease[17], so other polymorphisms of this gene are likely to be involved in the genetic susceptibility towards gall bladder stone formation. Identification of such polymorphisms might increase the understanding of the pathogenesis of this disorder. However, the reported frequencies of other polymorphisms of this gene vary from 0-20[24-30] in different populations. Therefore, genetic association studies of these polymorphisms with gallstone disease may require larger sample size.

In conclusion, our results showed no significant association of *SLCO1B1* 388A>G gene polymorphism with gallstone development. These results need to be further confirmed by other tests like bile salts concentration in the bile and correlating these with different genotypes in patient and controls. Further, other functional polymorphisms of this gene should be studied and this study may be repeated in other population groups with larger sample size to confirm the role of this polymorphism in gallstone disease.

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