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Original Article

Anatomical and molecular studies of cytochrome P450 family CYP7A1 gene polymorphism and its association with gallstone in north Indian population

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ABSTRACT

Introduction: In this study, we have mainly focused on the molecular and biochemical aspects to know the cause of gall stone diseases in human. To know about these problems, there may be necessitating initiating interest in researchers of this field and to set up easily available tools and techniques. The screening of polymorphism with molecular approaches of P450 super family *CYP7A1* gene was performed to explore its relation with gallstone diseases.

Methods: Total 300 samples (150 patients and 150 controls) were analyzed for the study. The polymorphisms were analyzed by PCR followed by RFLP with BsaI restriction enzyme. The lipid profile was estimated by using modified Roeschlau's method. The cholesterol content in gall stones was determined with Liebermann-Burchard reaction method.

Results: The cholesterol content of recovered gallstones was found 98.86 ± 25.43 (% by weight). BMI and serum glucose were found higher in patients than in the control group: 30.84 ± 8.13 kg/m^2 *vs* 28.61 ± 7.50 kg/m^2 (P = 0.032), and 121.03 ± 15.11 mg/dL *vs* 104 ± 21.05 mg/dL (P = 0.001) respectively. Statistical analysis showed no significant differences in genotypic frequencies of gene *CYP7A1* gene polymorphism between patients and controls. Frequencies of C allele (*CYP7A1* gene polymorphism) in patients and controls were obtained 24.24% *vs* 26.46% (P = 0.621, OR = 0.89). Genotypic frequencies between patients and controls were found 59.42% *vs* 54.97% for AA; 30.25% *vs* 34.72% for CA; and 10.33% *vs* 10.31 for CC.

Discussion: In this study by using of multiple logistic regression analysis the results have indicated that *CYP7A1* gene polymorphism may not play any significant role in gallstone disease.

1. Introduction

Gallbladder stone disease is prevalent in China and India with an increasing incidence rate. Cholesterol gallstone disease (GD) is the key appearance of gallbladder disease, and may be one the mainly general digestive disorders globally, generally in western populations.[1] In Mexico, the incidence of GD has been reported around 14.3%.[2] The development of gallstones is occurred by disturbed gallbladder functions (serum lipids), hyper-secretion of cholesterol into bile.[3,4] The pathogenesis of GD is dependent on multi-functional including environmental and genetic factors.[5,6] By the study of epidemiological data there is high occurrence of GD has been recorded in American, Indians and Hispanic populations the studies have suggested that for this disease genetic factors take placed a key role.

Many risk factors, namely obesity, diet, female gender, metabolic syndrome and type 2- diabetes are mainly associated with this disease.[7,8] There are several lithogenic genes are found in humans being such as ABC transporters for phosphatidylcholine (*ABCB-4*) and bile salts (ABCB-11), cholesterol-7-α-hydroxylase (*CYP7A1*), cholecystokinine type-A receptor (CCK1R), cholesteryl ester transfer protein (CETP)[4,5] *etc.*

The Cytochrome P450 enzymes are monooxygenases which involved in the catalyzing several reactions in drug metabolism and synthesis of cholesterol, steroids and other fatty compounds. The human liver the endoplasmic reticulum membrane P450 protein catabolic pathway for cholesterol is found, which are

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involved to change cholesterol in to bile. The reaction is the rate determining step and involved as the chief step of the regulation of bile acid synthesis. Mainly by this mechanism cholesterol is removed from our body.[9] Cytochrome P450 enzymes/proteins are encoded by several *Cytochrome P450* genes. There are 57 *P450* genes have been identified in human till now.[10] Researchers are trying to know about the properties of these genes and their roles associated in different diseases. As usual, *P450* genes have great medical significance and participated in the formation of important endogenous substrates. The regulations (up/down) of these genes may affect the expression levels of genes.[11] One of the well known gene; *CYP7A1* existed which is a member of the cytochrome P450 super family of enzyme. *CYP7A1* gene is known to encoding CYP7A1 enzyme which is located on the 8q11-12 chromosome and made up of 6 exons and 5 introns.[12] The key role of *CYP7A1* gene is usually in cholesterol metabolism. It has been shown that the *CYP7A1* genetic variants determine serum levels of LDL lipoproteins and triacylglycerols.[13–15] The *CYP7A1* gene polymorphisms have been examined for its potential effects on lipid metabolism. Large number of polymorphic variants has been reported of *CYP7A1* gene.[16] It has noted that there are typed six haplotype-tagging single nucleotide polymorphisms (htSNPs) has been reported in Caucasian population. In this report it has been mentioned for a constant genetic structure of *CYP7A1* gene and its associated sequences concerned to genetic variations in *CYP7A1* gene which is linked to cholesterol, bile acid metabolism.

Xiang et al[17] has reported that usually the individual gender become more responsible for variation in bile acid metabolism and gallstone diseases than *CYP7A1* gene polymorphism. In addition, it has been mentioned that normally genetic variability in *CYP7A1* gene is doubtful to play an important role in cholesterol metabolism and bile acid homeostasis under normal physiological conditions. Further, in the study of Chi et al. The CYP7A1 gene polymorphism has been found appreciably related in increment of serum lipid levels in population.[18] There are several studies have been mentioned in literature related to *CYP7A1* gene polymorphism and metabolic disorders of cholesterol and bile acid, counting hypercholesterolemia, hyper-triglyceridemia and GDs.[19–24]

Genetic variation in *CYP7A1* gene due to genetic or environmental influence has been reported to increase plasma LDL-cholesterol concentration level[21,22] and might be linked for GDs[22] during the study of single-nucleotide polymorphism (SNP) in the promoter region of *CYP7A1* gene. Furthermore, in another report it has been mentioned that *CYP7A1* gene polymorphism are playing a significant role in gallstone diseases due to the regular lipid responses of diets, pharmacological issues and surgical operations.[23]

In the another study of *CYP7A1* gene polymorphism, it has been mentioned that this gene has been involved in the regulation of bile acid and Chenodeoxycholic Acid (CDCA) pool size which is substantial smaller in women than in men in addition, the age, ethnicity and body size have not been recorded for the bile acid pool size metabolism.[24]

A few studies[25] have been attained to know the relationship between human gallstones diseases and certain genetic variations in *CYP7A1* gene for cholesterol metabolism. Although the amino acid sequences of CYP7A1 protein between the species is highly conserved (80–90% identical). As compared to controls, the activity of *CYP7A1* gene variations has been found in patients with gallstones[26,27] and up-regulation or down-regulation of this gene was observed.

In adult human being the biosynthesis of bile acids in liver is about 500 mg per day from cholesterol.[28] The biosynthesis of bile acids starting from cholesterol occurs through two major pathways: one is classic (neutral) pathway and another alternative (acidic) pathway. In human beings, the classic pathway is playing principal role in this process. More than 16 enzymes participate in the synthesis of bile acids.[29] The major role is played by cholesterol-7α-hydroxylase (CYP7A1) which catalyses the initial and rate-limiting reaction in the classic pathway to synthesis 7-α-hydroxycholesterol. 7-α-Hydroxycholesterol is then metabolized to 7-α-hydroxy-4-cholesten-3-one; by using several reaction steps it convert into two primary bile acids (chenodeoxycholic acid; CDCA) and cholic acid; CA). Blood plasma 7-α-hydroxy-4-cholesten-3-one concentration is commonly working as a biomarker of bile acid synthesis rate for CYP7A1 activity.[30] Since the 7-α-hydroxylation of cholesterol is essential in cholesterol and bile acid homeostasis. However, the effects of CYP7A1 variants on the fasting plasma concentrations of individual bile acid in Kanpur population might not studied till today. For example, hydrophobic bile acids CDCA and DCA (deoxycholic acid) have additional effective inhibitory activities on bile acid synthesis than the more hydrophilic ursodeoxycholic acid (UDCA).[31]

Therefore the aims and objectives of this study were to analyze the possible association between the *CYP7A1* gene polymorphism and gallstones diseases. We also examined whether genetic variants of the *CYP7A1* gene affect the plasma lipid/total plasma cholesterol concentrations *etc.* in the patients of gallstone. This study may help in better understanding of genetic background of *CYP7A1* gene polymorphism in Kanpur population, which is at high risk of the gallstone disease.

2. Material and Methods

2.1. Sampling

A total of 300 samples (150 patients + 150 controls) were collected for the study from June to September 2016. The collected samples were 75 males and 75 females suffered from stones or cholesterol crystals in their gallbladder in each group *i.e.* patients and controls. There was no any previous commencement of cholecystitis problems associated with the patients such as colic, fever with chills and jaundice. One hundred fifty healthy samples (75 males and 75 females) with normal liver, kidney and endocrine function were collected as controls. The average ages of patients and controls were 45 and 44 years correspondingly. B-mode ultrasonography with Siemens (Germany) 500/SSD connected with a transducer of 5–10 mHz was performed with all the overnight fasting donors. Around 5 mL venous blood was collected from each and half of the volume was instantly put in anti-coagulants containing EDTA vials for DNA extraction. The remaining half of the sample volume was set ready for biochemical analysis. Body mass index (BMI) was estimated by weight/height^2 (kg/m^2). All the issue was proper informed to donors; who have participated in this study and all the subject was approved by the Ethical Committee of Rama medical college and research centre, Rama University, Kanpur (India).

A 184-bp fragment containing *CYP7A1* gene polymorphism was digested with restrictive enzyme BsaI and electrophoresed on 1.5% agarose gel and stained with ethidium bromide.

2.2. Methodology

Estimation of plasma lipids, lipoproteins, plasma total cholesterol, triglyceride, HDL cholesterol, LDL cholesterol, APO and AI were performed by commercially available kits (Cell Biolabs Inc., San Diego, CA, USA) with an automatic analyzer (Hitachi 7060, Japan). Obtained gallstones were cleaned with distilled water and dried at 37 °C to determine the cholesterol content using the Liebermann-Burchard reaction method for the estimation of

Table 1
List of primers, Tm and used restriction enzyme in DNA digestion.

Forward primer	5′-GAGAAGGCAAACGGGTGAAC-3	Tm-54
Reverse primer	5′-GGTATGACAAGGGATTTGTGATGA-3′	Tm-54
Restriction enzyme	BsaI	

cholesterol in gall stone. All the stones were classified based on their colour chemical composition.[32,33]

Total genomic DNA was extracted from whole blood with using Qiagen (Germany) DNA isolation kit and followed protocol according to manufacturer's guidelines. The primers for *CYP7A1* gene were synthesized by GCC biotech. Pvt. Ltd. (Calcutta). The obtained primers were dissolved in TE buffer (1 mM, pH-8.0) and further diluted with nuclease free water and made them 10 pm/μl. PCR was conducted in 20 μl reaction volume with 10 μl master mix (Takara), 5 μl nuclease free water, 1 μl forward and reveres primer each and 30 μg DNA template. Conditions for PCR was initial denaturation 94 °C for 5 min, and then 34 cycle at 94 °C for 30 s for denaturation, 50 °C for 30 s for annealing for CYP7A1 gene then extension was performed at 72 °C for 1 min and final extension was performed at 72 °C for 7 min. 1% agarose gel was used for electrophoresis with using 1X TAE buffer.

DNA fragments of target polymorphic sequences of *CYP7A1* genes were amplified using polymerase chain reaction (PCR) on BIORAD T100 Thermal Cycler, Singapore. Primers of PCR are listed in Table 1. Single nucleotide polymorphisms of *CYP7A1*, the products of PCR were digested by restriction enzyme BsaI (Biolabs Inc, New England, USA). The enzymes are indicated in Table 1.

2.2.1. Statistics

Sample size has been calculated in order to control type I & type II error. Assuming a minimum power 80% and 95% significance level the sample size has been calculated using this formula:

$$n = \frac{2(p)(1-p)(Z\beta + Z\alpha/2)}{(p1-p2)2}$$

p-Incidence of the disease (gall stone disease), q- (1-p), $(P1\text{-}p2)^2$ or d^2 - is the difference which we want to detect at a specified power & level of confidence. Z_β - power of statistical test it was to be minimum 80% for which is Z_β is 0.84. $Z_{\alpha/2}$ -is the level of confidence we had choosen 95% confidence in this $Z\alpha/_2 = 1.96$. When P-value indicates the incidence of the clinical condition *i.e.* gallstone disease.

Following the literature the incidence of gallstone disease has assumed between 6%. The calculated minimum sample size for our study was 88. The calculated minimum sample size for control group was 88.

The results were expressed as means ± SD. The differences in concentrations of lipids between patients and controls and those among genotypes were calculated using *T* test. Statistical analysis was performed using the statistical software package IBM SPSS statistics 22 for Windows (USA) to evaluate the concentrations of lipids between patients and controls after adjustment for sex, age and BMI. Frequencies of alleles between controls and patients were evaluated for statistical significance using *Chi-square test*. A multivariate model was used to the relative odds of GSD with all the variables by multiple logistic regressions. Standard errors and logistic regression coefficients were estimated to know the determination of odds ratio (OR) 95% confidence intervals (CI) for calculating significant factors (Table 2).

Hardy-Weinberg's equilibrium was estimated by *Chi square* test. Multiple logistic regression analysis was performed to examine the autonomous factors connected with GD for data co-relation.

2.2.2. Inclusion criteria

Patients who had diagnosed as gall stone diseased patients by ultra sound scanning (USG). Patients who got operated for removal of gallstones and who were will to participate in the study.

2.2.3. Exclusion criteria

Patients who are not willing to participate in the study.

3. Results

3.1. Description of population

In this study patients with gall stones and controls were analyzed with a mean age of 45.38 ± 13.11 years and 44.51 ± 10.16 years respectively with P value 0.956. A normal distribution test was performed as mentioned by Kolmorov-Smirnov with P value 0.281 for the study. There were 50% of individual's belonged to male in both test and controls. The male/female ratio of patients and controls was taken 1:1 (Table 2). The cholesterol content of recovered gallstones was found 98.86 ± 25.43 (% by weight). The statistically significant differences were measured in the data between patients and controls (Table 2), with the exception of HDL-C (P = 0.086). Body mass index (BMI) and serum glucose were higher in patients than in the control group: 30.84 ± 8.13 kg/m^2 *vs* 28.61 ± 7.50 kg/m^2 (P = 0.032) for BMI; and 121.03 ± 15.11 mg/dL *vs* 104 ± 21.05 mg/dL (P = 0.001) for glucose. In contrast, serum lipids, cholesterol, LDL-C and triglycerides, were lower in patients than in controls (Fig. 1).

3.2. CYP7A1 *gene polymorphisms analysis*

Allelic and genotypic frequencies of *CYP7A1* gene with patients and healthy controls are demonstrated in Table 3. Statistical analysis showed no differences in genotypic frequencies of gene *CYP7A1* polymorphism between patients and controls. Frequencies of C allele (*CYP7A1* gene polymorphism) in patients and controls

Table 2
Demographic parameters of patients and controls.

SN	Parameter	Patients	controls	P-value
1.	No. (M/F)	75/75	75/75	–
2.	Age (yr)	45.38 ± 13.11	44.51 ± 10.16	0.956
3.	BMI (kg/m^2)	30.84 ± 8.13	28.61 ± 7.50	0.032
4.	Triglycerine (mg/dL)	120 ± 67.61	149 ± 28.91	0.028
5.	HDL cholesterol (mg/dL)	45.67 ± 10.95	47.22 ± 76.25	0.086
6.	LDL cholesterol (mg/dL)	128.33 ± 38.46	129.04 ± 52.81	0.024
7.	Cholesterol in stones (% by weight)	98.86 ± 25.43	–	0.002
8.	Serum glucose (mg/dL)	121.03 ± 15.11	104 ± 21.05	0.001
9.	Serum lipid (mg/dL)	640.84 ± 64.62	674.67 ± 39.60	0.004

Table 3
Allelic (af) and genotypic (gf) frequencies of *CYP7A1* gene in patients and controls with gall stone.

SN	Polymorphism *CYP7A1*BsaI gene	Patients		Controls		P-value	OR	95%CI
	Alleles	n	af	n	af	–	–	–
1.	A	101	75.76	76	73.54	0.621	1.13	0.70–1.69
2.	C	49	24.24	74	26.46	0.621	0.89	0.60–1.44
	Genotypes	n	gf	n	gf			
1.	AA	91	59.42%	83	54.97%	0.651	1.11	0.63–1.98
2.	AC	47	30.25%	50	34.72%	0.782	0.98	0.50–1.75
3.	CC	12	10.33%	17	10.31	0.827	0.90	0.40–2.36

were 24.24% *vs* 26.46% ($P = 0.621$, OR = 0.89). Genotypic frequencies between the groups were similar ($P = 0.90$) with the following distribution in patients and controls: 59.42% *vs* 54.97% ($P = 0.651$, OR = 1.11) for AA; 30.25% *vs* 34.72% ($P = 0.782$, OR = 0.98) for CA; and 10.33% *vs*10.31 ($P = 0.827$, OR = 0.90) for CC. The distributions of *CYP7A1* gene polymorphisms in both groups were in Hardy-Weinberg equilibrium were found all the $P \leq 0.05$. Multiple logistic regression analysis clearly indicated no direct significant involvement between the gene polymorphism frequencies and the gallstone diseases.

Distribution of genotypes and association of polymorphisms with GD Fig. 2 indicates the genotypes of *CYP7A1*gene polymorphisms. The distributions of genotypes for patients and controls are listed in Table 3. Using Chi-square test, there was a significantly higher frequency of A allele of *CYP7A1* gene was observed in patients compared with controls (A allele: 75.76% *vs* 73.54%, $P < 0.05$, Table 3).

There were no significant differences between patients and controls in the polymorphisms of *CYP7A1* gene.

4. Discussion

The result obtained from this study of *CYP7A1* gene polymorphisms with gallstone disease in the Kanpur population. Patients and controls were collected mainly in Kanpur and nearby districts, which were corresponding for both age and sex. The female/male ratio (1:1) and mean age for 45.38 ± 13.11 year for patients and for controls 44.51 ± 10.16 year were obtained. The statistical significant differences were ($P \leq 0.05$) in the data between the groups, with the exception of HDL-C (Table 2). BMI and serum glucose were found higher in patients than in controls, while serum levels of cholesterol, LDL-C, total lipids, and triglycerides were greater in controls. However, these mean differences were not measured clinically significant, since both groups were in the class-I obesity group, and had normal or borderline levels of serum glucose, cholesterol, LDL-C, total lipids, and triglycerides. In distinction to these results, several studies have verified an apparent correlation between BMI and hyperglycemia with gallstone disease,[34] while other studies has been mentioned that dyslipidemias, such as decreased HDL-C and increased triglycerides and LDL-C levels, correlated with increased risk for gallstone disease.[35–37] We found that both genotypic and allelic frequencies of CYP7A1 gene polymorphism did not show significant differences between patients and controls (Table 3). The frequency of the A allele in patients was 59.42%, similar to that observed in population.[38]

In epidemiological research there is clear relationship between plasma lipid concentration and gallstone disease. But in the present study it is found that patients with gallstone disease have lower plasma cholesterol. Data obtained from the study were found similar to the finding of Juvonen,[39] Scragg[40] and Attili[41] even though there was distinction with the finding of.[42] In the case of lowered plasma cholesterol levels in women,[43] suffered with gallstone disease. As in earlier studies it has explained by researcher, plasma lipid concentrations are varying in populations owing to diet habits, environmental factors, genetic factors, ethnicity, *etc.* In the available literature it is still a big question arises in mind that whether changes in plasma lipid concentration are main factors for gallstone disease or some other factors are involved in the gallstone formation which may be still unknown. Till today, the exact mechanism of changing in plasma lipid concentrations which may increases the risk of gallstone disease is not known. Nevertheless, epidemiological studies have indicated that genetic variations have close relation with gallstone disease.

In this study the Kanpur population may has verified that gallstone disease was genetically controlled by polygenetic factors. However, only polymorphism of *CYP7A1* gene may not be connected to gallstone disease. This study was performed to make a relationship between gallstone disease and the *CYP7A1* gene polymorphism at promoter region where dinucleotide repeat microsatellite polymorphism form. But in our finding the *CYP7A1* gene polymorphism may not be related to gallstone disease. We found a slightly higher frequency of A allele in patients than in controls (59.42% *vs* 54.97%, $P < 0.05$). Additionally, *CYP7A1* gene polymorphism did not alter the threonine residue at position 2488 though; it may be an indication of strong linkage.

The frequency of C allele in patients was 24.24% slightly less than the 37.20% observed in a population.[44] Other studies have confirmed the relationship between *CYP7A1* polymorphism with increased LDL-C levels and gallstone formation in a population.[45] In addition, *CYP7A1* deficiency has been correlated with gallstone disease phenotype.[46] In this study, the distribution of their genotypes in the groups was not significantly different from the expected distribution for a population in Hardy-Weinberg equilibrium. In addition, there was no correlation of these polymorphisms with BMI, glucose or lipid profile.

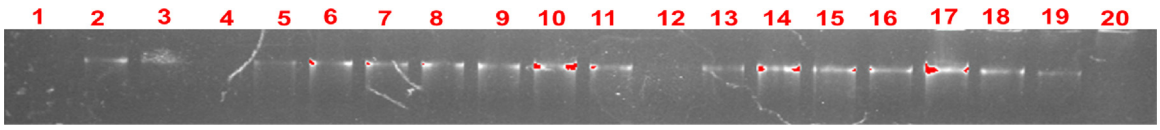

Fig. 1. Isolated DNA from whole blood by Qaigen kit method.

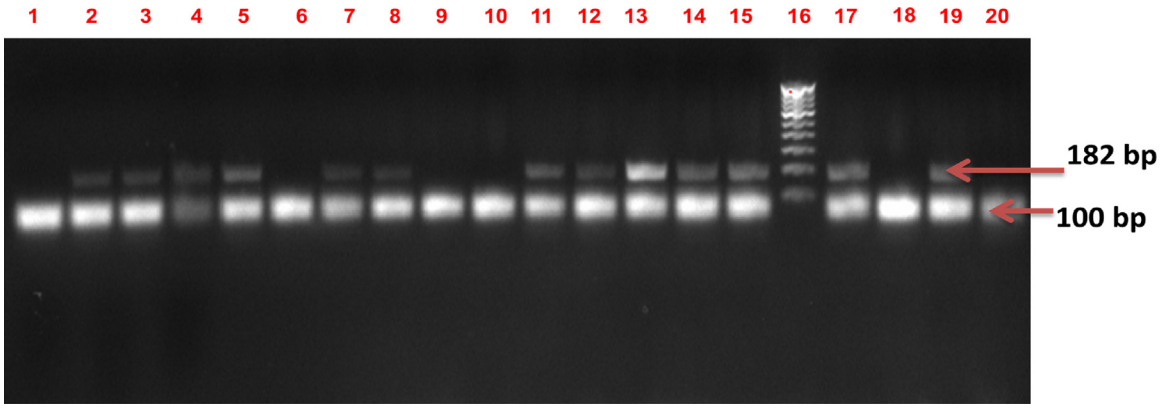

Fig. 2. DNA amplification using PCR, lane 2, 3, 4, 5, 7, 8, 11, 12, 13, 14, 15, 17, 19 are patients samples and lane 1, 6, 9, 10, 18, 20 are control samples, lane 16 is 100 bp ladder.

5. Conclusion

The results of this study showed that there may be not significant association existed between *CYP7A1* gene polymorphisms and gallstone disease in Kanpur. Nevertheless, there is need of further investigation of family pedigrees with gallstone disease. Since gene polymorphisms are heterogeneous among human populations, gallstone disease may be caused by different risk genes among different population. The key factor for gallstone disease is hyper saturation of biliary cholesterol. Hence it would give better understanding if all the genes participated should taken into consideration which are involved in the in the pathway of cholesterol metabolism. This study has provided moderate information about the gall stone diseases. Results of this finding may be helpful to encourage research and knowledge related to gallstone disease especially in the Kanpur population.

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