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RESEARCH ARTICLE

Role of Sequencing Genetic for 708 T>A of Cholesterol 7α Hydroxylase (CYPA17) in Patient with Gallstone Disease

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Abstract

AIM: To investigated the association of the CYP7A1 gene polymorphism and analyze the sequencing of CYP7A1 gene with development of gallstone disease in Iraq population. Methods: The polymorphism were analyzed by polymerase chain reaction followed by restriction fragment length polymorphism, in two group match by age and sex: patient with gallstone (n=140) and stone-free control (n=140).Result: Novelty single nucleotide polymorphism in upstream of CYP7A1 gene was founded for PCR amplification 708 T >A in patient with gallstone and conformed by sequencing data and allelic frequencies in patient and control for T of CYP7A1 gene 38.2% vs 54.6% were significant (p=0.0001).Conclusion: The result have been demonstrated that association between the polymorphism of 708 T> A for CYP7A1 gene and an effect on cholesterol 7 α hydroxylase serum level with high prevalent of gallstone disease.

Keywords: *CYP7A1, Gallstone, Polymorphism, Sequencing analyzing.*

Introduction

Gallbladder stone disease (GSD) is one of the most common diseases in many countries. The formation of GSD is multi-factorial, with a complex interaction between the environment factors and multiple susceptible genes[1].The formation of gallstones is accelerated by impaired gallbladder emptying, hyper secretion of cholesterol into bile, or destabilization of bile by kinetic protein factors[2, 3]. Several risk factors, such as obesity, diet, female gender, metabolic syndrome and type-2 diabetes, are usually associated with this pathology of gallstone [4].

Cholesterol 7-hydroxylase (*CYP7A*, EC 1.14.13.17), a cytochrome P-450 enzyme, is the rate limiting enzyme of hepatic bile acid synthesis, with its activity regulated by bile acids, cholesterol and hormones[5]. Although the amino acid sequence of CYP7A1 between species is highly homologous(80-90%sequence identity), species respond differently to diet cholesterol[6].

As compared with control subjects, the activity of CYP7A1 varied in patients with gallstones[7], and diminished or elevated patterns were observed. The heterogeneity of activities of CYP7A1 in patients with GSD may be related to CYP7A1 polymorphisms. The polymorphism of in the upstream of CYP7A1 gene was reported to affect its enzyme activity [8]. A number of studies have been focused on the association between the polymorphism CYP7A1gene and metabolic disorders of cholesterol and bile acid, including hypercholesterolemia, and GSD [9]. We investigated whether the CYP7A1 polymorphism is an additional genetic risk factor contributing to the expression or development of the gallstone disorder.

Material and Method

We studied two groups matched by age and sex: consecutive symptomatic patients (n = 140) with gallstone disease (GD) and healthy stone-free control subjects (n= 140) confirmed by abdominal ultrasonography. Patients were cholecystectomized at the Division of Gastroenterology of the Babylon Hospital from March 2018 to August. Subjects were also questioned about their past medical history, and their body mass index (BMI) was calculated.

In accordance with the World Health Organization's categories, subjects with BMI ≥ 25 kg/m2 were considered overweight and ≥ 27 as class-I obese. Those with renal or liver

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malfunction were excluded. The Ethical and Research Committee of the study have been approved in the department of biochemistry, Collage of Medicine Babylon University, Iraq.

DNA Amplification and Restriction Fragment Length Polymorphism

Genomic DNA was isolated from whole blood containing EDTA, using Wizard® Genomic DNA Purification Kit. The polymorphisms were analyzed by polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP). Reaction conditions, primers and restriction fragments are summarized in Table 1.In the same way, a region spanning the polymorphic site A-204C in the promoter region of CYP7A1 (chromosome 8), was amplified and cleaved with *Bsa* (1 U for 2 h at 37 ℃) to determine T (normal) and A alleles (27). Five random samples of each polymorphism were sequenced to confirm the results.

Table 1: Conditions and products of polymerase chain reaction followed by restriction fragment length polymorphism

Gene	Primers	Tm (°C)	RE	bp	Alleles
CYP7A1	Forward: TGGTAGGTAAATTATTAATAGATGT Reverse: AAATTAAATGGATGAATCAAAGAGC	58	*Bsa* I	948	A: 581, 367 bp C: 542, 367 and 39 bp

Forward and reverse primers in 5' to 3' directions Tm: Annealing temperature; RE: Restriction enzyme

DNA Sequencing of PCR amplicons

The resolved PCR amplicons were commercially sequenced from both (forward and reverse) termini according to instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from ABI sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed DNA sequences of local bacterial samples with the retrieved neighboring DNA sequences of the NCBI Blastn engine, the virtual positions and other details of the retrieved PCR fragments were identified.

Interpretation of Sequencing Data

The sequencing results of the PCR products of different samples were edited, aligned, and analyzed as long as with the respective sequences in the reference database using Bio Edit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The observed variations in each sequenced sample were numbered in PCR amplicons as well as in its corresponding position within the referring genome.

Checking the Novelty of SNPs

The observed SNP was submitted to the dbSNP database to check their originality. Each particular SNP was re-positioned according to its place in the reference genome subsequently; the determination of the presence of previous SNP was performed by viewing its corresponding dbSNP position. Then, the dbSNPs position for the detected SNP was documented.

Statistical Analysis

Data are presented as mean ± SD. Mean differences in covariates were analyzed by the Student's t-test. A sample size of 96 individuals per group was calculated to detect differences (delta) of 0.14 in polymorphism frequencies between the groups, with 80% power and 5% significance. Allelic frequencies observed in patients and controls were evaluated for differences using the Fisher's exact test when the number of observations in any cell was ≤ 5.

The P values were corrected by t-test for multiple comparisons, taking into account the number of alleles observed, and considered significant when $P < 0.05$. Odds ratios (OR) with 95% confidence intervals (CI) were used as the measure of association between specific genotypes and alleles with GD. Hardy-Weinberg's equilibrium was calculated by $\chi 2$ test. Multiple logistic regression analysis was performed to investigate the independent factors associated with GD. SPSS v16.0 software was used for data analysis.

Result

Sequencing of the 948 bp Region within the *CYP7A1* gene

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Within this locus, the samples were included in the present study that had shown to amplify *CYP7A1* genetic sequences in the chromosome number 8. The latter gene is responsible for encoding on cholesterol 7-alpha-monooxygenase; an enzyme catalyzes a rate-limiting step in cholesterol catabolism and bile acid biosynthesis by introducing a hydrophilic moiety at position 7 of cholesterol. Important for cholesterol homeostasis (https://www .uniprot.org/ uniprot/P22680).The sequencing reactions indicated that the exact identity after performing NCBI blastn for these PCR amplicons(https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).Concerning the supposed 948bp amplicons, NCBI BLASTn engine shown about 99%sequences similarities between the sequenced samples and the intended reference target sequences. By comparing the observed DNA sequences of these local samples with the retrieved DNA sequences (Gen Bank acc.NC_000008.11), the approximate positions and other details of the retrieved PCR fragments were identified (Fig.1).

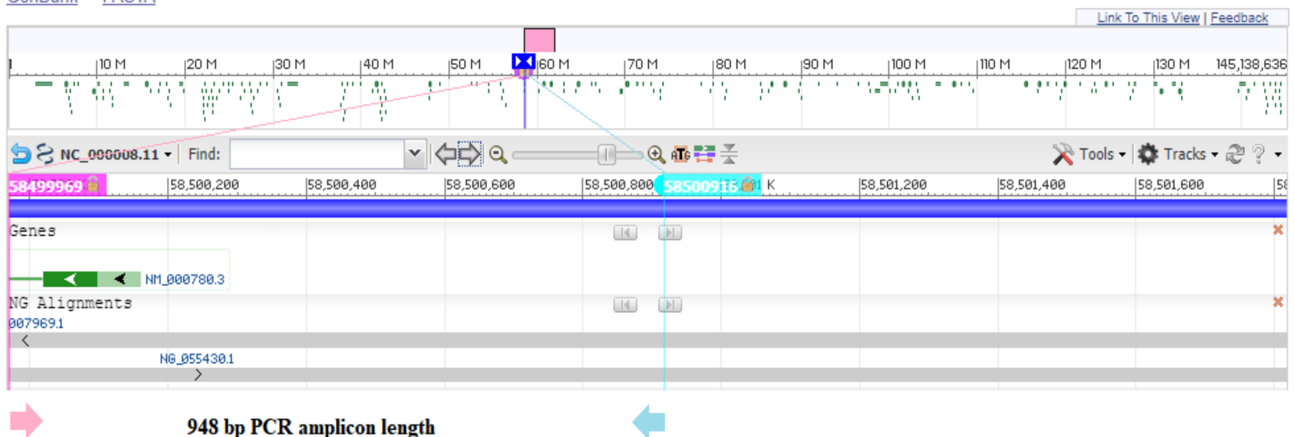

Fig. 1: The exact position of the retrieved 948 bp amplicon that partially covered a portion of the *CYP7A1* gene within chromosome 8(Gen Bank acc no. NC_000008.11).The violet arrow refers to the starting point of this amplicon while the cyan arrow refers to its end point

After positioning the 948 bp amplicons' sequences within the chromosome no. 8, the details of its sequences were highlighted, in terms of the positioning of both forward and reverse primers of the 948 bp amplified amplicon (Table 1).

Table 1: The position and length of the 948 bp PCR amplicons used to amplify a portion of the *CYP7A1* gene within chromosome no. 8 (GenBank acc. no.NC_000008.11). The grey colored sequences referred to the position of the reverse and forward primers, respectively

Amplicon	Referring locus sequences (5′ - 3′)	length
DNA sequences within the *CYP7A1*gene	*AAATTAAATGGATGAATCAAAGAGCAATTTAAAGATAAAACATTACTTACCTTCT CCTAATTCCAAGAATAAGCCATAGACAACAGCATGCTGCTATAGCAATCCCCCAAA TCAAAGATGTGGTCATCATTTTGCAAATCTAGGCCAAAATCTCTGAGGAAGAAAAT CTCTGATTAGAAAGGGAAGGATGCCACTGAAAAGAGACTCAAGCTAGGCTTTTTA TATACATAGTATCCAGATCCATTAACTTGAGCTTGGTTGACAAAGCAAACAATTAG CCATTTGTTCATTCTATTAGAAAAAAAAAAGTGGTAGTAACTGGCCTTGAACTAAG TCCACAGGTATCAGAAGTGGTTCCAAAGCAATCAGAGACCTGCAATACTTGATAA GTTGAAGGTCTCTCAAATATATGTTGACTTAACATTCGGACCTGGGGACAACAGC TAATATTAAGAGTTTGGTATGTGTAAAAAGAACAAATAAACCTGTTTAAGATGGGC ATAGCTAATAAATACATAAACTATAATCATTTAAAGAAAGATAAGAATGAGTTATT CATCAAGCTTATAATTTGGTCTTGTTAACTTTTAAAGAAGAATTAATTTAGGTGCT TTGCCAGAGAGACGGTGATCAAGTTCAGAGGAAAGAGAACTGGGAAAAACATTTC TGCTGCTTCATGTTTCAGTGCTTTTATAAAATCATAGATATTTTACTACATATAGTA ACATGTTACTCACCTAACATCCTCAAGAGAGTATTCTACATAAGTGCATCCTCTAG ATAAATATAACTTATTGGAAGCATCAAAATTTTATCTATCATTCTTGTGACTAAAAT GTTTATCAATGTTTCTAATTATTAAAGTAATAAATTTCATTTCAAGTAAGTTAAGAC TAACTAAAACATATCTTGGTGGAAAGTGAACATCTATTAATAATTTACCTACCA**	948bp

* refers to the reverse primer sequences
** refers to the forward primer sequences

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The alignment results of the 948bp samples revealed the presence of only one SNP occurred in this position in only two, (S1 and S2); out of six analyzed samples in comparison with the referring reference DNA sequences (Fig. 2).

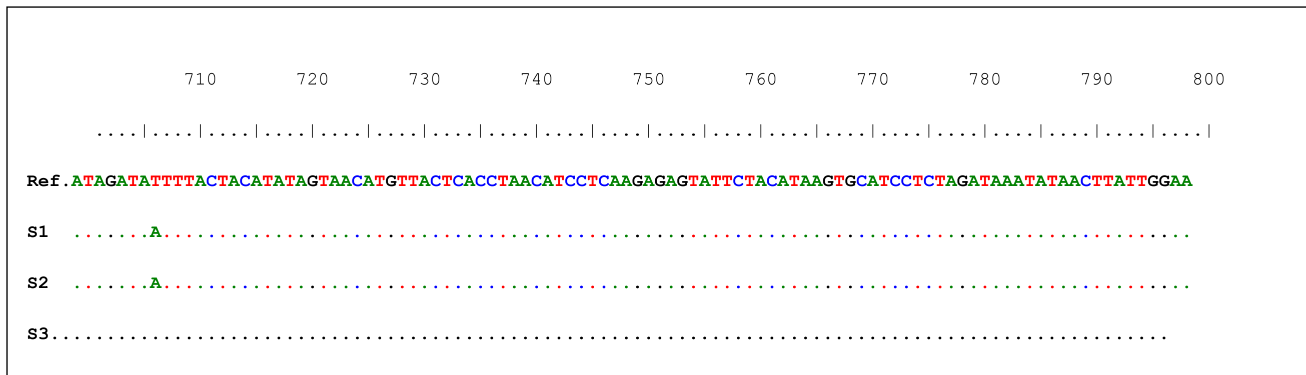

Fig. 2.DNA sequences alignment of three local samples with their corresponding reference sequences of the 948 bp amplicons of the *CYP7A1* genetic DNA sequences. Each substitution mutation was highlighted according to its position in the PCR products. The symbol "ref" refers to the NCBI referring sequence, "S1-S3" refer to the samples 1 to 3, respectively

The sequencing chromatogram of the observed substitution SNP, as well as its detailed annotations, was documented, and the chromatogram details of the observed SNP was shown according to its position in the PCR amplicon, in whichS1 and S2 samples had this T708A variant (Fig. 3).

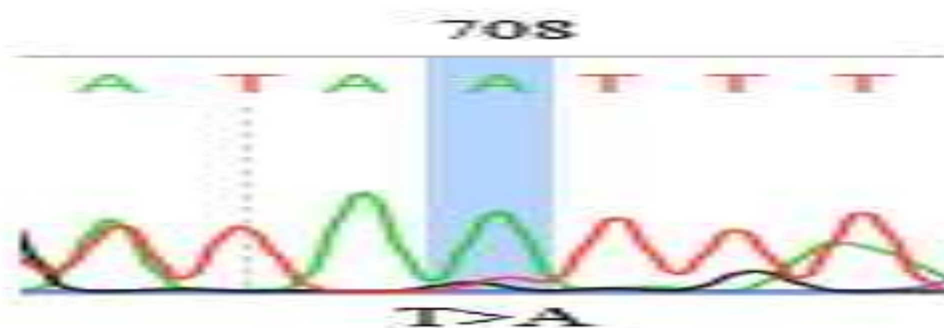

Fig. 3: The pattern of the observed substitution mutation within the DNA chromatogram of the targeted 948bpamplicons within the *CYP7A1* gene. The observed substitution mutation is highlighted according to its position in the PCR products. The symbol ">" refers to substitution mutation

SNPs Characteristics Check

To elucidate the positions of the observed SNP with regard to their deposited SNP database of the sequenced 948 bp fragment, the corresponding position of the *CYP7A1*gene was retrieved from the dbSNP server (https://www.ncbi.nlm.nih.gov/projects/SNP/). To find out the nature of the observed SNP, a graphical representation was performed concerning the*CYP7A1*dbSNP database within chromosome 8 (GenBank Acc. No. NT_008183.20).By reviewing the dbSNP engine, it was found that this SNP was found to be not known previously (Fig.4).

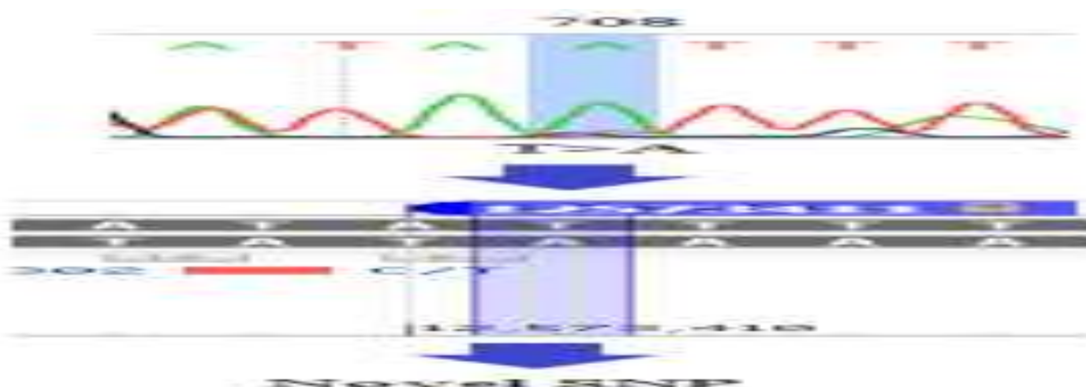

Fig. 4: The SNP's novelty checking of *CYP7A1*genetic single nucleotides polymorphism using dbSNP server. The identified SNP is marked with a blue color

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Therefore, it was found that this observed variant was novel and positioned in the intronic region within the *CYP7A1* gene. To summarize all the results obtained from the sequenced 948 bp fragments, the exact position of the observed variation was described in the NCBI reference sequences (Table 2).

Table 2: The pattern of the observed SNP in the 948 bp ampliconsin comparison with the NCBI referring sequences of the *CYP7A1* gene within chromosome 8 (Gen Bank acc. no. NT _008183.20)

Sample No.	Zygosity status	Native	Allele	Position in the PCR fragment	Position in the reference chromosome 8	Type of SNP	SNP novelty
S1, S2	Heterozygous	T	A	708	12573411	Upstream variant	Novel

Biochemical Characterization

Patient with gallstone and control with mean age of 41.31±13.48 years vs 43.84±10.81 years (p=0.085), and in both group 67.14% of population were female and 32.85% were male, while the cholesterol 7 α hydroxylase enzyme of recovered in patient gallstone was 28.85±9.74 compare with control that clinical significant (p=0.0001), and body mass index (BIM) were higher in patient than in the control group 30.10±5.54 kg/m² vs 26.15±4.94 kg/m² (p=0.0001) for BMI.

Table 3: Clinical characteristics in control and patient with gallstone (mean ±SD)

Variable	Patient (n=140)	Control (n=140)	p
Sex (F/M)	67.14%/32.85%	67.14%/32.85%	
Age	41.31±13.48	43.84±10.81	0.085
Body mass index (kg/m2)	30.10±5.54	26.15±4.94	0.0001
serum cholesterol 7αhydroxylase (CYP7A1) (ng/ml)	28.85±9.74	6.32±2.04	0.0001

Association and Distribution of CYP7A1 Gene Polymorphism with Gallstone

The genotypes of CYP7A1 gene polymorphism were indicated in Figure (5), while allelic and genotypic frequencies of gallstone patient and control were shown in Table (4) that the frequencies of T allele (CYP7A1) gene polymorphism in patient and control were 61.8% vs 45.4 % (p=0.0001, OR=1.9), while genotype percentage in patient and control as following: for AA, 37.9% VS 28.6 % (OR =1.5. P=0.128), for AT, 47.9% VS 33.6 % (OR =1.8, P=0.021), TT 14.2% VS 53% (OR= 0.27, P=0.00001).

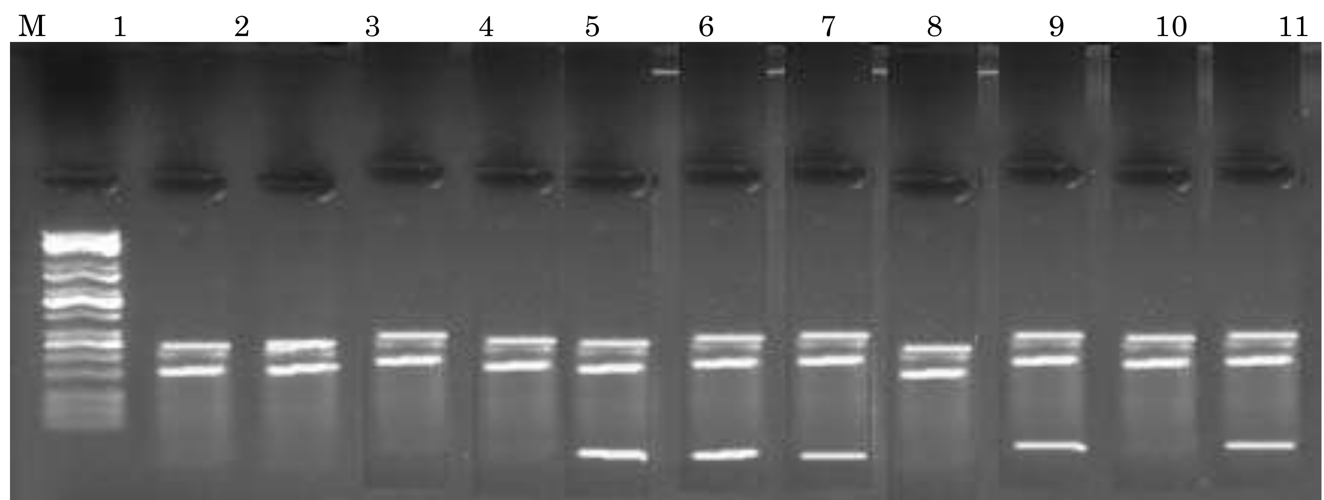

Figure 5: genotyping of CYP7A1 gene M: DNA Marker (1000bp) (iNtRON Biotechnology ,Inc), lanes 1,2,8 for TT Type , lanes 3,4,10 for AA ,lanes 5,6,7,9,11 for AT

Table 4: Statistical analysis of association between genotypes and alleles of CYP7A1gene (rs3808607) in gallstone patient and control

Genotype or Allele	Patients (No. = 140)		Controls (No. = 140)		Odds Ratio	95% Confidence Interval.	*p*-value	Corrected *p*
	No.	%	No.	%				
AA	53	37.9	40	28.6	1.52	0.92 - 2.51	0.128	NS
AT	67	47.9	47	33.6	1.82	1.12 - 2.94	0.021	NS
TT	20	14.2	53	37.8	0.27	0.15 - 0.49	0.00001	0.00001
A	173	61.8	127	45.4	1.95	1.39 - 2.73	0.0001	0.0005
T	107	38.2	153	54.6	0.51	0.37 - 0.72	0.0001	0.0005

p: Two-tailed Fisher's exact probability; NS: Not significant (*p*-value> 0.05)

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The distributions of CYP7A1 gene polymorphism in both groups were in Hardy-Weinberg equilibrium (all p≤ 0.05) in Table (5).

Table 5: Numbers and percentage frequencies (observed and expected) of CYP7A1 (rs3808607: T>A) genotypes and their Hardy-Weinberg equilibrium (HWE) in Gallstone patients and controls

Genotype	Patients (No. = 140)				Controls (No. = 140)			
	Observed		Expected		Observed		Expected	
	No.	%	No.	%	No.	%	No.	%
AA	53	37.9	53.4	38.1	40	28.6	28.8	20.6
AT	67	47.9	66.2	47.3	47	33.6	69.4	49.6
TT	20	14.2	20.4	14.6	53	37.8	41.8	29.8
HWE Analysis	*p*-value=0.873 [NS]							

P: Two-tailed Fisher's exact probability; NS: Not significant (p-value> 0.05)

Discussion

In the work that were reported on the association of CYP7A1 gene polymorphism and sequencing with gallstone in Iraq, patient and control were matched for both age and sex .The female/ male and mean age (41.31±13.48 years) of patient that supported the notion that female gender and age were risk factor. BMI and level of cholesterol 7α hydroxylase (CYP7A1) were higher in the patient than control, that mean significant differences (p =0.0001) who the result , anther studies have been demonstrated a clear correlation between BMI with gallstone were agreement with Sun H[10], while the level of CYP7A1 serum enzyme were elevated in gallstone disease that were agreement with W Gordon [11]. Statistical analysis were showed that CYP7A1 gene Bsa I polymorphism frequencies between patient and control were different in Table (4), and the frequency of A allele in patient was 61.8 % slightly higher in control 45.4% in Iraq population, the result have been confirmed by sequencing of CYP7A1 polymorphism in gallstone and agreement with kurzawski M [12].In the studied gene in the work , the distribution of the genotyping in the groups were significantly from the expected distribution for population in Hardy-weinberg equilibrium.

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