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Polymorphism of XbaI (rs 693) G/A and TLR4 – rs 4986791 C/T Genes with Gallbladder Disease

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ABSTRACT

Background: Gallstone disease (GSD) is a prevalent illness worldwide. Study aimed to investigate the association of XbaI (rs 693) G/A and TLR4 – rs 4986791 C/T genes with gallbladder disease.
Material and Methods: Over the course of three months, from October 2020 to December 2021, samples were taken from fifty healthy persons and one hundred patients with a diagnosis of gall bladder infection from Al. Hilla Teaching Hospital. The patients' ages ranged from fifteen to seventy-one years. TLR4 –rs 4986791 C/T and XbaI (rs 693) G/A genes were found using the tetra arms approach in both sick and healthy groups.
Results: In the event that polymorphisms exist in TLR4 rs4986791 C/T, T-ARMS-PCR was employed to genotype the variant. Based on whether the polymorphism is present or not, the TLR4 rs4986791 C/T distribution is as follows: In GG wild homozygous, the T-ARMS-PCR product only contains the C allele at 295 bp. For the lane (TT) mutant type homozygote, the 219 bp T-ARMS-PCR product showed only the T allele; for the heterozygote (CT), the C and T alleles were detectable in the 295 bp and 219 bp T-ARMS-PCR product. Using T-ARMS-PCR, the outer internal control at 468 bp was identified in order to genotype the XbI rs693 G/A in the presence of XbI rs693 G/A polymorphisms. Distribution of the XbI rs693 G/A in the following categories according to the existence or absence of polymorphism: the (GG) wild type homozygote only showed the G allele, as evidenced by a 216 bp T-ARMS-PCR result. The heterozygote (GA) at 216 bp and 150 bp displayed both the G and A allele, however the lane (AA) mutant type homozygote only displayed the A allele at 150 bp, according to T-ARMS-PCR. The external internal control was found in the 323 bp T-ARMS-PCR product.
Conclusion: Patients with GD may benefit from early detection and alertness due to the potential power of the APOB rs693 polymorphism as a predictor of GD risk. This makes it useful for the goal of detection in clinics. TLR4 and gallbladder disease, however, do not connect.
Key words: gall bladder disease, single nucleotide polymorphism, Apo lipoprotein toll-like receptor 4

INTRODUCTION

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Up to 15% of Americans suffer from gallbladder disease (GD), which is a relatively common ailment that has a substantial health care cost in the country (1,2). Over two times as many women as men will experience GD during their lives (3). and GD affects them more frequently than males (4). According to

recent data from ultrasonography surveys, ethnicity is a recognized risk factor for GD; especially, Hispanic individuals with a background in central and south America have the greatest rate of the disease (2,5). A high incidence of GD is also seen in the north Indian population, impacting 29.5% of men and 64.1% of women (5). Conversely, those of East-South Asian (China, Japan, India, and Thailand) and African American (African American) heritage exhibit a decreased prevalence of GD development (6).

In addition to race, aging, being in a particular sex group, eating a hypercaloric diet heavy in carbohydrates and low in fiber, and other characteristics are risk factors for the development of GD. Obesity is a significant factor in the development of GD. Hepatic cholesterol buildup, intestinal bile acid absorption, bile acid production, inflammation, and stasis are other factors that affect the start of GD. There is mounting evidence that genetic factors play a major role in the development of GD (3,7). The low risk of complications suggests that laparoscopic correction (LC) is a safe procedure that can be performed on a day case basis with high patient satisfaction and a low readmission rate. This is as a result of the fact that most problems were minor and easily handled. Additionally, LC will decrease both the rate of bed occupancy and the overall burden on hospitals (8). Ultimately, the same-day diagnostic and patient care can be provided more quickly thanks to the microwave approach's faster turnaround time than the previous method. For small biopsies, a microwave might be a better option than a larger one. The two main drawbacks of using the micro-wave approach in high throughput histopathology facilities continue to be its lack of automation and the need for additional personnel (9).

Because it is believed to affect lipid metabolism and composition, the apolipoprotein B (APOB) gene is essential for the development of GD (10). The APOB gene's exon 26 has the synonymous variation XbaI polymorphism site (rs693) (11). Synonymous single-nucleotide polymorphisms (SNPs) are known to be considered spurious occurrences under no to little selection. SNPs are defined as nucleotide changes at synonymous codons that preserve the encoded aminoacid (12). Rather of being distributed haphazardly across genes, synonymous SNPs are more likely to target conserved areas (13). Furthermore, synonymous mutations are thought to be the primary cause of several diseases' mutations and have a significant role in disease penetrance, accounting for a higher percentage of somatic mutations seen in human pathologies (14), for instance, GD. Numerous investigations have looked at the connection between this polymorphism and GD, but the results haven't been entirely obvious (15). The TLR4 gene is found on chromosome 9 and has three exons. At exon 3, two non-synonymous SNPs (+1196C/T rs4986791) and Asp299Gly (Thr399Ile), respectively, replace these positions. A decrease in ligand recognition, protein interaction, and lipopoly-saccharide response may arise from Asp299Gly alterations, which modify the extracellular region of TLR4's normal structure (16).

TLR4 is a well-known member of the TLR family, which includes both immunological and non-immune cells. Immune cell TLR4 signaling influences a variety of immune response mechanisms, including CD8+ T-cell cytotoxicity, antigen presentation, and dendritic cell (DC) maturation, all of which are essential components of anti-tumor immunity (17). Since the Tlr4 gene's coding sequence contains 15 polymorphisms, it is highly polymorphic (18). Asp299Gly and Thr399Ile are two co-segregating SNPs in this gene among its numerous SNPs. Many studies have looked into the relationship between these SNPs and the chance of developing various cancers, including leukemia (23), breast cancer (19), gastric cancer (20), prostate cancer (21), hepatocellular carcinoma (22), colorectal cancer (26), gall bladder cancer (24), and leukemia (23). This study aimed to investigate the association of Xbal (rs 693) G/A and TLR4 – rs 4986791 C/T genes with gallbladder disease.

MATERIALS AND METHODS

Sample Collection

Between October 2023 and June 2024, a case-control study was conducted in Babylon, Iraq. The 100 gallbladder patients from Al. Hila Teaching Hospital, whose ages ranged from 15 to 71 years, were divided into 50 controls for the study. The researcher used a questionnaire form she had created during an interview with these patients. For Xbal and TLR4 genotyping polymorphisms, the final three milliliters were placed in an EDTA tube.

Genotyping of Xbal and TLR4 Polymorphisms

In compliance with company guidelines, genomic DNA was extracted from blood samples using a gSYAN DNA kit extraction kit (frozen blood) from Geneaid, USA.ARMS-PCR, or amplification-refractory mutation system PCR, is a rapid and simple method

for geno-typing Xbal and TLR4. Primers for Xbal and TLR4 genotyping are included in *tables 1* and *2*.

Thermo cycling for TLR4 rs4986791 C/T and Xbl rs693 G/A was performed under settings that PCR conditions in *table 3*.

Statistical Analysis

The patients' and the control group's genotype distributions were compared using the chi-square test. Less than 0.05 was the threshold for a statistically significant p-value.

RESULTS

Genotype of TLR4 rs4986791 C/T by Arms Methods T-ARMS-PCR was used to genotype TLR4 rs4986791 C/T in the presence of polymorphisms in TLR4 rs4986791 C/T. The distribution of TLR4 rs4986791 C/T in groups according to the presence or absence of the polymorphism is as follows: only the C allele at 295 bp in the T-ARMS-PCR product is present in GG wild homozygous. Only the T allele was visible in the 219 bp T-ARMS-PCR product for the lane (TT) mutant type homozygote, while the C and T alleles were seen in the 295 bp and 219 bp T-ARMS-PCR product for the heterozygote (CT). *Fig. 1* shows that the outer internal control was detected at 468 bps.

Depending on whether the polymorphism is present or not, the genotyping result for Xbl rs693 G/A in the presence of Xbl rs693 G/A polymorphisms by T-ARMS-PCR determines the distribution of Xbl rs693 G/A in groups: the 216 kb T-ARMS-PCR result for the (GG) wild type homozygote only revealed the G allele. The lane (GA) heterozygote had both the G and A alleles at 216 bp and 150 bp, but the lane (AA) mutant type homozygote only had a single allele at 150 bp. T-ARMS-PCR product, 323 bp, showed the outer internal control (*fig. 2*).

Association of TLR4 rs4986791 C/T gene SNPs

In this case-control study, we looked at the distribution of alleles and genotype frequency of TLR4 SNPs genotypes (rs4986791 C/T) among Iraqi patients with gall bladder disease and healthy controls. The genotype and allele frequency patterns for the (rs4986791 C/T) SNPs are shown in *table 4*. When comparing genotypes TT and CC in gall bladder patients to control groups, we discovered no significant changes ($p > 0.4$ and $p > 0.1$, respectively) in the case of (rs4986791 C/T) SNPs. No significant differences were

Table 1 - The Tetra-ARMS-PCR Primers for Xbl rs693 G/A polymorphisms with their sequence and amplicon size

T-ARMS-PCR Primer	Sequence (5'-3')	Product size
Forward inner primer (G allele)	TTCGGTCTCGTGTATCTTCTCGG	216
Reverse inner primer (A allele)	AGGCCAAATTCCGAGAGCCT	150
Forward outer primer Reverse outer primer	TAGCAGCAAGAGTCCACCAATCAG TGGTGAAATTCAGGCTCTGGAAC	323

Table 2 - The Tetra-ARMS-PCR Primers for TLR4 rs4986791 C/T gene polymorphisms gene polymorphism with their sequence and amplicon size

T-ARMS-PCR Primer	Sequence (5'-3')	Product size
Forward inner primer (T allele)	CTCAAAGTGATTTTGGGACCAT	219
Reverse inner primer (C allele)	TCAGATCTAAATACTTTAGGCGGG	295
Forward outer primer Reverse outer primer	AATGTTTCTTCATTTTCCCTGGT ATTGAAAGCAACTCTGGTGTGAG	468

Table 3 - All PCR thermocycler conditions were done for genes (TLR4 rs4986791 C/T and Xbl rs693 G/A

PCR step	Temp.	Time	repeat
Initial denaturation	95°C	5 min.	1
Denaturation Annealing Extension	95°C 61°C 72°C	30 sec. 30 sec. 30 sec.	35 cycle
Final extension	72°C	5 min	1
Hold	4°C	Forever	-

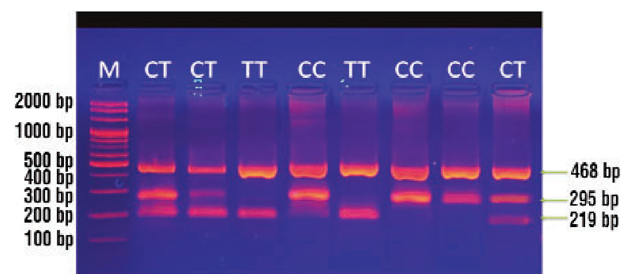

Figure 1 - Analysis of the TLR4-rs4986791 product using T-ARMS-PCR An picture of agarose gel electrophoresis showed the polymorphism of the C/T gene. There the marker, M (100-2000 bp), is found. The lane (CC) wild type homozygote's 295 kb T-ARMS-PCR result only showed the C allele. For the lane (TT) mutant type homozygote, the 219 bp T-ARMS-PCR product showed only the T allele; for the heterozygote (CT), the C and T alleles were detectable in the 295 bp and 219 bp T-ARMS-PCR product. The outer internal control was found in the T-ARMS-PCR product at 468 bps.

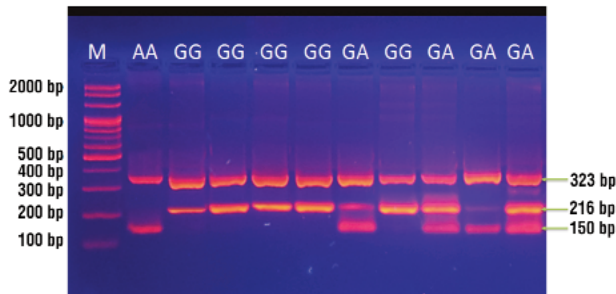

Figure 2 - An agarose gel electrophoresis image showed the results of the T-ARMS-PCR product analysis of the Xbal rs693 A/G gene polymorphism. There the marker, M (100-2000 bp), is found. For the lane (GG) wild type homozygote, the 216 bp T-ARMS-PCR result alone showed the G allele. While the heterozygote (GA) showed both the G and A allele at 216 bp and 150 bp, the lane (AA) mutant type homozygote's T-ARMS-PCR result only revealed the A allele at 150 bp. The outer internal control was found in the T-ARMS-PCR product at 323 bps.

seen with heterozygote CT, p≤0.06. While the frequency of CT genotype in the control group was 30%, the frequency in the gall bladder was 48.0%, with an odd ratio of 2.154 (0.948 - 4.894). The gall bladder's estimated TT genotype frequency was (20%), whereas the control group's frequency was (14%). The odd ratio was 1.535 (0.533 - 4.422). The frequency of the TLR4 SNP alleles and genotypes (rs4986791 C/T) is displayed in *table 4*. The genotyping results for the heterozygote mutant type TT were as follows: the genotype for CT was 2.154 (0.948 - 4.894) P = 0.06), and the genotype for TT was 1.535 (0.533 - 4.422) and p = 0.4.

Table 5 shows that when comparing female gall bladder patients to male groups, there were no significant differences in genotypes TT and CC (p > 0.5 and p > 0.9, respectively). With heterozygote CT, no significant differences were seen (p ≤ 0.3). In the female gall bladder group, the frequency of the CT genotype was 84.0%, with an odd ratio of 1.467 (0.464 - 4.638). On the other hand, 42% of the male group had the CT genotype frequency.In the female group with gall bladders, the estimated TT genotype frequencies were 26%, whereas the similar numbers in the male group were 26%. 0.539 (0.133 - 2.183) was the odd ratio.

Association of Xbal (rs 693) G/A Gene SNPs

Table 6 reports the genotype and allele frequency distributions for the (rs 693)G/A) SNPs. Regarding the (rs 693)G/A) SNPs, we discovered that, when compared to the control groups, patients with gall bladders had genotypes AA and GG that differed significantly (p=0.01* and p=0.005**, respectively). Heterozygote GA showed no significant changes (p=0.3). In the gall bladder, the prevalence of the GA genotype was 22.0%, with an odd ratio of 1.732 (0.611 - 4.912), compared to 14% in the control group. The estimated frequencies of AA genotype in the gall bladder were (32%), whereas the control group had a frequency of AA genotype of only 12%. The odd ratio was 3.451 (1.220 – 9.759).

In the instance of (rs 693) G/A) SNPs, *table 7* demonstrated that no significant differences were observed between genotypes AA and GG in female gall bladder patients when compared to male groups (p=0.9 and p=0.8, respectively). Heterozygote GA did not demonstrate any significant differences either (p= 0.8). When compared to the male group, the

Table 4 - Numbers and percentage frequencies of TLR4 gene SNPs (rs4986791 C/T) genotypes and their Hardy-Weinberg equilibrium (HWE) in gall bladder patients compared with control groups

	No. & %		Odds Ratio	p. value
	Patients	Healthy		
CC	16(32%)	28(56%)	0.537 (0.246 – 1.175)	0.1
TT	10(20%)	7(14%)	1.535 (0.533 - 4.422)	0.4
CT	24(48%)	15(30%)	2.154 (0.948 - 4.894)	0.06
C allele	56 (56%)	71 (71%)		
T allele	44 (44%)	29 (29%)		

Table 5 - Numbers and percentage frequencies of TLR4 gene SNPs (rs4986791 C/T) genotypes and their Hardy-Weinberg equilibrium (HWE) in gall bladder patient's male and female

	No. & %		Odds Ratio	p. value
	Male	Female		
CC	10(52%)	6(32%)	1.032 (0.3023 - 3.516)	0.9
CT	16(84%)	8(42%)	1.467 (0.464 - 4.638)	0.5
TT	5(26%)	5(26%)	0.539 (0.133 - 2.183)	0.3
C	28 (61%)	16 (53%)		
T	18 (39%)	14 (47%)		

Table 6 - Numbers and percentage frequencies of Xbal (rs 693) G/A genotypes and their Hardy-Weinberg equilibrium (HWE) in gall bladder patients compared with control groups

	No. & %		Odds Ratio	p. value
	Patients	Control		
GG	23(46%)	37(74%)	0.299 (0.129 – 0.694)	0.005**
GA	16(32%)	6(12%)	3.451 (1.220 – 9.759)	0.01*
AA	11(22%)	7(14%)	1.732 (0.611 – 4.912)	0.3
G allele	57 (57 %)	81 (81%)	0.3109 (0.644-0.568)	0.0003
A allele	43(43%)	19 (19%)		

Table 7 - Numbers and percentage frequencies of Xbal (rs 693) G/A genotypes and their Hardy-Weinberg equilibrium (HWE) in gall bladder patient's male and female

	No. & %		Odds Ratio	p. value
	Female	Male		
GG	14(45 %)	9(47%)	1.093 (0.348 - 3.435)	0.8
GA	7(22%)	4(21%)	0.914 (0.228 - 3.662)	0.8
AA	10(32%)	6(32%)	0.969 (0.285 - 3.302)	0.9
G allele	35 (56%)	22 (58%)		
A allele	44%))27	16 (42%)		

frequency of the GA genotype was (21%), but the frequency in the gall bladder female group was 22.0% and the odd ratio was 0.914 (0.228 - 3.662). An odd ratio of 0.969 (0.285 - 3.302) was found for the gall bladder female group, and the predicted frequencies of the AA genotype in that group were (32%).

DISCUSSION

TLRs are members of the family of pattern-recognition receptors (PRRs), which are expressed by antigen-presenting cells including T and dendritic cells, among others. Following their activation by pathogen-associated chemicals, TLRs transduce signals through several intracellular pathways, which in turn activate transcription factors such as NF-κB, AP-1, and interferon regulatory factors (IRFs). Furthermore, inflammatory reactions are brought on by these transcription factors' production of type I interferon and inflammatory cytokines (27,28). The phagocytosis process and other host defense systems, like pattern recognition in microbial infections, depend on TLRs. Receptors that identify conserved molecular patterns that are expressed by infectious diseases are known as pattern recognition receptors, or PRRs for short. They participate in the mediation of transcription factor activation, including NF-κB and pro-inflammatory cytokines (25). This causes inflammation. According to a study (29), polymorphisms in these TLR genes are not expected to be associated with an increased risk of bladder cancer. Additionally, there was no correlation found between the TLR3, 4, and 9 genes and bladder cancer risk. The investigation's conclusions support that assessment. Ethnically diverse populations' functional studies may provide light on changes connected to the onset of bladder cancer and changes brought on by the disease.

Several genes have been associated with an increased risk of GD thus far, including apolipoprotein E, mucin-like protocadherin, and ATP binding cassette subfamily G member 8 (30,31). Moreover, a growing body of research is associating the APOB rs693 polymorphism with a higher risk of GD. Because so few samples were utilized in each study, the findings from each one could not be entirely trustworthy. Dixit et al.'s study comprised 322 healthy controls and 214 GD patients; the rs693 polymorphism may not be connected to a higher incidence of GD (31,32). imply that people who have the A-allele or AA genotype of the rs693 polymorphism may be more susceptible to GD. The Asian population (OR: 1.58, 95% CI: 1.48–2.84 in the heterozygote model), the control group's hospital-based source (OR: 1.79, 95% CI: 1.13–2.84 in the dominant model), and the group as a whole (OR: 1.40, 95% CI: 1.05–1.87) all showed a significant correlation with the rs693 polymorphism and increased risk of GD. shows the possible connection between the GD risk and the APOB rs693 polymorphism, offering a helpful marker for identification in future large-scale treatment trials.

CONCLUSION

According to our current meta-analysis, the APOB rs693 polymorphism may be a strong predictor of GD risk. This means that it can be used as a diagnostic tool in clinics to identify and warn patients with GD early on. However, TLR4 is not linked to gallbladder illness.

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Conflict of interest

All authors declared there is no any conflict of interesting.

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Ethical Approval

This study approved by Ethical committee of Department of Biology (No.33 in 2023).

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