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Molecular Study of Celiac Patient in Basrah Province

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ABSTRACT

Celiac Disease (CD) is an autoimmune disorder triggered by gluten ingestion, leading to small intestine damage and potential malnutrition. The CD has a strong genetic basis, primarily linked to specific Human Leukocyte Antigen (HLA) gene variants, especially HLA-DQ2 and HLA-DQ8, essential in presenting gluten peptides to immune cells. **Objective:** The study aims to evaluate the molecular detection of HLA-DQA1, HLA-DQB1, and Tissue Transglutaminase (TGM2) genes by using conventional PCR technique with specific primers in CD patients, also to DNA sequencing of HLA-DQA1, HLA-DQB1, and TGM2 genes and compare them with standard genes to detect Single Nucleated Polymorphism (SNP) occurring in studied genes.

Methodology. This study used case control design with 118 total participants of whom 68 met the criteria for newly diagnosed CD and 50 comprised the control group. Participants were recruited from various teaching hospitals in Al-Basrah, Iraq, from December 2023 to June 2024. There were fifteen samples total five for each primer used for DNA sequencing. The PCR and Sanger sequencing were performed to detect genetic polymorphisms in these genes and the novel DNA sequences were aligned with the corresponding reference sequences from the NCBI.

Results. The study identified ten SNPS in the HLA-DQA1 gene, one in the HLA-DQB1 gene, and four in the TGM2 gene. These SNPs had different zygosity, with HLA-DQA1 having both homozygous and heterozygous forms.

Conclusion. The dbSNP database confirmed these spotted polymorphisms, enhancing the understanding of genetic variance in CD-associated genes. This research supports the necessity for genetic testing to identify CD predisposition and could contribute in further genomic and clinical investigations of autoimmune and immune-medical diseases.

Keywords: Celiac disease, HLA-DQA1gene, HLA-DQB1 gene, and TGM2 gene

INTRODUCTION

Celiac disease (CD) or gluten sensitive enteropathy is a complex immunological mediated disorder induced gluten intolerance from gliadin molecules in patients with genetic susceptible background [1]. The heterodimeric "major histocompatibility (MHC) class II molecules DQ2 and DQ8 located on antigen-presenting cells (APC)", which confer enhanced antigen binding with gliadin-derived peptides and ultimately lead to antigenic presentation to Cluster differentiation 4 (CD4) Lymphocyte (T cells), thereby inducing an immune response, are encoded by the "human leukocyte antigen (HLA)-DQ2 and HLA-DQ8" on APC [2]. Villous atrophy, cystic hyperplasia, intraepithelial lymphocyte proliferation, and, ultimately, jejunal and duodenal lesions are the outcomes of the subsequent immune response provocation [3].

Epidemiologists believe CD is underdiagnosed due to its 1.4% global prevalence. Symptoms like severe diarrhea, stomach discomfort, lethargy, bloating, weight loss, vomiting, and anemia often occur late in the disease's course and are not specific to CD. [4]. Under diagnosis, delayed diagnosis, and misdiagnosis of the disease condition remain the main challenges in the management of CD since patients do not usually exhibit persistent gastrointestinal problems prior to the suspicion of CD [5]. However, a number of medical

conditions, including hypergamma-globulinemia sprue, Whipple disease, Human Immunodeficiency Virus, Crohn's disease, and microscopic enterocolitis, have been linked to enteropathies [6]. The serological identification of anti-tissue transglutaminase (tTG) and anti-gliadin peptide antibodies is an additional option for CD diagnosis as it offers a noninvasive means of identifying individuals who may have CD [7]. In recent years, genetic testing has been used as a diagnostic approach for CD in patients suspected of having the disease. This testing looks for the existence of "HLA-DQ2 and HLA-DQ8" haplotypes associated with CD [2].

Indeed, the diagnosis of Celiac Disease was determined using histological examination and the Marsh classification to evaluate the extent of villous atrophy and intraepithelial lymphocytosis [8]. Clearly, it necessitated an upper gastrointestinal endoscopy accompanied by duodenal or jejunal biopsies conducted under sedation or general anesthesia, it is necessary to develop a biomarker that is more accurate, inexpensive, noninvasive and rapid for diagnosis of CD [9] .

MATERIALS AND METHODS

Study design/ subjects

A case-control study included 118 participants: 68 patients diagnosed with Celiac Disease and 50 healthy individuals serving as the control group. The patients in this study were newly diagnosed based on medical and laboratory investigations. All patients sought medical care at Al-Basrah Teaching Hospital and Al-Fayhaa Teaching Hospital, "Liver and Digestive System Disease and Surgery Hospital" and "Al-Sadar Teaching Hospital in Al-Basrah" province between December 2023 and June 2024. The average ages of the study population were 15 to 57 years. There were fifteen samples total five for each primer used for DNA sequencing. The relevant information from the patients and control group taking part in the study was obtained using the questionnaire. Every participant in the current study underwent an examination by hospital professionals.

Exclusion and inclusion criteria

A significant number of persons were omitted due to their failure to satisfy the inclusion requirements, such as indications of Celiac disease but without definitive confirmation by biopsy or serological test, the presence of other autoimmune disorders, and individuals aged below 15 years.

Ethical Considerations

The Training and Human Development Unit of the Al-Basrah Health Department, Ministry of Health, Iraq, granted full approval for the current study. The research committee's decision (number 490/2023) on December 3, 2023, approved the request in compliance with the Helsinki Declaration. The authors obtained each participant's signed agreement to conduct this study in accordance with international research ethics guidelines.

Sample Collection

A total of 2 mL of blood samples were collected from patients and controls and transferred into sterilized EDTA tubes for molecular analysis, specifically DNA extraction. DNA was isolated from the entire blood of participants with Celiac diseases (CD) and a control group using a standard DNA extraction kit protocol (Favorgen/ Taiwan). Each sample was extracted individually and then subjected to gel electrophoresis for result verification. As shown in **Figures (1).**

Figure (1): Agarose gel electrophoresis appearance demonstrating DNA extraction after 40 minutes at 110V over a 1% agarose solution stained with Ethidium Bromide.

Polymerase Chain Reaction Technique

All samples were tested for the presence of the HLA-DQA1, HLA-DQB1, and TGM 2 genes by PCR, per the instructions provided by Macrogen Inc. Geumchen. Then, using gel electrophoresis, diluted forward and reverse primers were used for the PCR, as shown in **Table (1).** The final working solution (10 pmol\$\mu$l) for each primer was created by diluting the stock solution with Tris-EDTA (TE) buffer.

Table (1): Specific primers used in the investigation.

PCR Thermocycling Condition

The thermal cycling settings for each gene were determined using a standard PCR thermocycler setup as outlined in **Table (2).**

Table (2): PCR Amplification Program for PCR

Only unambiguous chromatographs were acquired from ABI (Applied Biosystems). A direct Sanger dideoxy-sequencing method was performed for the amplified fragments. BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA) was used to edit, align, and evaluate the sequencing findings of the PCR products about the corresponding sequences provided in the reference database. To verify SNPs originality, the identified SNPs were uploaded to the dbSNP (Single Nucleotide Polymorphism Database) server, which is available at https://www.ncbi.nlm.nih.gov/projects/SNP/.

Statistical analysis

After reviewing and analyzing each sequence, it was uploaded to the NCBI Bankit site. The server's instructions were followed to the letter. The submitted sequences were uploaded to the NCBI database as nucleic acid sequences to assign a unique GenBank entry number to the sequences being studied.

RESULTS

Results of DNA amplification

Gel electrophoresis has been used to validate the results obtained from the DNA amplification PCR. The only bands seen are those with an anticipated molecular size of 726 base pairs, which is exclusive to the HLA-DQA1 primer. As seen in **Figure (2).**

Figure (2): The PCR products of the HLA-DQA1 primer

The bands with the anticipated molecular size of 293 base pairs have been detected, indicating their specificity for the HLA-DQB1 primer. As seen in **Figure (3).**

Figure (3): The PCR products of the HLA-DQB1

The bands with the anticipated molecular size of 895 base pairs have been detected, indicating their specificity for the TGM 2 primer. As seen in **Figure (4)**.

Figure (4): The PCR products of the TGM 2 primer

The DNA sequencing of HLA-DQA1, HLA-DQB, and TGM2 genes revealed ten SNPs in amplified HLA-DQA1 and HLA-DQB1 genes, with only one variant detected in amplified TGM2 sequences. The HLA-DQA1-based SNPs included rs3188502, rs17843560, rs9272721, rs1185554592, rs1253449846, rs35874654, rs9272724, rs35344500, rs34965214, and rs1554151707. TGM2-based SNPs included rs2067027, rs1019175686, rs2076381, and rs2076382.

The HLA-DQA1, HLA-DQB1, and TGM2 genes showed 99% to 100% sequence similarity. The NCBI BLAST engine partially covered this gene. DNA sequences were compared with Gen-Bank accounts and precise loci and specific information were determined. Ten SNPs were identified in the 724 base pair amplicons with the reference sequences of the HLA-DQA1 gene. Similarly, the study analyzed the HLA-DQB1 gene and TGM2 gene, identifying variants with varying degrees of heterozygous and heterozygous status. The HLA-DQB1 gene had a 149G>C variant in heterozygous status, while the TGM2 gene had four variants in homozygous and heterozygous states. Some SNPs were found in major homozygous and heterozygous states, while others were only found in rare homozygous status. The results highlight the importance of understanding gene variants for effective gene therapy.

The study analyzed sequenced sequence variants (SNPs) and their locations in relation to the HLA-DQA1 gene using the dbSNP server. The SNPs were found to be positioned in the intronic sequences of the TGM2 gene. The sequences were submitted to NCBI, and unique GenBank accession numbers were obtained for each sample. The study confirmed the novelty of the observed SNPs and identified several previously deposited SNPs in the genome.

DISCUSSION

The CD is an autoimmune condition triggered by gluten consumption in wheat, barley, and rye [10]. This leads to an immune reaction damaging the intestinal lining, preventing nutrient absorption and causing symptoms like diarrhea, bloating, fatigue, joint pain, and skin rashes, as well as non-digestive symptoms [11].

The current study reveals significant genetic factors contributing to celiac disease, specifically focusing on SNPs in the HLA-DQA1, HLA-DQB1, and TGM2 genes. Ten SNPs

were found on the HLA-DQA1 gene, indicating a strong genetic polymorphism. The HLA-DQB1 gene's one SNP (rs41561518) strengthens the relationship between HLA genetic diversification and celiac disease. These SNPs may also reveal disease-specific disparities or predisposition. Ivanova et al. 2023 searched and found new promising SNPs within the HLA-DQA1 and HLA-DQB1 regions not described earlier in the context of celiac disease. This suggests that the identified SNPs are relevant; the genetic landscape of celiac disease is continually evolving with discoveries [12]. Study by Martina et al. in 2018, HLA-DQA1 and HLA-DQB1 SNPs, Significance in CD, variants in these genes are known to be highly associated with celiac disease risk [13]. The SNPs like rs3188502, rs9272721, and rs41561518 increase the risk of developing CD in the population. The TGM2, an enzyme in tissue Transglutaminase, plays a role in gluten modification and is a primary autoantigen in CD. Polymorphisms affect TGM2 gene functions and disease risk [14]. Our results of sequencing TGM2 SNPs: The Four SNPs were detected in the TGM2 gene (rs2067027, rs1019175686, rs2076381, and rs2076382).

A recent study in 2020 explored genetic variations in TGM2 and their correlation with disease severity in celiac patients [15]. The study found consistent associations between TGM2 gene variations and disease markers, confirming the role of TGM2 in celiac disease. Amundsen's 2023 study further explored genetic variants in TGM2 and their impact on disease susceptibility. While most research supports TGM2's role, some variants may have different effects depending on the population studied [16].

The HLA-DQB1 gene suggests a potential polymorphism at the 149 locus in the 294 bp PCR region as associated with celiac disease susceptibility. Four SNP positions of the TGM2 gene, located on the 82nd, 444th, 475th, and 547th loci of the 895 bp PCR amplicon, suggest that genetic variability in CD8 may also affect the enzymatic activity of transglutaminase 2. Changes in TGM2 could either worsen or lessen the disease's course [17].

Bowcock's cross sectional study in 2019 showed that certain genotype variations in TGM2 influence the severity of the disease and serological reactions in patients [18]. This is consistent with our previous results showing variations in the TGM2 gene, which may potentially affect clinical result. The allocation of SNP in HLA-DQA1, both central homozygous and heterozygous rabbits squarely exonerates the genetic baseline as pertains to celiac disease that has links with influence in the (HLA-DQA1) gene [19]. Homozygous conditions might point to increased susceptibility to the disease than in heterozygous configurations, although this depends on other genetic or environmental factors [20].

The HLA-DQB1 heterozygous genotype raises questions about celiac disease's functional effects and potential protection. TGM2 Variants have varying genotype zygosity

status, with homozygous for some SNPs indicating higher risk, suggesting a more comprehensive genetic mechanism underlying the disease's development.

In a study conducted in 2022, Ludvigsson et al. concluded that some HLA-DQ haplotypes were always associated with disease risk, particularly in persons of a certain genetic background familiar with Celiac disease [21]. Furthermore, Katri et al. (2019) brought up the study about the variability of TGM2, where several homozygous variants are presumably linked to a severe disease state [22]. A meta-analysis by Lerner et al. found that while some HLA-DQ alleles have a significant association with CD, non-HLA SNPs like TGM2 may not have the same significant value as previously suggested due to variations in expressivity and other gene or environmental effects on disease phenotypic expression [23].

The field currently employs research to understand environmental change factors (like exposure to gluten) and genetics factors [24]. Studies such as those aiming to understand these interactions better and could provide further context for the findings on SNPs in HLA-DQA1, HLA-DQB1, and TGM2 [25].

The polymorphic markers of celiac disease are highly complex; however, some SNP is particularly present in genes HLA-DQA1, HLA-DQB1, and TGM2 [26]. The definitional literature in the last five years essentially supports the relevance of these SNPs, although due to differences in methodology and population structure, the genetic aspect of celiac disease is more complicated [27].

CONCLUSION

This research reveals many SNPs that may influence the immunological response and genetic susceptibility to CD. By correlating these SNPs with recognized databases, such as dbSNP, the research validates their frequency and consistency across groups, highlighting their potential significance in Crohn's disease risk. These results highlight the significance of genetic screening in evaluating susceptibility to CD and provide essential insights for both clinical and demographic investigations.

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7. Authors' contribution: Duaa Faiz Othafa, Dr. Dawood Salman Mahdi and Dr. Ihsan Edan Alsaimary contributed to every aspect of the research.

8. Conflict of Interest:

The authors declare no conflicts of interest.

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Each sample was extracted individually and then subjected to gel electrophoresis for result verification. As shown in **Figures (1).**

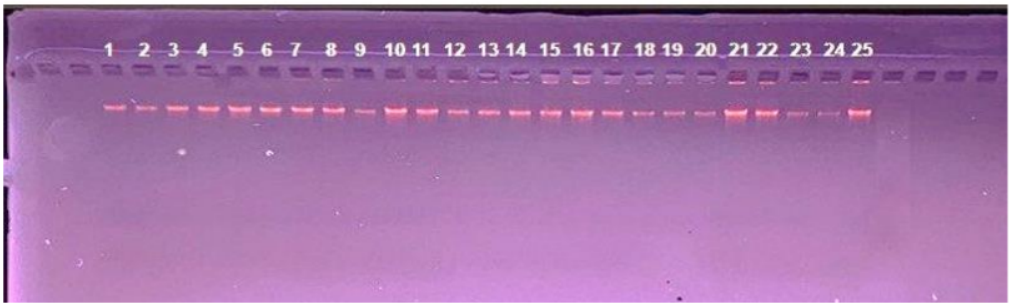

Figure (1): Agarose gel electrophoresis appearance demonstrating DNA extraction after 40 minutes at 110V over a 1% agarose solution stained with Ethidium Bromide.

Results of DNA amplification

Gel electrophoresis has been used to validate the results obtained from the DNA amplification PCR. The only bands seen are those with an anticipated molecular size of 726

base pairs, which is exclusive to the HLA-DQA1 primer. As seen in **Figure (2).**

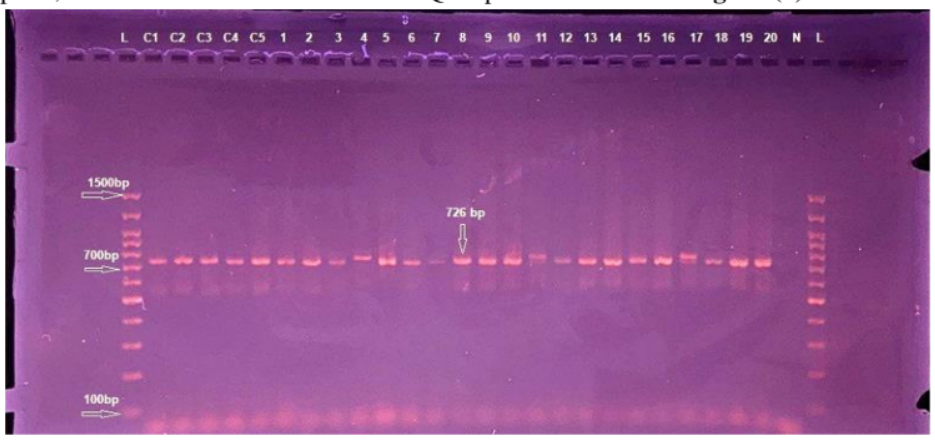

Figure (2): The PCR products of the HLA-DQA1 primer

The bands with the anticipated molecular size of 293 base pairs have been detected, indicating their specificity for the HLA-DQB1 primer. As seen in **Figure (3).**

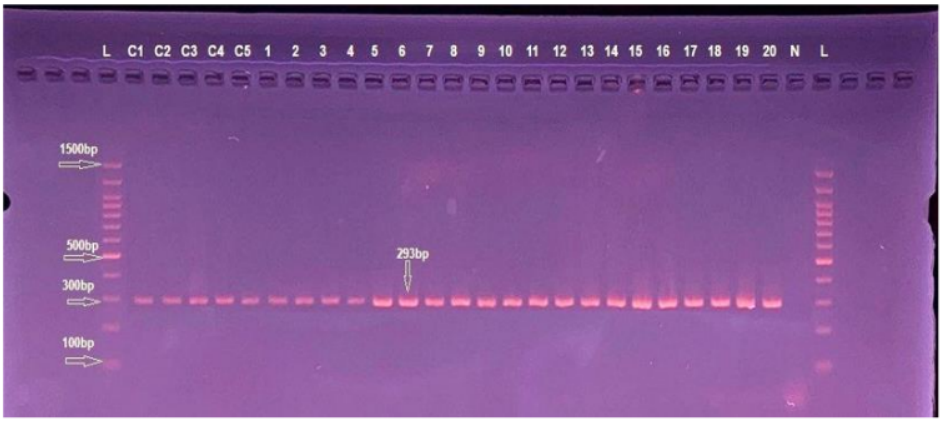

Figure (3): The PCR products of the HLA-DQB1

The bands with the anticipated molecular size of 895 base pairs have been detected, indicating their specificity for the TGM 2 primer. As seen in **Figure (4)**.

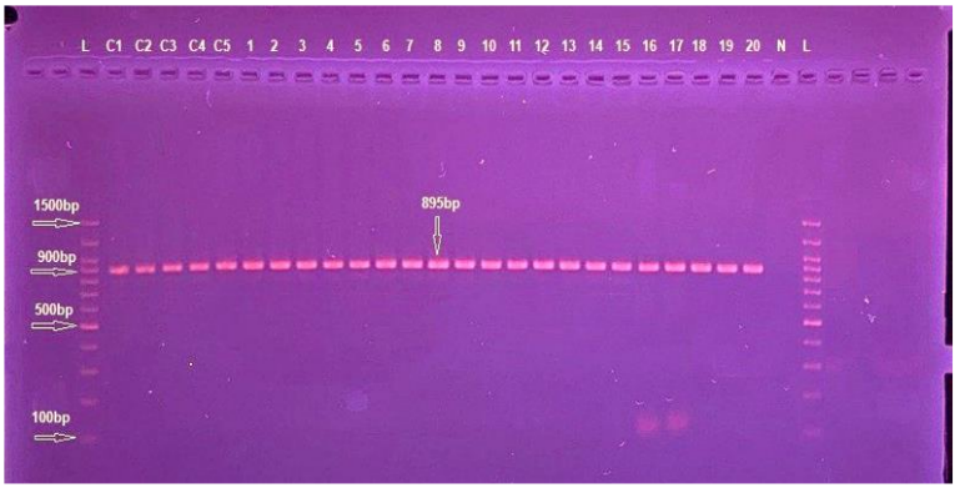

Figure (4): The PCR products of the TGM 2 primer

Table (1): Specific primers used in the investigation.

Gene	PRIMER SEQUENCES 5……..'3	PCR Product	TM	Reference
HLA-DQA1	F ATCTTCACTCATCAGCTGACCA R GCTGACCCAGTGTCACGGGAG	726bp	57°C	http://dx.doi.org/10.4236/ojgen.2014.42013
HLA-DQB1	F TCCCCGCAGAGGATTTCGTG R GGCGACGACGCTCACCTC	293bp	61°C	http://dx.doi.org/10.4236/ojgen.2014.42013
TGM 2	**F GCAGTGTGGATGGGGAAACT** **R CTGGCTGTGTCAGGCTGTAT**	895bp	55°C	This study NC_000020.11 Homo sapiens chromosome 20, GRCh38.p14 Primary Assembly Range38127385 – 38168475

Table (2): PCR Amplification Program for PCR

Step	Temperature(˚C)	Time	No. of cycles cycle
Initial denaturation	94	3 min	1
Denaturation	94	30 sec	35
Annealing	Variables according to (primer's TM)	30 sec	
Extension	72	1 min	
Final extension	72	5 min	1
Hold temperature	-4	∞	-