

## Sorting Intolerant from Tolerant and PolyPhen-2 Algorithms: A Variation in Exon 14 of ATP7B Gene among 4 West Iraqi Families with Wilson's Disease

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### ABSTRACT

**Background:** Wilson's disease (WD) is a genetic disorder (autosomal recessive) that affects copper metabolism. A favorable prognosis for WD can be achieved with early diagnosis and treatment. It is also strongly advised to conduct a family screening.

**Objectives:** To find the relationship between genotype and phenotype to facilitate the diagnosis of the disease as well as using modern methods in diagnosing WD.

**Materials and methods:** This study included nine WD patients and fifteen healthy participants. Members of patients with WD and healthy members provided whole blood. Blood DNA was extracted, and Exon 14 was amplified using specific primers using polymerase chain reaction (PCR). The results of the PCR products were aligned to the published human genome database using the BLAST tool.

**Results:** Three different variations have been recorded as a result of the experiment. All of the changes point to a deficiency in the ATP7B protein, which has been identified as a cause of WD.

**Conclusion:** The ATP7B gene mutation spectrum in Iraqi patients has been enhanced as a result of our research, and this information could be used to develop gene therapy and clinical/prenatal diagnosis to prevent WD in Iraq.

**Keywords:** Sift; Polyphen-2; Variation; Exon; Wilson's disease.

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### INTRODUCTION

Wilson's disease (WD) is an autosomal recessive which causes the accumulation of copper in various body organs such as the liver and brain. It is caused by around 800 allelic variations of the gene ATP7B. WD carries an incidence of one in every 30,000 people in Europe. The clinical signs of WD vary greatly, but most commonly include liver and neurological problems. The majority of cases begin between the ages of five and 35 years, however, WD can affect any age [1]. Although the condition is monogenic (caused by a single gene), there are significant disparities in clinical manifestation and age at the onset of first symptoms among patients with the same genotype, even within the same family [2].

Although the disease is caused by distinct allelic variations of the ATP7B gene, research is ongoing to understand the

disease's diversity. Several studies have been conducted to investigate the implications of ATP7B gene allelic variations on the function of the ATP7B protein. There have been multiple research on the genotype-phenotype relationship within the ATP7B gene, some with favorable findings [3], and some with negative findings [4, 5]. If detected early, WD is a condition that can be treated with medications, reducing morbidity and mortality. It is still poorly understood, even though it has been identified and classified for more than a century. Many people go untreated, and those who are treated are routinely disregarded for their symptoms and dysfunctions [6].

There are more than nine hundred known variants of the ATP7B gene, which produce WD [7]. Pediatric patients are often identified after three years of age, with liver involvement being the most common manifestation [8]. Each patient over the age of three who arrives with unexplained liver disease of any severity should be evaluated, and WD is not ruled out.

Recent whole genome sequencing studies suggest that molecular diagnosis could affect up to 1 in 7026 people [9]. Despite the discovery of the disease's genetics, scientists are still unable to predict the phenotypic variation of WD. Nu-

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merous studies have shown that there is no relationship between genetics and phenotype. The aim of all the studies that have taken place in the past has been to show a relationship between genotype and phenotype to facilitate the diagnosis of the disease, but the researchers were unable to find such a relationship [10]. Consequently, the symptoms of WD may be caused by the interaction of genetic and metabolic factors [11, 12]. If a person is suspected of having WD, the proportion of copper in the body can be measured to indicate the presence or absence of the disease [13–15]. There is lacking Iraqi studies to accurately diagnose WD using bioinformatics algorithms. Hence, we conducted this study aiming to use bioinformatics algorithms in the diagnosis of genetic diseases, where the most common algorithms in this field were used to improve the accuracy of the diagnosis of the WD.

## MATERIALS AND METHODS

This cross-sectional study was conducted during the period from 2018 to 2019. Patients diagnosed with WD (n = 9) were recruited from private medical laboratories in West Anbar province, Iraq. Informed consent was taken from the patient's parents. The current study was approved by the Ethical Approval Committee of the University of Anbar (reference number 80 on 4-6-2023).

### Study design

Of approximately 24 individuals, nine patients with WD and 15 healthy individuals from their relatives were present. Five milliliters of whole blood were drawn from family members' peripheries for DNA isolation. Exon 14 of the ATP7B gene was tested for mutations.

### Molecular analysis

#### COLLECTION OF BLOOD SAMPLES

Approximately 5.0 ml of peripheral blood samples were collected from the WD patients and their family members. The target population was all families that had at least one WD patient. The blood samples were placed in the EDTA tubes and then in the refrigerator at -20°C until the sample was used for examination.

#### DNA Extracted

Genomic DNA was extracted from blood samples from patients and members of their families using a ready kit (G-Spin DNA Extraction Kit, Intron Biotechnology, Cat. No. 17045). The procedure was done according to the manufacturer's instructions.

#### POLYMERASE CHAIN REACTION

The exon 14 regions of the ATP7B gene were amplified using the primers described in Table 1. Polymerase chain reaction (PCR) amplification was carried out in a 25-ml volume containing 5 ml of Taq PCR premix, 10 picomoles per l of forward primer, 10 picomoles per l of reverse primer, 1.5 ml of DNA, 16.5 l of distilled water [13].

#### Sequencing and sequence alignment

Homology searches were performed using the Basic Local Alignment Search Tool (BLAST) program, which is available at the National Center

for Biotechnology Information (NCBI) online at [https://nicem.snu.ac.kr/main/?en\\_skin=index.html](https://nicem.snu.ac.kr/main/?en_skin=index.html).

## BIOINFORMATICS TOOLS

In this study, we used two commonly used pathogenic prediction tools freely available on the Web: SIFT (Sorting Intolerant from Tolerant), SIFT can be used to detect non-synonymous SNPs and lab-induced missense mutations based on sequence homology and amino acid physical characteristics, and PolyPhen-2 (Polymorphism Phenotyping v2) is a method that predicts the effect of an amino acid substitution on the structure and function of a human protein based on simple physical and comparative considerations.

## RESULTS

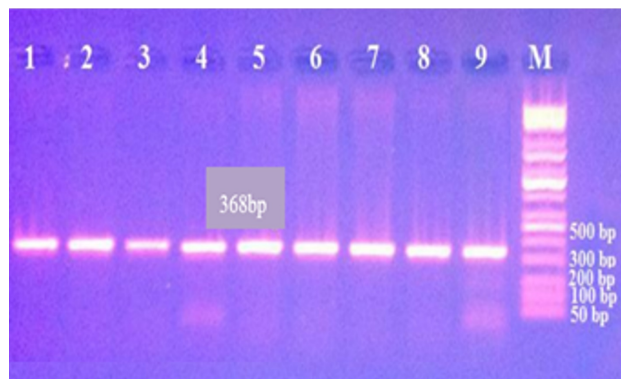
As shown in Table 2, the sex ratio (male: female) was 2:1, seven patients were from urban regions, and paternal consanguinity was relatively common in all of these patients.

In exon 14 of the ATP7B gene, 18 alleles from 9 WD patients and (positive control) 30 alleles from 15 healthy people were analyzed for mutations. Figure 1 shows gel electrophoresis of the amplified gene.

Variations were detected in 7 WD patients out of 9 patients, while healthy people have not detected any variations. Variations have been recorded, which are point mutations in the coding region (CDS) in the forward allele (T176G), in the reverse allele (CDS) (A176C), and the non-coding region (Intron) (G204A). The majority of mutations in exon 14 of the ATP7B gene were missense, and the type of substitution was transversion. This confirms that WD is caused by a change in amino acids (Table 3).

As shown in Figure 2, the results obtained from the use of the algorithm PolyPhen-2 as Number 0 have shown that the mutation is benign (does not affect the normal function of proteins). The red color denotes that Number 1 will change, where the structure and function of proteins will have an impact on amino acids.,

As shown in Table 4, the results obtained from the use of two types of algorithms, SFIT and PolyPhen-2, showed that while the results obtained from the use of the SFIT algorithm are similar to the results of the PolyPhen-2 algorithm, all results are not affected by the protein function, which means that other factors have an impact on the protein function that



**Figure 1.** Gel electrophoresis for Exon 14 products visualized under ultraviolet light after staining with ethidium bromide. M: 1000 bp marker. The product size is 368bp.

**Table 1.** The used Primers.

Exon	Forward	Reverse
14	GGTGTCTTGTTCCTGTCTGAG	TAGGAGAGAAGGACATGGTGAG

**Table 2.** Demographic characteristics of the patients.

No.	Gender	Age	Resident	Consanguineous
Patient 1	Male	14	Rural	Present
Patient 2	Male	13	urban	Present
Patient 3	Female	8	urban	Present
Patient 4	Male	5	urban	Present
Patient 5	Male	4	urban	Present
Patient 6	Male	6	urban	Present
Patient 7	Male	9	urban	Present
Patient 8	Female	10	Rural	Present
Patient 9	Female	9	urban	Present

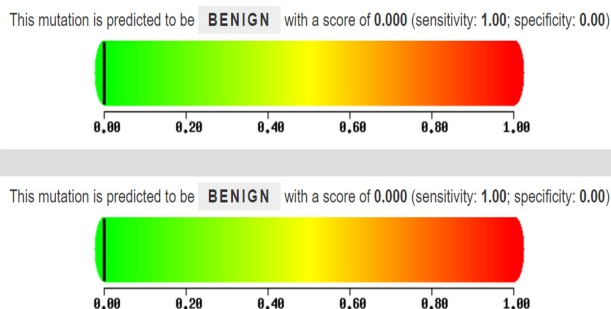
**Table 3.** Characteristics of mutations in intron and exon 14 of the ATP7B gene in patients with Wilson’s disease.

Number of patients	Type of substitution	Amino acid change	Nucleotide change	Type mutation
P1-7-E14-F	Transversion	Phe59Cys	T176G	Missense
P1-7-E14-R	Transition	-----	G204A	Intron
P2-7-E14-F	Transversion	Phe59Cys	A176C	Missense
P2-7-E14-R	Transition	-----	T176G	Missense
P2-7-E14-R	Transversion	Phe59Cys	G204A	Intron
P3-7-E14-F	Transversion	Phe59Cys	A176C	Missense
P3-7-E14-R	Transversion	Phe59Cys	T176G	Missense
P4-7-E14-F	Transversion	Phe59Cys	A176C	Missense
P4-7-E14-R	Transversion	Phe59Cys	T176G	Missense
P5-7-E14-F	Transversion	Phe59Cys	A176C	Missense
P5-7-E14-R	Transversion	Phe59Cys	T176G	Missense
P6-7-E14-F	Transversion	Phe59Cys	A176C	Missense
P6-7-E14-R	Transition	-----	T176G	Missense
P6-7-E14-R	Transversion	Phe59Cys	G204A	Intron
P7-7-E14-F	Transversion	Phe59Cys	A176C	Missense
P7-7-E14-F	Transversion	Phe59Cys	T176G	Missense
P7-7-E14-R	Transition	-----	G204A	Intron
P7-7-E14-R	Transversion	Phe59Cys	A176C	Missense

may be non-genetic. Two bioinformatics algorithms, SFIT and PolyPhen-2, were used to predict whether there would be a future effect of the variations detected in the gene code segment sequences.

**DISCUSSION**

Because the disease is the result of inherited mutations, genetic, endogenous, and environmental factors can all have an impact on a person’s ability to withstand oxidative injury. Typically, the WD is discovered between the ages of 5 and 40 years. However, ATP7B gene changes in people beyond the age of 70 have been identified by molecular research [16]. In addition, persons in their fifth and sixth decades [17] were found to have clinical indicators of the disease in addition to molecular investigations being used to make the diagnosis. Consuming copper or other minerals like zinc, which may prevent enterocytes from absorbing copper, are examples of such impacts [18], that could affect the person’s capacity to fend off oxidative damage. There wouldn’t be a difference between male and female WD in a perfect society. However, several organizations reported differing proportions. In South India, men made up more than two-thirds of the cases. This disparity was related to the higher prevalence of medical attention among Indian men [18]. On the other hand, it is widely recognized that men and women experience the disease’s clinical



**Figure 2.** PolyPhen-2 (Polymorphism Phenotyping V2) results.

**Table 4.** Predicting the effect of mutations intron and exon 14 of ATP7B gene in Wilson's disease patients using programs SIFT and PolyPhen-2.

Number of samples	Amino acid change	Sift score	Prediction effect	Polyphen-2 score	Prediction effect
P1-7-E14-F	Phe59Cys	0.22	TOLERANT	0.000	BENIGN
P1-7-E14-R	Intron	----	-----	----	-----
P2-7-E14-F	Phe59Cys	0.22	TOLERANT	0.000	BENIGN
P2-7-E14-R	Intron	----	-----	----	-----
P3-7-E14-F	Phe59Cys	0.22	TOLERANT	0.000	BENIGN
P3-7-E14-R	Phe59Cys	0.22	TOLERANT	0.000	BENIGN
P4-7-E14-F	Phe59Cys	0.22	TOLERANT	0.000	BENIGN
P4-7-E14-R	Phe59Cys	0.22	TOLERANT	0.000	BENIGN
P5-7-E14-F	Phe59Cys	0.22	TOLERANT	0.000	BENIGN
P5-7-E14-R	Phe59Cys	0.22	TOLERANT	0.000	BENIGN
P6-7-E14-F	Phe59Cys	0.22	TOLERANT	0.000	BENIGN
P6-7-E14-R	Intron	----	-----	----	-----
P7-7-E14-F	Phe59Cys	0.22	TOLERANT	0.000	BENIGN
P7-7-E14-R	Intron	----	-----	----	-----
	Phe59Cys	0.22	TOLERANT	0.000	BENIGN

manifestations differently [19]. Besides, the study found that women are more likely to develop the hepatic form of the disease than men and that women develop the neuropsychiatric form of the illness almost two years later than men [19]. The higher male-to-female ratio in the present study has two likely causes. First, with a large part of the country's patients coming from the south, most families decide to treat male patients with less consideration for ladies, as is the case in South India. The second reason is that some of the affected women are pregnant and might use zinc supplements at that time. Popular WD treatments include zinc. As a result, the disease's symptoms will lessen, and no further medical attention will be required.

It is believed that WD is more prevalent in rural regions than urban ones due to the high rate of consanguinity among rural populations [20]. The study's contrary conclusion may be relevant to the kinds of medical treatments. Urban areas in Iraq have easy access to specialized medical clinics, whereas rural areas never have such facilities, and patients, particularly those with this type of ailment, must travel great distances to receive a diagnosis and regular medication.

Single nucleotide polymorphisms (SNPs) are regarded as the primary cause of the majority of inherited traits, including a sizeable portion of hereditary illness susceptibility. SNPs are the most common type of genetic variation among individuals. Over 1000 proteins have been identified as being implicated in the link between a single nucleotide change and monogenic illness, which has been described in several cases. Together with genome sequence and other proteomics data, a large number of recently discovered human SNPs (about 4 million distinct SNPs; <http://www.ncbi.nlm.nih.gov/SNP/index.html>) enable far more comprehensive knowledge of the relationship between genotype and phenotype at this level. The precise mechanisms by which an SNP can cause a phenotypic change, however, are still poorly known.

Consanguineous marriage has drawn a lot of attention because it is a significant contributor to the prevalence of some hereditary diseases. An estimated 20% of people on Earth reside in societies that encourage consanguineous unions. As

a result, this type of marriage is present in about 8.5% of all marriages worldwide. Consanguineous marriage is incredibly common in several regions, notably in nations where Islam is the dominant religion, like many in Asia and Africa. However, this kind of union is not typical in Western nations [21, 22]. All of the WD patients in this study had consanguineous parents. Numerous investigations carried out in numerous communities have amply demonstrated the effect of consanguinity on the prevalence of genetic disorders. Because they inherit the same genomic segments from both their parents, people born through consanguineous marriage have a large number of homozygous genomic segments. In comparison to the typical predicted homozygosity in a random marriage, long-term parental inbreeding raises homozygosity by 5% [23]. Consanguinity does not affect the allele frequencies from a statistical perspective, but it does increase the likelihood of marriage between two people who are heterozygous for the same dangerous recessive allele. As a result, compared to non-consanguineous marriage, the likelihood that the offspring of the first-cousin marriage will have the malformations is anticipated to be very high. This is particularly clear for the genes that cause uncommon autosomal recessive disorders [24].

The Iraqi WD patients' exon 14 was analyzed for changes. In a different investigation, patients with WD were found to have the following three exon 14-point mutations: (T176G), (A176C), and (G204A). These mutations, which are most prevalent in Iraqi WD patients, could be the cause of its severe symptoms and early onset. They can also be utilized in conjunction with other common mutations to make a molecular diagnosis of WD [25]. This finding emphasizes how important to look into the whole ATP7B gene's mutation patterns in our community to predict risk. The creation of a mutational database for the WD population in Iraq will benefit from broader research [26].

Based on sequence homology and the physical properties of amino acids, the SIFT approach assesses whether a substitution of an amino acid impacts the function of a protein. The PolyPhen-2 algorithm predicts how a change in an amino acid

will affect the structure and function of a human protein. It does this by using sequence homology and mapping the replacement site to known three-dimensional protein structures [25–27]. The prediction of all variants by popular bioinformatics algorithms (SIFT, Polyphen2) suggests that there may not be a phenotypic change in people regardless of the prevalence of these polymorphisms, as the two amino acids they replaced had the same structural, physical, and chemical properties. As a result, further gene changes in other locations may have contributed to the illness, or there may have been small variations that contributed to the disease's cumulative occurrence [27]. These results advance our knowledge of ATP7B mutations in WD patients and may help in the design of efficient therapeutic approaches for these people. SNPs in Alzheimer's disease, however, may exist, according to research [28], and may disrupt the ATPase protein's capacity to bind metal correctly, causing abnormalities in copper homeostasis. Likely, the decreasing effect of this minor and frequent allele of this SNP is caused by linkage disequilibrium with one or more polymorphisms in the same gene because this effect was not observed in WD [29].

A small sample size (due to the difficulty in the diagnosis of WD as well as the fact that it is so rare) is considered a limitation of the current study. Therefore, a future study with a large sample size is warranted.

## CONCLUSION

Protein dynamics, both local and global, are essential for protein function. Disease-causing mutations tend to destabilize proteins, weaken protein binding, and increase flexibility. Our research increased the library of ATP7B mutations involved in the development of WD. Bioinformatics can be used to screen mutations in the ATP7B gene and assess the SNP's influence on ATP7B.

## ETHICAL DECLARATIONS

### Acknowledgements

All of the study participants and their guardians deserve our gratitude. Besides, I appreciate Dr. Yassin. H. Majeed (Consultant of Internal Medicine and Liver Diseases and Professor in the Department of Internal Medicine/Gastroenterology, College of Medicine, University of Anbar, Ramadi City, Anbar, Iraq) for his kind help and kind effort.

### Ethics Approval and Consent to Participate

The Ethical Approval Committee of the University of Anbar (reference number 80 on 4-6-2023) gave its approval to the current study. The sample members and their parents were interviewed directly by the researcher, and informed consent was obtained from each participant and their parents to participate in the research, and the study was conducted as a result.

### Consent for Publication

Not applicable (no individual personal data included).

### Availability of Data and Material

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

### Competing Interests

The author declares that there is no conflict of interest.

### Funding

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### Authors' Contributions

Yaseen OQ designed the study and wrote the manuscript. Yaseen OQ read and approved the manuscript.

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