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Detection of Mutations in Exon 10 and 13 of *ATP7B* Gene among Iraqi patients with Wilson Disease

A Thesis

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Dedication…

To All Patients Suffering from
Wilson's disease

Ahdaf - 2016

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Summary

Wilson disease (WD) is a rare inherited autosomal recessive disorder of copper metabolism due to mutations in copper transport gene *ATP7B* resulting in hepatic and neuropsychiatric manifestation. The aim of this study is to investigate the extent to which mutations in gene *ATP7B* involved in development of WD and assessment of parameters related to copper metabolism in patients with Wilson disease. Accordingly, blood samples were obtained from 35 patients with WD (20 male and 15 female) with a mean age of 9.12 years and age range of 5 – 40 years old Other 10 apparently, healthy individuals were also included. The serum level of ALT, AST, ALP, and Bilirubin were investigated which were (132.14±23.12 U/L, 96.93±11.21 U/L, 184.29±48.45 U/L and 3.6±1.13 mg/dl) respectively that differed significantly from their counter control groups. Serum level of copper among WD patients of ranged from (78 – 214 mg/dl) with average of (127.18±64.432 µg/dl), this average does not differ significantly from that of control groups.

A total of 70 alleles belong to 35 WD patients and other 20 alleles belong to healthy individuals have been examined for mutations in the exon 10 and exon 13 of *ATP7B* gene. Seven different mutations have been recorded. These includes three nucleotide polymorphisms (SNPs) : Lys832Arg, Pro840Leu and Thr991 Thr with 22.86% , 25.71% and 4.29% frequencies respectively; two point mutations : Ala1003 Val and Lys1010Arg which had 8.57% and 11.42% frequencies respectively and two frameshift mutations: c.2977-2978insA and c.2457delA with frequency of 24.29% and 27.14% frequency among WD patients.

These data strongly indicate that both c.2519C>T polymorphism and the frameshift mutation c.2977-2978insA could be exploited in the development of molecular diagnosis of WD. However, further studies are required to find out the most prevalent mutations in other exons.

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List of Abbreviations

Symbol	*Definition*
ALF	**Acute Liver Failure**
ALT	**Alanine Transaminase**
ALP	**Alkaline Phosphatase**
ALS	**Amyotrophic Lateral Sclerosis**
Apo E	**Apolipoprotein E**
AST	**Aspartate Aminotransferase**
ATOX1	**Antioxidant 1 copper chaperone**
ATP7A	**ATPase copper Transporting Alpha**
ATP7B	**ATPase copper Transporting Beta**
ATX1	**AnTioXidant**
Cβ	**C- Beta**
CCS1	**Copper Chaperone for Superoxide dismutase 1**
CNS	**Central Nervous System**
COMMD1	**The Copper Metabolism MURR1 Domain protein 1**
CT	**Computerized Tomography**
CTR-1	**Copper Transporter Protein**
Cu^{+2}	**Copper**
DNA	**Deoxyribonucleic Acid**
ELISA	**Enzyme-Linked Immuno-sorbent Assay**
GG	**double-glycine**
Hb	**Hemoglobin**
IL1B	**Interleukin -1β**
IL-1RN	**Nterleukin -1 Receptor antagonist gene**
K-F	**Kayser–Fleischer rings**
MRI	**Magnetic Resonance Imaging**
mSOD1	**mutant SOD1**
PCR	**Polymerase Chain Reaction**
PIB	**P-type ATPase of class IB**
PINA	**Pineal night-specific ATPase**
RBCs	**Red Blood Cells**
SNP	**Single Nucleotide Polymorphism**

Symbol	*Definition*
SOD1	**superoxide Dismutase 1**
TGN	**trans-Golgi network**
TSB	**Total Serum Bilirubin**
UTR	**Untranslated Region**
WD	**Wilson Disease**
XIAP	**X-linked inhibitor of apoptosis**

Chapter One

Introduction and Literature Review

1.1. Introduction

Wilson disease (WD) , also called hepatolenticular degeneration , is a rare inherited autosomal recessive disorder of copper metabolism due to mutations in copper transporter gene *ATP7B*,resulting in hepatic and neuropsychiatric manifestations (Mathur *et al.*,2015; Chen *et al.*, 2015) . Although it has been recognized and described for nearly a century, it still far from completely understood. If diagnosed early, WD patients can be managed by medicines which results in reducing morbidity and mortality. However , many patients remain undiagnosed , while in the diagnosed patients' various symptoms and dysfunctions are often neglected (Mathur *et al.*, 2015; Chen *et al.*, 2015) .

Wilson disease is characterized by excessive accumulation of copper in different tissues and organs such as liver , central nervous system (CNS) , kidneys and other tissues , therefore , it is often fatal if not diagnosed and treated when symptomatic(Beinhardt *et al.*, 2014; Wu *et al.*, 2015). Patients with WD may present with a broad spectrum of clinical symptoms (Beinhardt *et al.*, 2014), mostly with liver and neuropsychiatric manifestation . Moreover , both the type of hepatic as well as neurologic symptoms can be highly variable , making a challenge in the diagnosis of WD and often delaying diagnosis (Kodama *et al.*, 2012; Beinhardt *et al.*, 2014).Frequent early symptoms include difficulty speaking, excessive salivation,ataxia, masklike facies, clumsiness with the hands and personality changes. Late manifestations include dystonia , spasticity, grand mal seizures , rigidity, flexion contractures and psychiatric features which occur in 10-20% of patients (Schilsky, 2014).

The excessive deposition of the copper in brain may cause neurological disorders such as Parkinson-like symptoms , including bradykinesia , tremor and dystonia , or neuropsychiatric symptoms , such as hypomnesia, dysgnosia , and personality abnormalities(Schilsky,2014).

Wilson disease is a relatively rare disease , although reliable data on the prevalence of the disease is scarce , it is estimated to be 1 case in 30,000 live births in some populations to 1/ 100000 in most populations with carrier frequency of 1 in 90 to 122 , however , the prevalence of WD has been re-evaluated in different clinical studies (Li *et al.*, 2011; Cocoş *et al.*, 2014) .Wilson's disease may present at any age , nonetheless , the majority of cases presents between ages 5 and 35 year (Liovet and Ducreux ,2012). The underlying molecular mechanisms for WD have been extensively studied. It is now believed that a defect in the copper-transporting P-type adenosine triphosphatase (ATPase) (Wu *et al.*, 2015)causes impairment in the processes of incorporation of copper into ceruloplasmin and excretion of excess copper into bile . This defect occur secondary to one of several mutations in the *ATP7B* gene (Schilsky, 2009). The genetic linkage studies narrowed the assignment of the WD locus to 13q14-q21 (Javed *et al.*, 2008; Dong &Wu, 2012; Kodama *et al.*, 2012; Mathur *et al.*, 2015).Furthermore; molecular genetic analysis reveals at least 300 distinct mutations. While most reported mutations occur in single families, only few mutations are more common. However , there is a wide range of mutations and the frequency of each of them varies considerably from country to country (Ferenci , 2006).

Genotype–phenotype association studies continue to provide ever larger sets of single-nucleotide polymorphisms (SNPs) linked to diseases or associated with responses to vaccines, medications and environmental factors. Such as-sociations provide an important step in studies of the genetic underpinnings of human diseases. When a SNP is localized within a gene or in the close neighborhood of a gene, then it is generally assumed that it affects the phenotype through changes at the expression level, the function or other properties of this particular gene. However, the molecular mechanisms that lead to the change are

usually not obvious. In the case of non-synonymous SNPs, where the underlying mutation occurs in the gene coding region and changes an amino acid, it is usually expected that this amino acid change affects protein function, expression, conformation or stability(Salari *et al.*, 2013). To determine genotype in siblings who do not harbor the prevalent mutations, the optimal choice is to use intragenic single-nucleotide polymorphisms (SNPs) markers with high heterozygosity values in the study population. New opportunities are opening up with the discovery of large numbers of SNPs in the human genome that could be used for tracking disease loci, for association studies, or simply as markers for monogenic disorders. Sets of SNP markers are highly heterozygous across most world populations and could be used in combination with analysis of prevalent mutations as a comprehensive strategy for determining presymptomatic and carrier sibs of WD patients. However, to date, limited information is available on the SNPs in *ATP7B* in any population(Gupta *et al*., 2005; Gupta *et al*., 2007)

Worldwide there is a dramatic improvement of analytic tools and the genetic testing became an integral part for the diagnosis of WD (Ferenci, 2006). The identification and distribution of particular mutations will help to design shortcuts for genetic diagnosis of WD. Therefore the current study designed to determine the most common mutations in the *ATP7B* gene in Iraqi patients with WD which can open a new era for the possibility for molecular diagnosis of Wilson disease.

1.1.1 Aims and Research Strategy

Determine the mutational profile of exon 10 and exon 13 in *ATP7B* gene among Iraqi patients with Wilson disease and investigate the more common mutations in this exon to be used in molecular diagnosis of the disease, In addition, estimate of serum level of copper and ceruloplasmin and their diagnostic value in WD patients.

1.2. Literature Review

1.2.1. Historical background

Wilson disease was first described by an American-born neurologist, Samuel Alexander Kinnier Wilson, although he was not the first to recognize the disease process (Pfeiffer,2007). Wilson provided the first detailed, and masterfully, coherent description of both the clinical and pathological details of the entity that now bears his name when he published a part of his thesis that submitted for the degree of Medicine Doctor at the University of Edinburgh in July 1911 for which a gold medal was awarded (Compston,2009). In the published article of Dr. Wilson in Brain in 1912 he described, for the first time so far as he can ascertain, a disorder that is often familial, affects young people, and is invariably fatal. Dr. Kinnier Wilson has encountered four cases (two of whom were siblings), making the diagnosis during life in three; but he acknowledges that the literature may contain six previous reports and he has located the case notes of two other affected individuals, previously not described from these same families. Moreover, Dr. Wilson d called it progressive lenticular degeneration (Pfeiffer, 2007; Compston ,2009).

Many other physicians and investigators, both before and after Wilson's paper, made contributions to various aspects of WD. Some authors attribute to Frerichs the first case report of WD, in 1861. Westphall, in 1883, reported main neurological manifestation in two cases similar to that seen in patients with multiple sclerosis. Westphall used the term "pseudosclerosis" for this new disease. In 1898 and 1899 Strümpell reported, three other cases of pseudosclerosis and the pathology of the third one revealed the presence of cirrhosis of the liver(Barbosa, *et al.*, 2009).

During 1902 to 1921, different manifestations and features had been reported including corneal pigment rings, which was first described by Kayser, excess copper in the liver was reported by Rumpel in a patient dying of WD , while Hall postulates autosomal recessive inheritance of the disease . After 13 years Gerlach and Rohrschneider documented an excess copper in the corneal pigment rings. On the next decades at the late 40s, Cumings documented excess deposition of copper in both brainand livers of patients with WD. For this reason Cumings suggested a possible beneficial effect for the treatment with Dimercaprol. In 1952, four scientist; Bearn, Kunkel, Scheinberg and Gitlin independently reported a deficiency of the serum copper protein, caeruloplasmin. Three years later the use of penicillamine was proposed by Walshe for treatment of WD. In 1961 zinc salts postulated to have a value in treatment. Near the end of 60s Walshe reported that triethylenetetramine 2HCl (trientine) was a valuable copper chelating agent as an alternative to penicillamine. In 1982 Starzl and colleagues first report of liver transplantation for Wilson disease, and in 1984 Walshe describes the ability of tetrathiomolybdate to mobilize copper from the liver with improvement in the histological picture. Finally, three separate groups report the identification of the gene for Wilson disease; a P-type ATPase ,*(ATPase7B)* located on chromosome 13q14 encoding for protein the controls the movement of copper through cell membranes(Walshe ,2006 ; Pfeiffer,2007; Compston, 2009; Roberts,2011; Chabik, Litwinand Członkowska ,2014).

1.2.2. Epidemiology

Wilson's disease is a relatively rare disease. Although reliable data on the prevalence of the disease is scarce, it is estimated to be 1/40000 to 1/30000. However, in most European countries this number is much

lower 12-18 per million (Olsson *et al.*, 2009), whereas in countries where consanguineous marriages are common this number is relatively high. In Costa Rica this number exceeds 60/one million(Jiménez *et al.*, 2009)and the prevalence is as high as one in 10 000 in China, Japan, and Sardinia (Shimizu *et al.*, 2010; Patil *et al.*,2013).

It has long been known that Wilson disease was transmitted by autosomal recessive inheritance, and so consanguinity is relatively common in affected families. The heterozygote carrier state has a prevalence of approximately 1 in 90 (Thijeel *et al.*, 2011; Chabik *et al.*, 2014) .

The disease has been rarely described before 3 years of age (Wilson *et al.*,2006). Presentation in 40–60% of patients is with primary features in the second decade of life. The remainder of patients comes to clinical attention during the third and fourth decades with a primarily neurologic (34%) or psychiatric (10%) presentation (Walshe 2006; Korman *et al.*,2008;Taly *et al.*, 2009). In Iraq about 76% of the patients had positive family history and Wilson disease hepatic insult is the most common in children below 8 years (Al-Karboli *et al.*,2007; Thijeel *et al.*, 2011) .

From other point of view different worldwide geographical distribution of *ATP7B* gene mutations had been reported. The most common mutation in patients from central, eastern, and northern Europe is the point mutation H1069Q (exon 14). About 50-80% of Wilson disease (WD) patients from these countries carry at least one allele with this mutation with an allele frequency ranging between 30 and 70%. Other common mutations in central and eastern Europe are located on exon 8 (2299insC, G710S), exon 15 (3400delC) and exon 13 (R969Q). The allele frequency of these mutations is lower than 10% (Caca *et al.* 2004; Ferenci ,2006; Firneisz *et al.*,2008). The c3400delC mutation is the most common in Brazil, and c.2333G>T (p.Arg778Leu) in Asian

countries(Liu *et al.*, 2004; Deguti *et al.*, 2004; Ye *et al.*,2007).In Mediterranean countries there is a wide range of mutations, the frequency of each varies considerably from country to country(Ferenci, 2006).

1.2.3. Etiology

1.2.3.1. Genetics

Wilson disease is a monogenic disorder. Genetic linkage of Wilson disease to the locus of the gene for the red blood cell esterase D indicated that the Wilson disease gene was on the long arm of chromosome 13. The mutation affecting the *ATP7B* gene is localized to chromosome 13q14.3-q21.1. The copper-binding ATPase protein, encoded by this gene, is a member of the P type-ATPase family (Alonso *et al.*,2007).

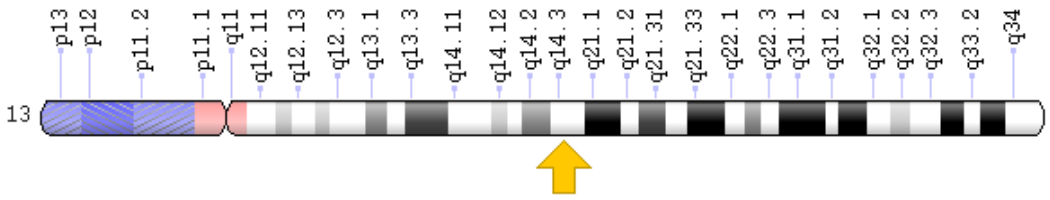

Figure 1-1: *ATP7B* gene location (Alonso *et al.*, 2007).

ATP7B (OMIM # 606882), located on 13q14.3, has a total genomic length of 80 kb and contains 21 exons encoding a copper-transporting P-type ATPase (Wilson ATPase) which consists of 1,465 amino acids. The gene is synthesized in the endoplasmic reticulum and then localized in the trans-Golgi network (TGN) of hepatocytes. Different levels of *ATP7B* expression are also detectable in the brain, kidney, lung, and placenta. There are over 500 mutations of the *ATP7B* gene, and most of them are extremely rare. Missense or nonsense mutations caused by single nucleotide variant are very common (60%), followed by insertions/deletions (26%) and splice-site mutations (9%). Compound heterozygote appears in the majority of WD patients. The mutations differ

greatly in different geographic regions: H1069Q and R778L (Wu *et al.*, 2001; Li *et al.*, 2011)are relatively common mutant alleles in European and Asian populations, respectively, and the proportion of other reported mutations is mostly lower than 10%. The hotspots for WD gene mutations in European population are located in exons 8-18, while mutations in exons 2-5 that are associated with some severe phenotypes are found in Indian population.

The *ATP7B* gene is expressed in the liver and brain (Chabik *et al.*,2014) . In 1999 a pineal night-specific ATPase (PINA), a splice variant of the *ATP7B* gene was identified in rats (Borjigin *et al.*, 1999). The defect of the *ATP7B* gene in the liver results in reduced excretion of copper into bile, and consequently in the accumulation of copper and organ damage in the liver, brain and other organs(Lalioti *et al.*, 2010; Li *et al.*, 2011). The copper transporting ATPase is needed for the biliary excretion of copper as well as the incorporation of copper into ceruloplasmin(Lalioti *et al.*, 2010). The database maintained by the University of Alberta lists over 300 distinct mutations identified from patients with Wilson disease(Alonso *et al.*,2007) .

Although missense mutations are most frequent, deletions, insertions, nonsense, and splice site mutations all occur. Most affected individuals are actually compound heterozygotes, having inherited different mutations from each parent. The large number of mutations has made commercial genetic testing for Wilson's disease impractical(Loudianos *et al.*, 2002; Cox *et al.*, 2005).Whether the profusion of mutations accounts for the prominent variability in clinical presentation and age of symptom onset in Wilson's disease patients is unclear. The H1069Q mutation, which is the most frequent mutation in USA and northern Europe, has been reported to be associated with later onset of symptoms and less severe disruption of copper metabolism(Gromadzka *et al.*, 2005),

although not all studies support this assertion(Vrabelova *et al.*, 2005). In contrast, nonsense and frameshift mutations may correlate with earlier onset of symptoms and more severe disturbance of copper metabolism(Gromadzka *et al.*, 2006). Individuals with the same mutation, even homozygotic twins(Senzolo *et al.*, 2007), may demonstrate wide variability in age of symptom onset and clinical presentation, which suggests that additional factors are also operative. For example, recent reports propose that methionine homozygosity at codon 129 of the prion-related protein gene may influence the onset of symptoms in WD (Grubenbecher *et al.*,2006; Merle *et al.*, 2006). It has been generally assumed that WD who possesses a mutation in only a single allele does not develop symptoms of the disease. However, the development of depression and parkinsonism, recently described in three elderly sisters who were found to be heterozygotes for a nucleotide deletion at the 50 UTR region of the *ATP7B* gene(Sechi *et al.*, 2007).

1.2.3.2. Inheritance

Wilson disease is a fully-penetrant, autosomal recessive, inherited disease, which means that the patient have to be homozygous or a compound heterozygote for harmful mutations in *ATP7B*gene, in order to develop the disease. However, not all mutations are complete gene knockouts, which accounts for the wide range of severity of the disease. Furthermore, it has been suggested that WD on occasion may become a dominant disease, this theory was adopted by Brewer in 2000, where he summarized the data in heterozygous carriers of the WD gene defect, and found that the gene really is codominant in many respects, with intermediate abnormalities of ceruloplasmin release into the blood and copper accumulation abnormalities in liver and urine. He suggested that under environmental circumstances of increased copper ingestion, WD

could be made into a dominant disorder, that is, copper toxicity could be seen in heterozygous carriers and the affected children are heterozygous carriers of the WD gene defect (Brewer ,2000; Senzolo *et al.*, 2007).

1.2.4. Pathophysiological mechanisms and role of copper

Copper is an essential element for cellular function, yet free copper is extremely toxic and can produce irreversible cellular damage. Copper is essential for a number of biological reactions, including important metalloenzymes, such as tyrosinase and cytochromeoxidase. It plays a role in many processes, including mitochondrial energy generation, melanin formation, scavenging of oxygen radicals and the cross-linkage of collagen and elastin. In addition, copper is a constituent of the dopamine-ß-hydroxylase, an important enzyme in the catecholamine biosynthetic pathway(Hassan and Masood, 2004).

Normal copper homeostasis is maintained by the balance between intestinal absorption and biliary excretion. Metallothionein, which is a cystein-rich zinc- and copper binding protein, plays a crucial role in the regulation of copper absorption (Kucinskas *et al.*, 2008; Li,X.-H.*et al.* ,2011). However, the regulation of copper excretion via bile is not completely understood. Since copper is an essential trace element, a certain amount is required in the diet, which normally contains about 1.00 mg/day copper. However, the daily intake is a little more than it is required, and the excess copper must be eliminated. Under normal conditions, the majority of copper is tightly bound to the ceruloplasmin protein, whereas a small amount of copper is loosely bound to plasma albumin. Ceruloplasmin contains 0.3% copper. The concentration of plasma free copper is 0.1 mg/l(Portala ,2001).In WD patients free copper concentration in plasma is approximately 0.30 mg/l(Hassan and Masood ,2004; Chen *et al.*, 2015).As a result of positive copper balance, copper

accumulates in most organs, primarily in the liver and the central nervous system. The toxicity of copper is related to the induction of free radical formation, which causes cell injury, inflammation and finally cell death. Copper is also harmful to mitochondria and inhibits numerous enzyme activities, e.g. glutathione reductase (Liovet and Ducreux ,2012; Medicus *et al.*,2010; Gu *et al.*, 2013).

Copper is absorbed in the proximal small intestine and carried across the enterocyte by ATP7B into the portal circulation where copper is bound loosely into the albumin. Copper is transported into the hepatocytes via copper transporter protein (CTR-1), situated on the sinusoidal aspect of hepatocytes(Patil ,2013).Within the cell, it binds to metallochaperones. CCS1 (copper chaperone for superoxide dismutase 1) deliver the copper to superoxide dismutase (SOD1) a principally cytoplasmic defense against oxidant stress. In case of copper excess, *ATP7B* moves toward the canalicular aspect, where it promotes biliary copper excretion. *ATP7B* dependent biliary copper excretion is the principal homeostatic mechanism for copper metabolism. Biliary excretion of copper also occurs by conjugation with glutathione; however it is a low affinity pathway in comparison to *ATP7B* dependent biliary copper excretion. Thus the liver utilizes some copper for its metabolic needs, the excess of copper is excreted in the bile in normal individuals and not in Wilson disease patients(Patil, 2013).

1.2.5. Clinical manifestations

There is a wide variability in clinical manifestations, patients with WD presented with different presentations(Machado *et al.*, 2006; Soltanzadeh *et al.*, 2007; Noureen and Rana, 2011).

Although the fundamental pathogenetic defect of WD lies within the hepatobiliary system, the consequences of the relentless copper

accumulation are played out on a multisystemic battle field. The clinical manifestations of Wilson disease are predominantly hepatic, neurologic, and psychiatric, with many patients having a combination of symptoms(Pfeiffer ,2007) .

The clinical phenotype includes myriad presentations varying from asymptomatic state to chronic liver disease, neuropsychiatric manifestations or acute liver failure. Individuals usually becomes symptomatic between the ages of 5 and 35 years, which reflects the potential of the liver to store excess copper, but both younger individuals and older individuals have been well describe (Ferenci, 2005).

Hemolysis is also a common finding in patients with WD, particularly among patients with acute liver failure.

1.2.5.1. Liver disease involvement

Liver disease is common in children and younger age groups with WD. Some patients may have non-immune mediated (Coomb's negative hemolytic anemia) with transient episodes of jaundice or low-grade hemolysis, even when liver disease is asymptomatic (Roberts and Schilsky, 2008).

Acute liver failure (ALF) constituting 30% of all WD cases and associated with higher mortality rate reaches up to 95%(Squires *et al.*, 2006; Devarbhavi *et al.*,2012; Patil *et al.*, 2013) .

Regarding the gender variation in the ALF presentation, previous studies revealed conflicting results; where in some series females in general are overrepresented in patients with WD.

Other studies supported the idea that WD disease seen more often among boys (Dhawan *et al.*, 2005; Ghaffar *et al.*, 2011; Devarbhavi *et al.*,2012).

Other presentations of liver involvement are summarized as Jaundice, anorexia, vomiting present in(15-44%)of patients, Ascites/edema(14-50%), Hepatomegaly/splenomegaly (15-49%) , Acute liver failure (17 - 30%), Asymptomatic (5-23 %), Variceal hemorrhage(3-10 %),Hemorrhagic diathesis(3-8 %), Hemolysis(5-20 %) .

1.2.5.2. Neurologic manifestations

Neurological manifestations are the present features in (40–50%) of patients with WD. The reported neurologic manifestations of WD are broad, and can be challenging because patients present in a myriad of ways. Neurologic symptoms may be very subtle or may be rapidly progressive, leading to severe disability over the course of months (Liovet and Ducreux, 2012) . In patients with known cirrhosis, neurologic manifestations may be mistaken for hepatic encephalopathy.

The majority of patients with neurologic signs fall into one of several categories: dysarthric, dystonic, tremulous, pseudosclerotic (tremor with or without dysarthria), or parkinsonian(Lorincz ,2010). Initially, only one symptom may be present (often unilaterally), but as the disease progresses, complex combinations of neurologic signs and symptoms may develop.

Some of the more common neurologic manifestations include dysarthria (85 to 97 %), gait abnormalities/ataxia (30 to 75 %) , dystonia (11 to 69%) , tremor (22 to 55 %) , parkinsonism (19 to 62 %) , drooling (48 to 86 %) of patients with neurologic Wilson disease , other neurologic manifestations include chorea, athetosis, cognitive impairment/dementia, seizures, hyperreflexia, myoclonia and urinary incontinence(Stapelbroek *et al.*, 2004; Machado *et al.*,2006 ; Javed *et al.*, 2008 ; Lorincz 2010; Kearney , 2011; Noureen and Rana ,2011; Mihaylova *et al.*, 2012) .

1.2.5.3. Behavioral and psychiatric symptoms

Behavioral and psychiatric symptoms are more common in patients with neurologic involvement than in patients with hepatic involvement and could occur in 10 to 100 % of WD patients. Patient with WD suffers from one or more of the following organic dementia , psychosis , psychoneurosis, and behavioral disturbances characterized by impulsivity occasionally extending to unlawful behavior (Shanmugiah *et al.*,2008; Gavarini *et al.*, 2010; Zimbrean and Schilsky, 2014) .However , behavioral and psychiatric symptoms due to WD are often misdiagnosed (eg , they may be attributed to puberty).

1.2.5.4. Hemolysis

Hemolysis in WD is uncommon. It occurs due to deficiency of ceruloplasmin, which results in excessive inorganic copper in blood circulation, much of it which accumulates in red blood cells. However, the exact mechanism of hemolysis is not known. While not a common finding in isolation, Coombs-negative hemolytic anemia may be the initial symptom of WD. Nonetheless hemolytic anemia with liver failure should make suspicion of WD as a cause (Padma and Kumar ,2014; Kumar and Radhakrishnan, 2014) .

1.2.5.5. Ocular manifestations

Kayser-Fleischer (K-F) rings are brownish or gray-green rings that are due to fine pigmented granular deposits of copper in Descemet's membrane in the cornea. K - F rings are considered to be the hallmark of WD and are seen in more than 90% of patients with neurologic manifestations and 50 to 60 % of patients with hepatic manifestations.

1.2.5.6. Other manifestations

Wilson disease can cause abnormalities related to copper deposition in other organs. Other manifestations of Wilson disease that are less common include:

Fanconi syndrome, in which proximal tubular dysfunction leads to lucosuria, aminoaciduria, hypouricemia, and proximal renal tubular acidosis(Selvan *et al.*,2012). Nephrolithiasis secondary to distal renal tubular acidosis , hypercalciuria and Nephrocalcinosis are early feature of WD onset and might be present as the unique sign of WD (Di Stefano *et al.*, 2012).

Other manifestations include gigantism, cardiomyopathy , myopathy, hyperparathyroidism , pancreatitis, impotence, infertility or repeated spontaneous abortions and a variety of dermatologic disorders (Liovet and Ducreux, 2012). Also patient with WD might presented with congenital scoliosis (Li *et al.*, 2014) .

1.2.6. Diagnosis

Early diagnosis and prompt treatment of WD is critical for complete recovery which is otherwise quite fatal .The diagnosis of WD starts with recognition of the clinical features suggestive of the disease or identification of family members who require screening(Liovet and Ducreux ,2012) .

Testing begins with liver biochemical tests, a complete blood count, serum ceruloplasmin level, an ocular slit-lamp examination, and 24-hour urinary copper excretion. Magnetic resonance imaging (MRI) or computed tomographic (CT) scanning of the brain may reveal structural abnormalities in the basal ganglia in patients with neurologic Wilson

disease(Machado *et al.*, 2006; Das & Ray 2006; Barbosa *et al.*, 2009; Rosencrantz &Schilsky, 2011).

1.2.6.1. Level of ceruloplasmin (<20 mg/dL or 200 mg/L)

Levels of ceruloplasminare abnormally low (<200mg/L) in 80–95% of cases. It can, however, be present at normal levels in people with ongoing inflammation as it is an acute phase protein. Low ceruloplasmin is also found in Menkes disease and aceruloplasminemia, which are related to, but much rarer than WD (Roberts and Schilsky 2003 ; Ala *et al.*, 2007) .

1.2.6.2. Hepatic copper concentration

A diagnosis of WD is established if the hepatic copper concentration is ≥250 mc/g dry weight (4 micromol/g dry weights).WD is excluded if the hepatic copper concentration is <50 mc/g dry weight (0.8 micromol/g dry weight). Although , a 5-fold increase of hepatic copper concentration is considered as the best available test for diagnosis of hepatic WD , the sensitivity and specificity of this test have never been fully investigated (Ferenci *et al.*, 2005; Patil *et al.*, 2013).

1.2.6.3. Molecular diagnosis

The European Clinical Practice Guidelines for WD referred that detection of the positive gene has the major role in the diagnosis of WD. (Liovet and Ducreux, 2012). In the past, genetic testing was considered impractical because of the great numbers of WD causing mutations reported in literature and most of these mutations are very rare. Recently, it has been documented that molecular genetic testing detects WD earlier

and more securely in very young children who frequently do not meet the diagnostic criteria of laboratory tests for WD. With the reduction in the cost, molecular genetic testing, become more actively used particularly in young children (Seo ,2012).

Direct molecular-genetic diagnosis is difficult because of more than 500 possible mutations vast majority of them are rare. Comprehensive molecular-genetic screening takes several months, which makes this an impractical method. Nevertheless, it is reasonable to perform molecular analysis of the *ATP7B* gene in any patient who has a provisional diagnosis of Wilson's disease, both for confirmation purposes and to facilitate the subsequent screening of family members(Liovet and Ducreux ,2012) .

The method for detection include haplotyping and direct gene sequencing. Haplotyping acquires genetic information according to the molecular markers inside the target gene or in the lateral wing of the target gene. Direct gene sequencing is now the standard method of diagnosis, this method can help in identifying the type of mutations. As a results of the fact that exons of WD gene disperse over a 4.3-kb region, in general, only mutational hotspots are sequenced(Liovet and Ducreux, 2012; Coffey *et al.*,2013; Wu *et al.* 2015; Gupta, 2015).

In haplotyping, determination of the type of mutations isn't needed, and the single-nucleotide polymorphisms in the *ATP7B* lateral wing are used for haplotyping. In screening of relatives of WD patients, haplotyping is suitable; nonetheless, if haplotyping is used for low probability gene recombination false positive rate will increase. In the recent years, by using novel sequencing technique, all exons even the whole genome could be sequenced.

This open the era for more efficient and comprehensive gene diagnosis of WD (Coffey *et al.*, 2013; Wu *et al.*, 2015).

Wilson's disease is conventionally diagnosed according to its clinical symptoms and biochemical indices. However, a few WD patients present with decreased ceruloplasmin level (usually 95%), nervous system problem and Kayser– Fleischer rings (+) in the cornea simultaneously. In most affected patients, WD manifests as liver dysfunction or decreased ceruloplasmin level with unknown reasons. At this time, diagnosis is made mainly based on gene detection. WD has an extremely high fatality rate without treatment, and screening family members of patients with diagnosed WD can avoid the unnecessary treatment of patients with heterozygote genotype(Ala *et al.*, 2007) .

The diagnosis of WD is conventionally according to its clinical symptoms and biochemical indices, however, a few WD patients present with typical, WD manifests as liver dysfunction or decreased ceruloplasmin level with unknown reasons. Therefore the diagnosis is mainly made on gene detection(Ala *et al.*, 2007). From other point of view, discrimination of heterozygotes from asymptomatic patients is essential to avoid inappropriate lifelong therapy for heterozygotes. Genetic testing, either by haplotype analysis or by mutation analysis, is the only definite solution for differentiating heterozygote carriers from affected asymptomatic patients(Seo ,2012).

It is worth mentioning that, a negative result of gene detection does not exclude a diagnosis of WD and the accuracy and predictability of gene diagnosis of this disease still needed further investigations and genetics studies to clarify the mechanism underlying WD at the molecular level which might helping the emergence of new treatment method including stem cell transplantation and gene therapy(Chen *et al.*, 2015).

1.2.6.4. Screening family members

First-degree relatives of any patient newly diagnosed with WD must be screened for WD. Assessment should include: brief history relating to jaundice, liver disease, and subtle features of neurological involvement; physical examination; serum copper, ceruloplasmin, liver function tests including amino transferases, albumin, and conjugated and unconjugated bilirubin; slit-lamp examination of the eyes for Kayser-Fleischer rings; and basal 24-hour urinary copper. Individuals without Kayser-Fleischer rings who have subnormal ceruloplasmin and abnormal liver tests undergo liver biopsy to confirm the diagnosis. Molecular testing for*ATP7B* mutations or haplotype studies should be obtained and may be used as primary screening. Treatment should be initiated for all individuals greater than 3 years old identifiedas patients by family screening(Das and Ray ,2006; Roberts and Schilsky, 2008).Newborn screening. Measurement of ceruloplasmin in Guthrie dried-blood spots or urine samples from newborns may promote detection of individuals affected with WD, but further refinement of methodology involving immunologic measurement of ceruloplasmin is required before wide-scale implementation can be advised(Owada *et al.*,2002; Aoki ,2004; Kroll *et al.*,2006) .

1.2.7. ATP7B protein structure and function and the Influence Different Mutation

Wilson's disease gene-encoded *ATP7B* protein belongs to P-type ATPase super family. There are 11 classes in the P-type ATPase super family. P-type ATPase of class IB (PIB) is responsible for transporting $Cu^{2+}$and other heavy metalions across biological membranes. Human PIB-type ATPases consist of *ATP7A* and *ATP7B*(Olsson *et al.*, 2009).

The characteristic domains of PIB include actuator domain (A-domain), phosphorylation domain (P-domain), nucleotide-binding domain (N-domain), and the M-domain which are composed of eight transmembrane-spanning helices. The core structures of *ATP7B* protein are highly conserved, for example, TGEA in the A-domain, DKTGT motif in the P-domain and SEHPL in the N-domain. In addition, there are six CXXC motifs that can bind to Cu2+ in the heavy-metal binding domain (Hatori *et al.*, 2007). The analysis of the LpCopA protein that is homologous to *ATP7B* by dividing the M-domain into six core helices M1–M6 and two PIB‐specific helices MA and MB(Gourdon *et al.*,2011).

The active transmembrane transport of Cu2+is complex and involves a series of conserved domains: Intracellular free Cu2+binding to the heavy metal binding domain first approaches the double-glycine (GG) motif in the MB helice, then these ions are transported onto Cys-Pro-Cys motif in the M4 helice, and finally discharged across the membrane by phosphorylation in the A-domain(Gourdon *et al.*, 2011).

Interestingly, although E1064 is very close to its downstream H1069 site, its mutation does not alter the protein folding in the N-domain. Furthermore, the thermal stability and intracellular localization of the E1064A mutation are just slightly different as compared to wild-type(Dmitriev *et al.*, 2011). R778L mutation is located in exon 8, with an allele frequency of 14–19%(Ferenci, 2006)leucine replaces the conserved Arginine in the M2transbilayer helix, which possibly affected thetransmembrane transport of Cu2+. There is a high similarity between the East‐Asian‐specific mutationsR778Land L770L which originate from a single ancestor. On the other hand, it had been concluded that R778L mutation was related to hepatic manifestations in WD(Liu *et al.*,2004). Previous studies referred that copper metabolism defect is

associated with the presence of two mutations ,G943S and M769V, in the M-domain, but the ceruloplasmin level almost normal(Liu *et al.*, 2004).Mutations in promoter regions –441/–427del, possibly lead to the occurrence of WD by altering *ATP7B* expression and these are more common in Sardinian.

1.2.8. Modifiers

Modifiers are a group of genes that aggravate or relieve the phenotypes of other virulence genes and make an important contribution to neurological disease phenotypes(Kearney,2011; Hamilton and Yu ,2012). The well-known modifier that had strongest correlation with WD is the Apolipo-protein E (ApoE) protein gene, which located in 19q13.2. ApoEprotein plays an important role in lipid metabolism. It consists of three major alleles, designated as ε2, ε3, ε4. ApoE ε3 and ε3/3 are the most common alleles and genotypes. ApoEε4 is considered to be related to lipid disorder and neurodegenerative diseases (for example, Alzheimer's disease). There is evidence that the onset age of WD is late in patients with ε3/3, possibly because apoE3 has anti-oxidative and neuroprotective properties(Wang *et al.*, 2003).On the other hand, ApoE ε4 is related to the early onset for WD, particularly in homozygous for H1069Q. Additionally, it is suggested that individuals with ApoE ε3/ε3 genotype, estrogen exerts neuroprotective effects on nerve growth, while in those ApoE ε4-positive genotype the protection by estrogen is impaired(Litwin *et al.*, 2012).

In vitro prion-related protein is likely to participate in the regulation of copper homeostasis and has been shown to bind copper in the human body(Merle *et al.*,2006). Prion protein gene codon129 has polymorphism. If the 129M allele is replaced by 129V allele, the onset of WD symptoms will advance about 5 years, but the phenotype of the symptoms would not

alter. Methylene tetrahydrofolate reductase is a key regulatory enzyme in folate and homocysteine metabolism (Chen *et al.*, 2015) Two common polymorphisms C677T and A1298C decreased the Methylene tetrahydrofolate reductase activity, and thus increased homocysteine level, which influences copper homeostasis *in vivo* and intracellular Cu2+toxicity.Individuals carrying double wild-type homozygotes 677CC/1298AA manifested WD symptoms 6 years later than those non carriers(Gromadzka *et al.*, 2011; Chen *et al.*, 2015). Some genes encoding cytokines possibly influence the phenotypes of WD, nterleukin-1 receptor antagonist gene (IL-1RN) polymorphism have been found to affect disease susceptibility and activity in several inflammatory diseases (Chen *et al.*, 2015).Individuals carrying interleukin-1β (IL1B) C-511T gene had increased blood copper level and ceruloplasmin level, and those carrying the IL-1RN 2 gene had increased ceruloplasmin level and manifested WD symptoms about 3.5 years earlier(Gromadzka *et al.*, 2011; Rafiei *et al.* 2013).

Other potential modifiers includemurr1 (commd1) and atox1 genes (Chen *et al.*,2015).

The Copper Metabolism MURR1 domain protein 1 (COMMD1) is a protein involved in multiple cellular pathways, including copper homeostasis. Acting as a scaffold protein, COMMD1 mediates the levels, stability and proteolysis of its substrates (e.g. the copper-transporters *ATP7B* and *ATP7A*) (Vonk *et al.*, 2014). Previously, COMMD1 was identified as a regulator of copper homeostasis as deletion of COMMD1 resulted in an extensive accumulation of copper in the liver of Bedlington terriers and mice (Vonk *et al.*, 2011) .

An interaction between the Cu/Zn superoxide dismutase 1 (SOD1) and COMMD1, resulting in a decreased maturation and activation of SOD1. Mutations in SOD1, associated with the progressive neurodegenerative disorder Amyotrophic Lateral Sclerosis (ALS) , cause

misfolding and aggregation of the mutant SOD1 (mSOD1) protein.(Van de Sluis *et al.*, 2010)In addition, identify COMMD1 as a novel regulator of misfolded protein aggregation as it enhances the formation of mSOD1 aggregates upon binding. Interestingly , COMMD1 co-localizes to the sites of mSOD1 inclusions and forms high molecular weight complexes in the presence of mSOD1 (Ke *et al.*, 2010). The effect of COMMD1 on protein aggregation is client-specific as, in contrast to mSOD1, COMMD1 decreases the abundance of mutant Parkin inclusions, associated with Parkinson's disease. Aggregation of a polyglutamine-expanded Huntingtin, causative of Huntington's disease, appears unaltered by COMMD1. Altogether, this study offers new research directions to expand our current knowledge on the mechanisms underlying aggregation disease pathologies (Miyayama *et al.* 2010; Vonk *et al.* 2014).

Copper metallochaperone protein that is encoded by the ATOX1 gene in humans. In mammals, ATOX1 plays a key role in copper homeostasis as it delivers copper from the cytosol to transporters *ATP7A* and *ATP7B*(Maret and Wedd,2014). Homologous proteins are found in a wide variety of eukaryotes, including Saccharomyces cerevisiae as ATX1, and all contain a conserved metal binding domain. ATOX1 transfers Cu^{+2} to transporters *ATP7A* and *ATP7B.* Transfer occurs via a ligand exchange mechanism, where Cu^{+2} transiently adopt3-coordinate geometry with cysteine ligands from ATOX1 and the associated transporter. The ligand exchange mechanism allows for faster exchange than a diffusion mechanism and imparts specificity for both the metal and transporter. Since the ligand exchange accelerates that transfer and the reaction has a shallow thermodynamic gradient, it is said to be under kinetic control rather than thermodynamic control (Maret and Wedd, 2014; Roy-Choudhury*etal.*,2015).

Chapter Two

Materials and Methods

2. Materials and Methods

2.1. Materials

2.1.1.Apparatus

Various apparatuses used in this study are listed in table 2-1.

Table 2-1: Apparatuses used in this study.

No.	Apparatus	Company	Country
1	Autoclave	Tomy	Japan
2	Bench centrifuge	VEB	Germany
3	Electrophoresis equipments	Consort	Belgium
4	Hood	Labogene	Denmark
5	Micro centrifuge	Eppendorf	USA
6	Nanodrop/UVS-99	ACTGenel	USA
7	Oven	Sanyo	Japan
8	PH meter	WTW	Germany
9	Sensitive balance	Sartorius	Germany
10	Shaker	Bioneer	Korea
11	Spectrophotometer	Shimadzu	Japan
12	Thermocycler	Bioneer	Korea
13	U.V. transilluminator and camera	Flowgen	U.K.
14	Vortex	Lab Kites	Korea
15	Water bath	Labtech	Korea

2.1.2. Chemicals and biological materials

Chemicals and biological materials used in this study are listed in Table 2-2

Table 2-2 Various Chemicals and biological materials used in the study.

No.	Chemicals	Company	Country
1	Absolute ethanol	Pharmacia	Sweden
2	Agarose	Biobasic	Canada
3	Deionized water	Bioneer	Korea
4	DNA ladder	KAPA Biosystem	USA
5	Ethidium bromide	Biobasic	Canada
6	Loading dye	Biobasic	Canada
7	Premix	Bioneer	Korea
8	Primers	Bioneer	Korea
9	Tri-Borate EDTA	Biobasic	Canada

2.1.3. Kits

Table kits used in this study table 2-3

Table 2-3 Various kits used in this study.

No.	Kits	Company	Country
1	Alanine Aminotransferase (ALT)	Randox	U. K.
2	Alkaline Phosphatase (ALP)	Biolabo	France
3	Aspartate Aminotransferase (AST)	Randox	U. K.
4	Copper (Cu++)	Randox	U. K.
5	DNA extraction from blood	gSYNC™DNA Mini Kit Whole Blood Protocol/ Geneaid	Korea
6	Human Ceruloplasmin ELISA	Cusabio	China
7	PCR Product purification kit	Bioneer	Korea
8	Total Bilirubin	Randox	U. K.

2.2. Methods

2.2.1. Study subjects

Patients attending hospitals to undergo periodic checks to follow progress their condition with Wilson disease from 8 months were eligible for this study. Two hospitals in Iraq were included: Baghdad Medical City - Digestive diseases hospital and educational liver, Immaim AL-Kadhm main medical city. Ethical clearance to conduct the research was sought and obtained from these hospitals. Selections of patients were accomplished with assistance of pediatric physician.

Thirty - five patients were selected to be investigated in this study. All had Wilson disease of different grades and stages, 20 male and 15 female with a mean age of (9.12) years.

Data were collected through direct interview with the patient, and by seeking his/her hospital record as well as previous medical reports (a copy of data sheet is provided in appendix1).

Ten controls were selected randomly from apparently healthy individuals. Individuals who have been previously diagnosed with WD at the time of enrollment were excluded from this group.

Informed consent from patients as well as control was taken which included age, residence, and first relative family history of WD.

2.2.2. Blood Samples

Five – milliliter of blood was taken from patients and controls these divided as 2 ml of which was kept in EDTA tube (used in polymorphism SNPs study and kept - 20 º C) and the other 3 ml was put in plan tube and underwent centrifugation where the serum was obtained and preserved at - 20 º C until be used .

2.2.3. DNA Extraction from blood samples:

DNA was extracted from blood samples using ready kit (gSYNCTM DNA Mini Kit Whole Blood Protocol / Geneaid / Korea) procedure was done according to the manufacturer's instructions as follow:

1. A total of 200 µl of blood was transferred to 1.5 ml micro centrifuge tube, and the volume was adjusted to 200 µl with PBS.
2. 20 µl of proteinase K was added to the sample , mixed by pipetting , and incubated at 60 º C for 5 min , after which a GBS Buffer 200 µl was added to the 1.5 ml micro centrifuge tube and mixed by shaking vigorously .
3. The tubes were incubated at 60 º C for 50 minutes. During incubation, they were inverted every minute (it is essential that the sample and GBS Buffer are mixed thoroughly to yield a homogeneous solution). At this time, the required elution Buffer, 200 µl /Sample was pr-heated at 60 º C (for step 5 DNA elution).
4. Absolute ethanol (200 µl) was added to the sample lysate and immediately mixed by shaking vigorously for 10 sec , if precipitate appears , it was broken up as much as possible with a pipette .
5. The GD column was placed in a 2 ml collection tube, and the lysate from step 7 (including any precipitate) was transferred to the GD column, and centrifuged at 14000 rpm for 1 minute, or until the mixture pass the GD column completely.
6. The 2 ml collection tube containing the flow – through was discarded , and the GD column was placed in a new 2 ml collection tube , where 400 µl W1 buffer was added to the column which was centrifuge at 14000 rpm for 30 sec.
7. The flow – through was discarded , and the GD column was placed back in the 2 ml collection tube , where 600 µl wash buffer was

added to the GD column , which was again centrifuged at 14000 rpm for 3 sec.

8. The flow – through was discarded, and the GD column was placed back in the 2 ml collection tube.
9. To dry the column matrix, the GD column was centrifuged at 14000 rpm for 3 minutes.
10. The dried column was transferred to a clean 1.5 ml micro centrifuge tube, and 100 µl of pre-heated elution buffer was added to center of the column matrix , and let stand at least 3 minutes to ensure the elution buffer is absorbed by the matrix .
11. Micro centrifuge tube containing the GD column was centrifuged at 14000 rpm for 30 sec to elute the purified DNA. The Eluted DNA was stored at - 20 º C until be used for PCR.

2.2.4. Measurement of DNA concentration and purity:

Ananodrop was used to estimate the concentration and purity of the extracted DNA according to the following procedure:

2.2.4.1. Blanking

i. After lifting the sampling arm, 2 µlof elution buffers was pipetted onto the measurement pedestal.
ii. The sampling arm was laid down and the solution was observed bridging the gap between both optical fibers.
iii. When the ″ Blank ″ button clicked, the apparatus measured the solution with both 1mm and 0.2 mm path lengths, and then the system recorded both results automatically.
iv. When the measurement completed, the sampling arm was opened and blanking buffer was wiped from pedestal using laboratory wipe.

2.2.4.2. Sample measurement

i. The sample ID was typed; we select ″ Sample Type ″.
ii. For nucleic acid sample, 2 µl of the sample was pipette on to the measurement pedestal while the sampling arm opened.
iii. The sampling arm was laid down and the solution was observed bridging the gap between both optical fibers.
iv. The button ″ Measure ″ was clicked and the measurement result appeared on the screen within 3sec.

When any sample gives purity less than 1.5 and / or concentration less than 10 ng/µl, DNA extraction from the blood of that was sample re-performed until the desired purity and concentration was obtained.

2.2.5. Primers and PCR protocols:

2.2.5.1. Exon 10

For amplification of Exon 10 of *ATP7B*gene, the following primers were used.

The forward primer 5'- GTGACCGAATGAGTGGC-3', and reverse primer 3'-TTTCCCAGAACTCTTCACA-5 '. With fragment length of 510 (bp) (Dastsooz *et al*, 2013).

2.2.5.2. Exon 13

For amplification of Exon 13 of*ATP7B*gene,the following primers were used.

The forward primer 5'-GAAATGTCCTTATGTGATT-3', reverse primer 3'-AGTAAACAGATACTACTTTCATC-5'.With fragment length of 332 (bp) (Dastsooz *et al.*,2013).

The PCR protocol was as the following:

No.	Steps	Temperature and duration	
1	Initial denaturation	95 ºC for 7 min	
2	Denaturation	94 ºC for 30 sec	
3	Annealing	60 ºC for 30 sec	35 cycles
4	Elongation	72 ºC for 1 min	
5	Final elongation	72 ºC for 10 min	

A ready-mode 50 µl PCR master-mix (Bioneer / Korea) was used for amplification for both genes. Components of eachmaster-mixare shown in table 2- 4.

Table 2-4: Components of each Master-mix

No.	Components	Concentration	Quantity
1	TaqDNA polymerase	2.5	U
2	dNTP (dATP , dCTP , dGTP , dTTP)	250	µM
3	Tis-HCl (pH 9.0)	10	mM
4	KCL	30	mM
5	$MgCl_2$	1.5	mM
6	Stabilizer and tracking dye	No specific constration	

Template DNA (10 µl) from each sample and primers (5µl from each) were added to each master-mix tube. The mixture was then put in the shaker and spinner 10 cycles for better mixing. After mixing, the master – mix tubes were transferred to the thermo cycler (My Genie 32 thermal block / Bioneer/ Korea) who is previously programmed with the above protocol according to gene to be amplified.

2.2.6. Agarose gel electrophoresis

Gel was prepared by dissolved 1 gmof agarose (Biobasic / Canada) in 100 ml. Of 1xTris Borate EDTA (TBE) (Biobasic / Canada), then was put in oven for 5 min. After about 10 min, the gel was poured in the tray, when the gel solidifies it was transferred into the tank and enough amount

of TBE was poured to just cover the gel. The comb was removed and a 10 µl aliquot of PCR product was mixed with 2µl loading dye and was loaded in to the wells. Power supply was adjusted into 100 volt and run for 1 hour. The gel then was stained with 0.5µl/ml ethidium bromides (Biobasic / Canada) for 20 min and examined using UV transilluminator equipped with camera. The amplified products were determined by comparison with a commercial 1000 bp ladder (kappa). In case that any PCR product has negative result in agarose gel electrophoresis , there will be re-examine of extracted DNA using nanodrop , and DNA re-extraction if the purity is less than 1.5 and/or concentration was less than 10µl/ml , otherwise the amplification was re-performed and the cause of negativity was attributed to the processing.

2.2.7. PCR Product Purification:

PCR Purification product kit was used (gSYNC™ PCR Purification product Kit/Gene aid/ Korea) according to the manufacturer's instructions as follows:

1. Five volumes of PCR binding buffer was added to the PCR product. Mixed completely by vortex.
2. The mixture was transferred to the DNA binding column tube and centrifuged for 1 minute at 13.000 rpm.
3. The flow -through was poured off and the DNA binding filter column with the 2.0 ml collection tube was re-assembled.
4. Amount of 500 ml of washing buffer was added to the DNA binding column tube and centrifuged for 1 minute at 13.00 rpm to removes salts and soluble impurities in the DNA binding column tube.
5. The flow -through was poured and the DNA binding filter column with the 2.0 ml collection tube was re-assemble.

6. Step 4 and 5 were repeated.
7. The products were dried by additional centrifugation at 13.000 rpm for 1 minute to remove the residual ethanol and the DNA binding filter column was transferred, and wait for at least 1 minute at room temperature for elution.
8. Thirty micro liter of Elution buffer was added to the center of the DNA binding filter column, and wait for at least 1 minute at room temperature for elution.
9. The DNA fragment was eluted by centrifugation at 13.000 rpm for 1 minute.

2.2.8. DNA Concentration of Purified PCR Products

DNA concentration of the Purified PCR products was measured using /Nanodrop/UVS-99 (ACT Gene/USA). All products gave concentration more than 100ng/µl and were sent to DNA sequencing.

2.2.9. DNA sequencing

PCR product which was purified and detected on agarose gel electrophoresis were sent to (Macro gene company /Korea) for DNA sequencing , and analysed for the presence or possible either mutation of any polymorphism .The obtained sequences were aligned using "Clustalw" software with normal sequence from Gen Bank (Appendix III).

2.2.10. Biochemical tests

2.2.10.1. Alanine Aminotransferase (ALT)

Randox kit is a kit for quantitative in vitro determination of ALT in serum. It is measured by monitoring the concentration of

pyruvatehydrazone formed with 2, 4-dinitrophenyl-hydrazine. Absorbance was measured by spectrophotometer at wave length of 450nm (Davidsohn and Nelson, 1974).

2.2.10.2. Aspartate Aminotransferase (AST)

The principle of AST measurement is based on the reaction of R-Aspartate and 2- Oxaloacetate toyield R-Glutammate and Oxaloacetate in the presence of AST. Eventually, the Oxaloacetate is converted into L-malate by malate dehydrogenasein the presence of NADH. The oxidation of NADH to NAD^+ is directly proportional to the AST activity (Thomas , 1998).

2.2.10.3. Alkaline Phosphates (ALP)

Colorimetric determination (Kind *et al.*, 1954; Belfield *et al.*, 1971) of the ALP activity which reaction scheme is as follows:

Phenyl phosphate $\xrightarrow{\text{Alkaline Phosphates}}$ Phenol + Phosphate

Free phenol librated by hydrolysis of the substrate reacts then with 4-amino-antipyrine in the presence of alkaline potassium ferricyanide to form a red-coloured complex which absorbance measured at 510nm is directly proportional to the ALP activity in the specimen. Sodium arsenate in corporated in the reagent abolishes further enzyme activity and prevents the dilution of the colour inherent in earlier methods.

2.2.10.4. Total Serum Billrubin (TSB)

Total Bilirubin Linear chemicals kit is a colorimetric assay. In the reaction is converted to colored azobilirubin by diazotized sulfanilic acid and is photometrically measured. Of the two bilirubin fractions in serum bilirubin glucuronid and free bilirubin bound to albumin only the former

reacts directly, while the free albumin reacts after being displaced from protein by an accelerator. The total bilirubin measured with the accelerator (Young, 1997).

2.2.10.5. Copper (Cu++) Concentration

Estimation of Copper concentration

Spectrophotometer method was used to estimate serum levels of copper using a commercial kit (Randox/ United Kingdome).

Principles:

Copper, which is bound to ceruloplasmin at pH 4.7, is released by reducing agent. It is then reacts with a specific colour reagent, 3,5-Di-Br-PAESA[4-(3,5-Dibromo-2-pyridylazo)-N_Ethyl-N-(3-sulphopropyl) alanine] , to form a stable coloured chalate. The intensity of the colour is directly proportional to the amount of the copper in the sample.

Reagent composition :	
Contents	**Initial Concentrations**
R1a. Buffer Acetate Buffer Non reactive stabilizers	0.2 mol/l , pH 4.7
R2. Chromogen Acetate Buffer Complexant 3,5-Di-Br-PAESA	0.2 mol/l , pH 4.7
R1b. Reagent Ascorbic Acid Standard (CAL)	

Procedure:

Temperature	37°C
Wavelength	580 nm (570 – 590 nm)
Path length	1 cm
Reacting	Endpoint
Measurement	against reagent blank

Pipette into test tube:

Materials	Reagent blank	Standard	Sample
Double distilled H_2O	120 µl	-	-
Sample Supernatant	-	-	120 µl
Standard Supernatant	-	120 µl	-
Reagent	1000 µl	1000 µl	1000 µl

Materials weremixed and allowed to stand 60 second at 37°C, and initial absorbance (A_1) of sample and standard against reagent blank was read. Chromogen (R2) was added (250 µl) to each tube and then incubated for 5 min at 37°C and .The final absorbance (A_2) was read against reagent blank.

2.2.11. Measurement of Ceruloplasmin Activity

A ready ELISA kit (Cusabio / China) was used to measure CP activity Principle of the Assay

Reagent preparation:

1. All reagents were brought to room temperature (18 – 25 ºC) before be used for 30 min.
2. Fresh standard for each assay was prepared and used within 4hours and discarded after use.
3. Standards were reconstituted according to the instruction manual Foaming was avoided.
4. Biotin – antibody was 100 – fold diluted with Biotin – antibody diluent.
5. HRP – avidin was 100 – fold diluted with HRP – avidin diluent.

Preparation of standards:

The standard vial was centrifuged at 10000 rpm for 30 sec, and then reconstituted with 1.0 ml of sample diluents .This reconstitution produce a stock solution of 10 mg/ml. The standard was mixed to ensure complete reconstitution.

A total of 250 µl of sample diluent was pipetted in each tube (50 – 56). The stock solution was used to produce a 2 – fold dilution series. Each tube was mixed thoroughly before the next transfer. The undiluted standard serves as the high standard (10mg/ml). Sample diluent serves as the zero standards (0 mg/ml).

Assay procedure:

1. One hundred µl of standard and sample per well were added, and the microplate was covered with adhesive strip and incubated for 2 hours at 37 º C.
2. The liquid was removed from each well, and 100 µl of Biotin – antibody was added to each well. The microplate was covered with a new adhesive strip and incubated for 1 hour at 37 º C.
3. Liquid was aspirated from each well and microplate was washed three times .After the last wash , the remaining wash buffer was removed by aspiration , and the microplate was inverted and bloted against clean paper towels .
4. To each well, 100 µl of HRP – avidin was added. The microplate was covered with a new adhesive strip and incubated for 1hour at 37 º C.
5. The aspiration of wash process was repeated five times.
6. Ninety µl of TMB substrate was added to each well at the microplate, and then incubated for 30 min at 37 º C.
7. Stop solution (50 µl) was added to each well and the microplate was gently tapped to ensure thorough mixing.

8. The optical density was read at 450 nm as auto dragmatic reader (Spectrophotometer/ Shimadzu / Japan).

2.2.12. Statistical Analysis

The Statistical Package for the Social Sciences (SPSS, version 14) was used for statistical analysis. Risk association between the genotype and Wilson disease was estimated by the calculation of adjusted odd ratio and 95% confidence intervals using logistic regression. For this analysis, subjects who were homogenous for the wild type allele were considered a reference, and polymorphisms as dependent variables, age, sex, family history, co variables in the model. Chi- square was used for testing the deviation from Hardy-Weinberg equilibrium, distribution of different alleles between patients and control.Independent of test was used to compare mean of serum levels of ALT , AST , ALP , Bilirubin , Copper and Ceruloplasmin between patient and control .

Chapter Three

Results and Discussion

3. Results and Discussion

3.1. Demographic Data

During the period from January 2015 to August 2015, 35 patients with WD were investigated for mutational analysis of exon 13 and exon 10 for ATPase gene. The mean age at diagnosis was 9.12 years.

The disease is typically diagnosed between the ages of 5 and 40 years old. However, molecular studies have found *ATP7B* gene mutations occur in 70-year old patients (Mak *et al*., 2008). Further, the diagnosis not only restricted to molecular studies but also the clinical manifestations of the disease were recorded in patients in their fifth and sixth decade (Zhang *et al*., 2011). As the disease is caused by inherited mutations, this disparity in age-incidence could be explained only by the interaction between genetic, endogenous, and environmental factors. Such factors may involve the consumption of copper or other minerals like zinc, where the latter may inhibit the ability of enterocytes to absorb copper. Other factors include those which may influence the ability of an individual to resist oxidative injury (Schilsky, 2007). The sex ratio (male/female) was 4/3 as indicated in figure (3-1).

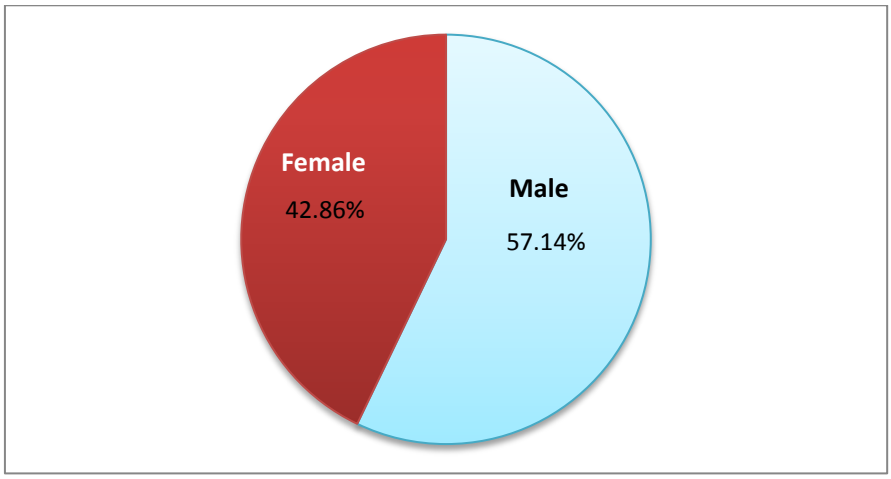

Figure 3-1: Male to female ratio of patients with Wilson disease

Ideally, there is no difference between male and female in respect of WD. However, different communities reported different proportions. In South India, more than two-thirds of patients were male. This disparity was attributed to the fact that Indian males were more likely to be brought for medical attention (Schilsky, 2007). On the other hand, the differences in clinical manifestation of the disease between male and female are well documented. Litwin *et al.* (2012) found that hepatic form of disease occurs more frequently in women, and women develop the neuropsychiatric form of disease almost 2 years later than men. Furthermore, Acute liver failure due to Wilson's disease occurs predominantly in young females (female: male ratio 4:1) (Walshe, 1987).These differences are attributed to the protective effect of estrogens and iron metabolism.

Two possible explanations for the higher proportion of male than female in the current study. First , as that in South India, most families keen to treat male with less care toward female, especially large percentage of the patients are from south parts of the country. The second explanation refers to the fact that of course some affected women are pregnant and they may take zinc as a supplemental drug for their pregnancies. Zinc is

one of drug of choice to treat WD. Thus, the symptoms of the disease will dim and there is no need for medical care.

Twenty-four patients (68.43%) were from urban area while 21 (31.43%) were rural area resident (figure 3-2).

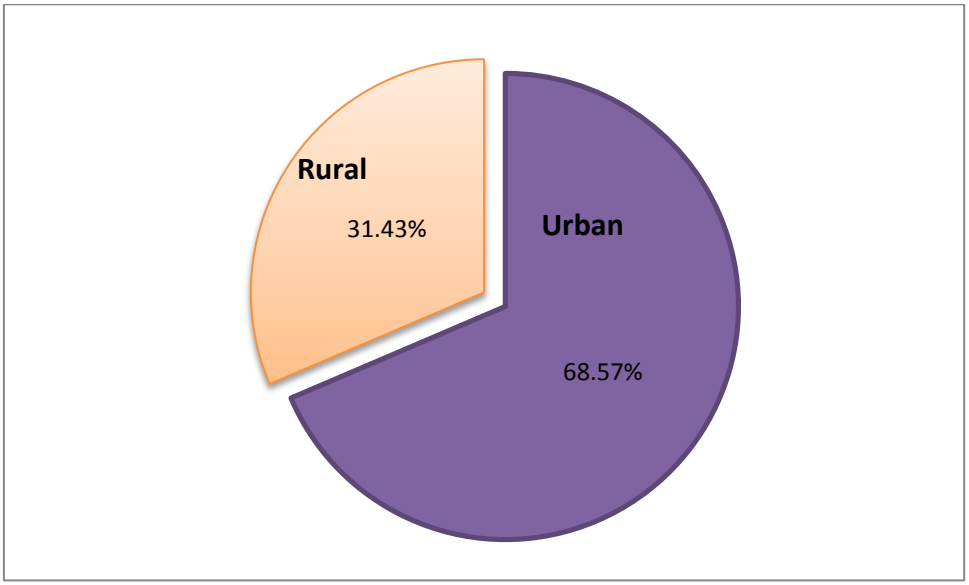

Figure 3-2: Rural/urban ratio of Wilson disease patients

It is supposed that WD is more prevalent in rural than urban area because the high incidence of consanguinity among rural residents (Moraru, 2015). The reverse result in this study may refer to kind of medical services. In urban area, people can easily access to the specialized medical centers, while rural areas in Iraq almost lack such centers, and the patient has to travel long distance to get the diagnosis and regular medication especially with this kind of diseases.

The frequency of parental consanguinity is relatively high 14 out of 35 patients (40%) as indicated in figure (3-3).

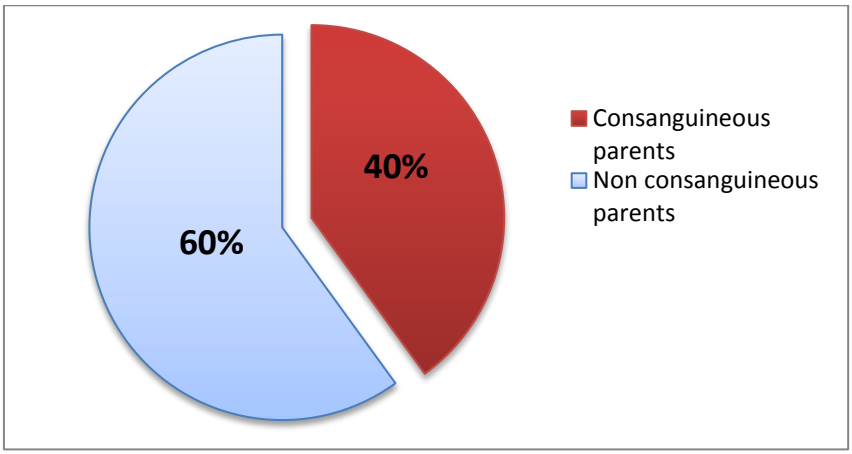

Figure 3-3: Consanguineous/ non-consanguineous ratio of Wilson disease patients

A considerable attention has been focused on the consanguineous marriage as an important factor associated with the prevalence of certain genetic disorders. World-wide, it is estimated that 20% of the world population reside in communities where consanguineous marriage is encouraged. Therefore, over 8.5% of the children in the world have parents with this type of marriage. In fact, consanguineous marriage is widely practiced in definite geographical areas like some countries in Asia and Africa, particularly when the dominant religion is Islam. On the other hand, the type of marriage has low prevalence in Western countries (Madi *et al*., 2005; Mehrabi and Zeyghami, 2005; Ali et al., 2008).In this study, WD patients who have consanguineous parents were 40% which is a very high percentage when compared with control whom only one individual who has consanguineous parent (10%).

The influence of consanguinity on the prevalence of genetic disorders is well-documented in several researches achieved in different populations. Genomes of individuals born from consanguineous marriage have many segments which are homozygous as these individuals inherit identical genomic segments from their father and mother. Hence, long time

parental inbreeding will lead to 5% increase in the homozygosity compared to the usual predicted homozygosity in random marriage (Woods *et al.*, 2006).From statistical aspect, consanguinity has no effect on the allele frequencies; however, consanguinity enhances the chance of marriage between two persons who are heterozygous for the same recessive harmful allele. Thus the offspring opportunity of the first-cousin marriage to get the defects is expected to be very high in comparison to non-consanguineous marriage. This especially obvious in genes responsible for rare autosomal recessive disease (Al-Gazali *et al.*, 2006).

3.2. Biochemical analysis:

3.2.1. Serum levels of ALT, AST, ALP and Bilirubin

Mean values of serum level of ALT, AST, ALP and bilirubin in patients with WD were 132.14±23.12 U/L, 96.93±11.21 U/L, 184.29±48.45 U/L and 3.6±1.13 mg/dl respectively which differed significantly from their counter parts in control group that were (45.12±4.6 U/L, 32.54±8.23 U/L, 87.08±22.39 U/L and 0.82±0.16 mg/dl respectively) as shown in (table 3-1).

Table 3-1: Serum levels of ALT, AST, ALP and Bilirubin in patients with WD and control group.

Index	WD patients	Control	P-values
ALT (U/L)	132.14±23.12	45.12±4.6	0.012
AST(U/L)	96.93±11.21	32.54±8.23	0.005
ALP(U/L)	184.29±48.45	87.08±22.39	0.019
Bilirubin (mg/dl)	3.6±1.13	0.82±0.16	0.022

The study revealed significant elevation in enzymes which indicate liver function in WD patients compared to controls. These results suggest

different degree of injury to the liver tissue. In fact, hepatocyte injury is characteristic of severe un-treated stages of WD. This injury is caused by excess free copper which binds to sulfhydryl groups and inactivates enzymes such as glucode-6-phosphate and glutathione reductase (Ibrahim, 2013). Furthermore, free copper may interact with oxygen species (e.g. superoxide anions and hydrogen peroxide) and catalyzes the production of toxic hydroxyl radicals (Ellenhorn *et al*., 1997). Of course, among those patients are many who have normal serum levels of these enzymes, but according to the present result the vast majority had elevated levels. This indicates that most patients either in severe stage of the disease or the drugs they receive are not fully effective.

On the other hand, elevation of bilirubin in WD patients is mainly attributed to hemolytic anemia accompanied many cases of this disease. The mechanism of this anemia is not fully understood, but accumulation of copper in the red blood cells (RBCs) may causes damage to the cell membrane, accelerate oxidation of hemoglobin (Hb) and inactivate enzymes of pentose phosphate and glycolytic pathways (Shrama *et al*., 2010). Supporting this hypothesis is spherocytes which characterized this anemia and indicate the involvement of cell membrane.

3.2.2. Serum Copper and Ceruloplasmin

Serum levels of copper among WD patients ranged from 78 to 214 µg/dl with an average of 127.18±64.432 µg/dl despite that many cases exceeded the normal range (70-155 µg/dl), this average does not differ significantly from that of control group which ranged from 75 to 129 µg/dl with an average of 113.01± 48.16µg/dl. On the other hand, mean serum levels of ceruloplasmin in patients ranged from 2.4 to 56 mg/dl

(average 18.14±11.2 mg/dl) compared with 36.79±13.72 mg/dl in control without significant difference (figure 3-4).

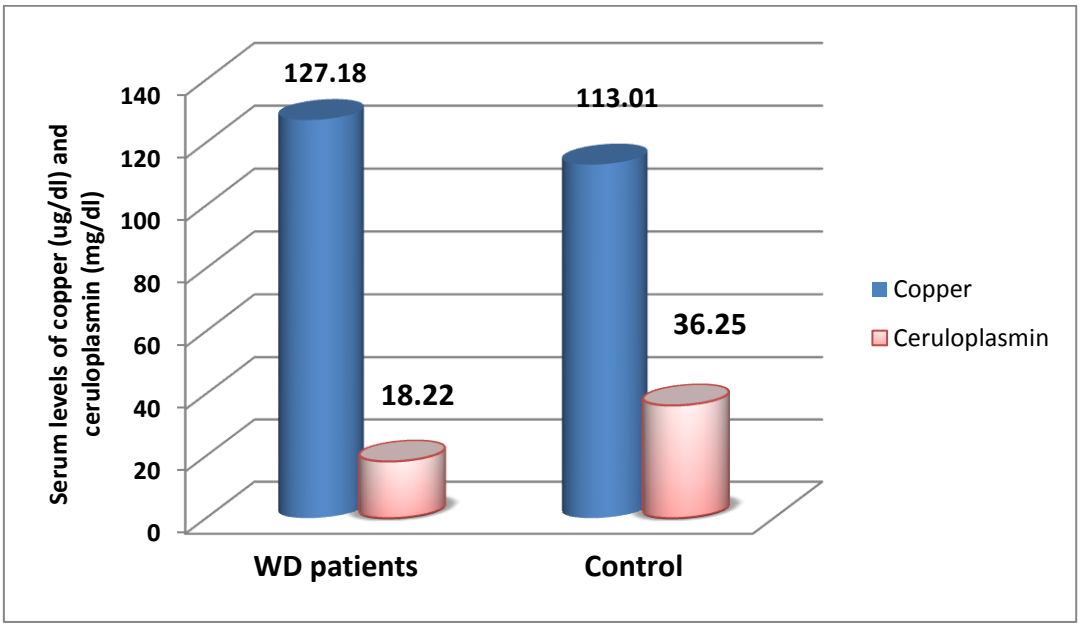

Figure 3-4 : Serum levels of copper and ceruloplasmin in WD patients and controls

Normally, excess copper deposit in hepatocytes. However, free copper exceed the storage capacity of hepatocyte, variable quantities of this metal spill out of the liver entering the blood circulation to eventually deposit in different organs. As mean serum level of copper is within normal limits, it is reasonable to conclude that the disease is not in advance stage. Elevation of liver enzymes and dropping of copper can be explained by the fact that increased copper concentration inside the hepatocytes caused reduction in a protein called X-linked inhibitor of apoptosis (XIAP). This results in an acceleration of caspase 3-initiated apoptosis with eventual cell death (Mufti *et al*., 2006). Dead hepatocytes release their contents among which liver enzymes.

Ceruloplasmin deficiency is well documented in patients with WD (Mak *et al.,* 2008) although the current study did not revealed significant

dropping in ceruloplasmin in WD patients; the average of this protein is less than that in controls. In fact, with the presence of defective ATP7Bprotein, it is expected to find low levels of serum ceruloplasmin. That is because defective ATP7B (due to gene mutations) is unable to incorporate copper to apoceruloplasmin, which is converted to ceruloplasmin when binds copper. Thus there are low levels of ceruloplasmin (Pfeiffer, 2007). However, there are two main reasons that ceruloplasmin levels are not diagnostic feature for diagnosis of WD.

The first reason is decreased serum levels of ceruloplasmin could be detected in large numbers of non-WD patients such as those with decompensated liver failure in whom there is a high reduction in synthetic liver function. Furthermore, low ceruloplasmin levels have been recorded in many pathological conditions like nephritic syndrome, Menkes disease, potein calorie malnutrition, acquired copper deficiency, hereditary aceruloplaminemia, and protein losing enteropathy (Hellman and Gitlin, 2002). The second reason is referred to ATP7B protein which has dual roles: copper transport and cellular trafficking. Many mutations affecting the trafficking of ATP7B inside the cell may not cause reduction in serum levels of ceruloplasmin. Hence, it cannot depend only on the concentration of cerulplasmin for diagnosis of the disease.

3.3. Genetic Analysis

A total of 70 alleles belong to 35 WD patients and other 20 alleles belong to healthy individuals have been examined for mutations in the exon 10 and exon 13 of *ATP7B* gene figure (3-5) and (3-6) shows gel electrophoresis of amplified gene containing exon 10 and exon 13 respectively.

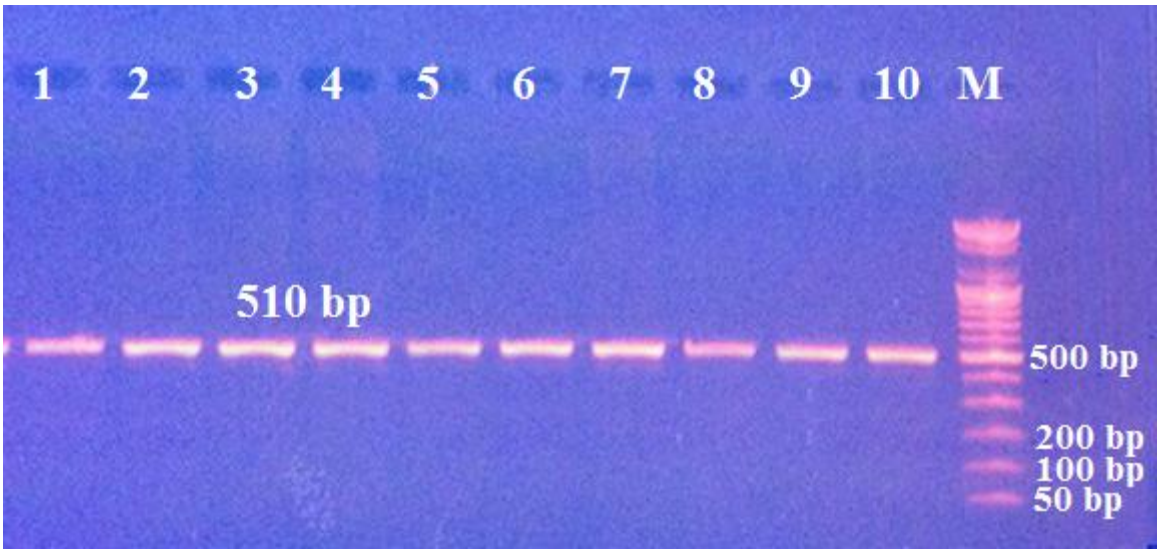

Figure (3-5): Gel electrophoresis for Exon 10 products visualized under U.V light after staining with ethidium bromide M: 1000 bp marker .The size of product is 510 bp ..(Cycling condition involves an initial denaturation at 95 C for 7 min followed by 35 cycles of denaturation at 94 ºC for 30 Sec, annealing at 60 ºC for 30 Sec , extension at 72 ºC for 1 min and final extension at 72 ºC for 10 min) .

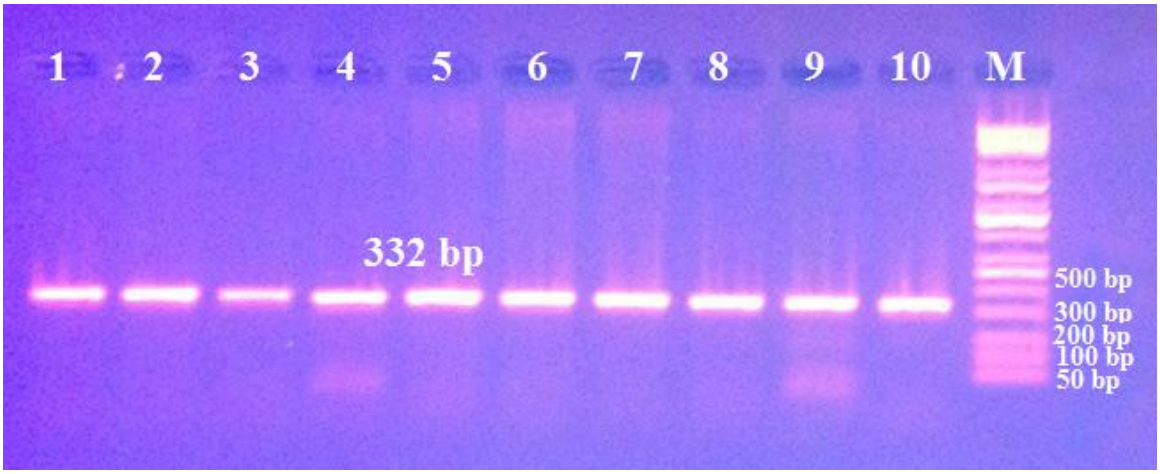

Figure (3-6) : Gel electrophoresis for Exon 13 products visualized under U.V light after staining with ethidium bromide M: 1000 bp marker .The size of product is 332bp .(Cycling condition involves an initial denaturation at 95 C for 7 min followed by 35 cycles of denaturation at 94 ºC for 30 Sec, annealing at 60 ºC for 30 Sec , extension at 72 ºC for 1 min and final extension at 72 ºC for 10 min) .

Mutations were detected in 19 WD patients out of 35 (54.29%). seven different mutations have been recorded, three of which are single nucleotide polymorphisms (SNP) (Table 3-2).

Table 3-2: Characteristics of the mutations and the affected domain of *ATP7B* gene in WD patients.

No	Mutation	Nucleotide change	Type	Exon	No of alleles (%)
1	Lys832Arg*	c.2495C>T	Missense	10	16 (22.86%)
2	Pro840Leu*	c.2519C>T	Missense	10	18 (25.71%)
3	Thr991Thr*	c.2973A>G	Silent	13	3 (4.29%)
4	Ala1003Val	c.3008C>T	Missense	13	6 (8.57%)
5	Lys1010Arg	c.3029A>G	Missense	13	8 (11.42%)
6	c.2977-2978insA	Insertion A	Frameshift	10	17 (24.29%)
7	c.2457delA	DeletionA	Frameshift	10	19 (27.14%)

***: single nucleotide polymorphism,**

The variant Lys832Arg (rs1061472) appeared in three genotypes; CC, CT and TT (figure 3-7). Six patients had the heterozygous form and two homozygous form of the SNP. On the other hand three individuals from control group were carriers for homozygous mutant allele while two of them were carrying hetrozygous mutant allele of this SNP.

Globally, this polymorphism is one of the most recorded variants in mutational analysis of *ATP7B* gene. For example, Gupta *et al.* (2007) were considered it among the four most common variants in Indian patients with WD which were c.1216 TCT_GCT/p.Ser406Ala, c.2495 AAG_AGG/ p.Lys832Arg, c.2855 AGA_AAA/p.Arg952Lys, and

c.1544-53A_C, and recommend to use these for molecular diagnosis of the disease. In Iran, Zali *et al.*, (2011) recorded 0.31 frequency of this variant among Iranian patients, however, the authors found it more prevalent among control group (0.38). Recently, Papur *et al.*, (2015) recorded this SNP among five other polymorphisms in the entire *ATP7B* gene of Turkish patients with WD. More recently, in China, Dong *et al.* (2016) conducted the largest study in this regard. They sequenced the *ATP7B* gene from 632 WD patients and 503 unrelated phenol typically normal individuals. Among the 161 variants recorded in this study, the homozygous mutant genotype of the SNP rs1061472 was reported in 105 WD patients, while the heterozygous genotype in 59 patients. On the other hand, 144 healthy individuals were bearing the homozygous mutant genotype vs 47 carrying heterozygous genotype with insignificant difference (odds ratio=0.988, 95%confidence interval=0.819-1.192).

Regardless of the prevalence of this polymorphism, it appears to have no causal relationship with the disease because similar or even higher prevalence of the mutant allele were recorded among normal population.

However, growing body of evidences linked this SNP with Alzheimer's disease (Buccosi *et al.*, 2011) suggesting that amino acid substitution lys 832 to Arg (Tanzi *et al.*, 1993) may interfere with normal function of ATPase protein in term of metal binding capacity, which can eventually result in copper homeostasis abnormalities. As this effect was not evidenced in WD, it can be postulated that linkage disequilibrium with one or more polymorphism in the same gene is responsible for the deteriorating effect of minor frequent allele of this SNP.

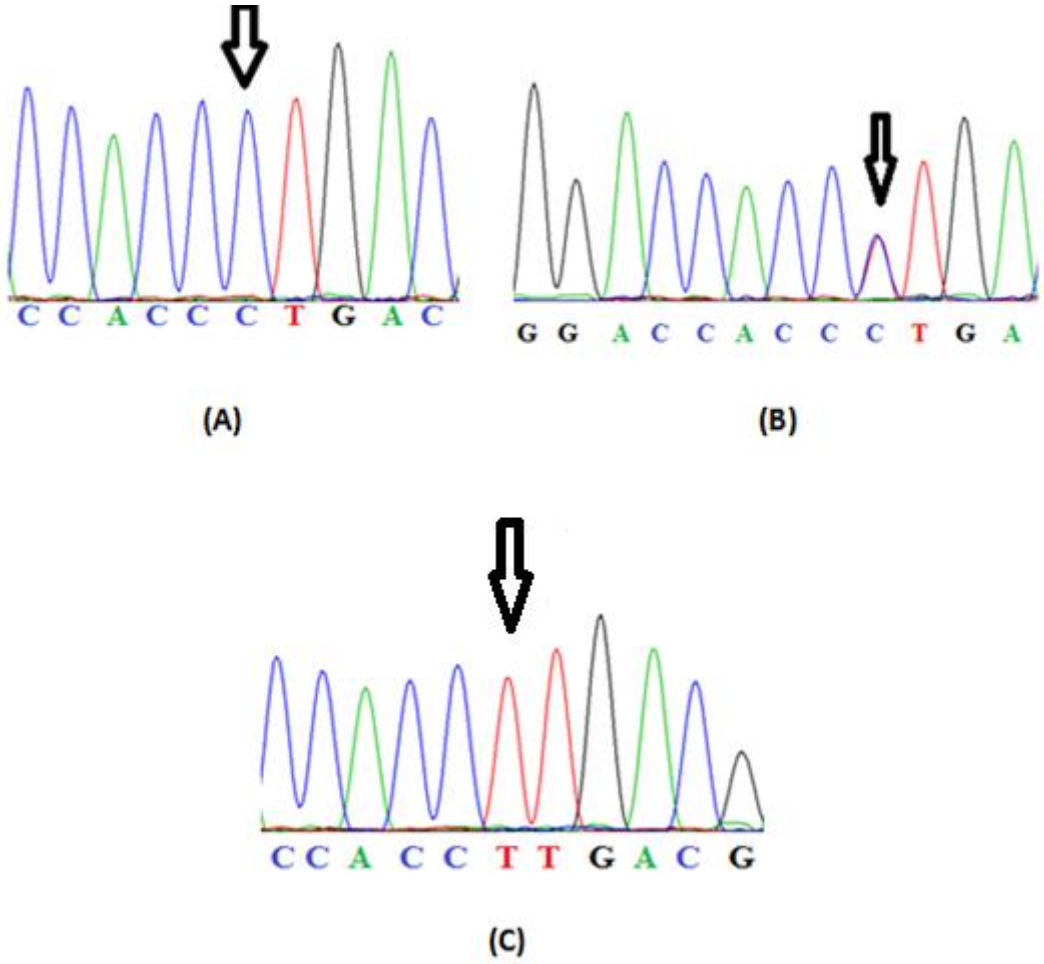

Figure 3-7: Different genotypes of the variant Lys832Arg (rs1061472), forward strand. A: homozygous mutant type allele (CC), B: heterozygous allele (CT), C: homozygous wild type allele (TT).

Similarly, the SNP rs768671894 (c.2519C>T, p.Pro840Leu) appeared in three genotypes (GG, AG and AA, figure 3-8).8 homozygous mutant genotype and 2 heterozygous genotype, while all healthy control groups had homozygous wild type genotype. This fact reflects the importance of this polymorphism as an effective variant in the etiology of the disease among Iraqi population. Among the available researches, very rare reports which pointed out this variant in the analysis of mutations of *ATP7B* gene in WD patients. This variant was previously reported by Zali *et al.*, (2011) in one Iranian patients with WD. Interestingly, Dong *et al.*, (2016) did find this variant neither in WD patients nor in healthy control of Chinese population.

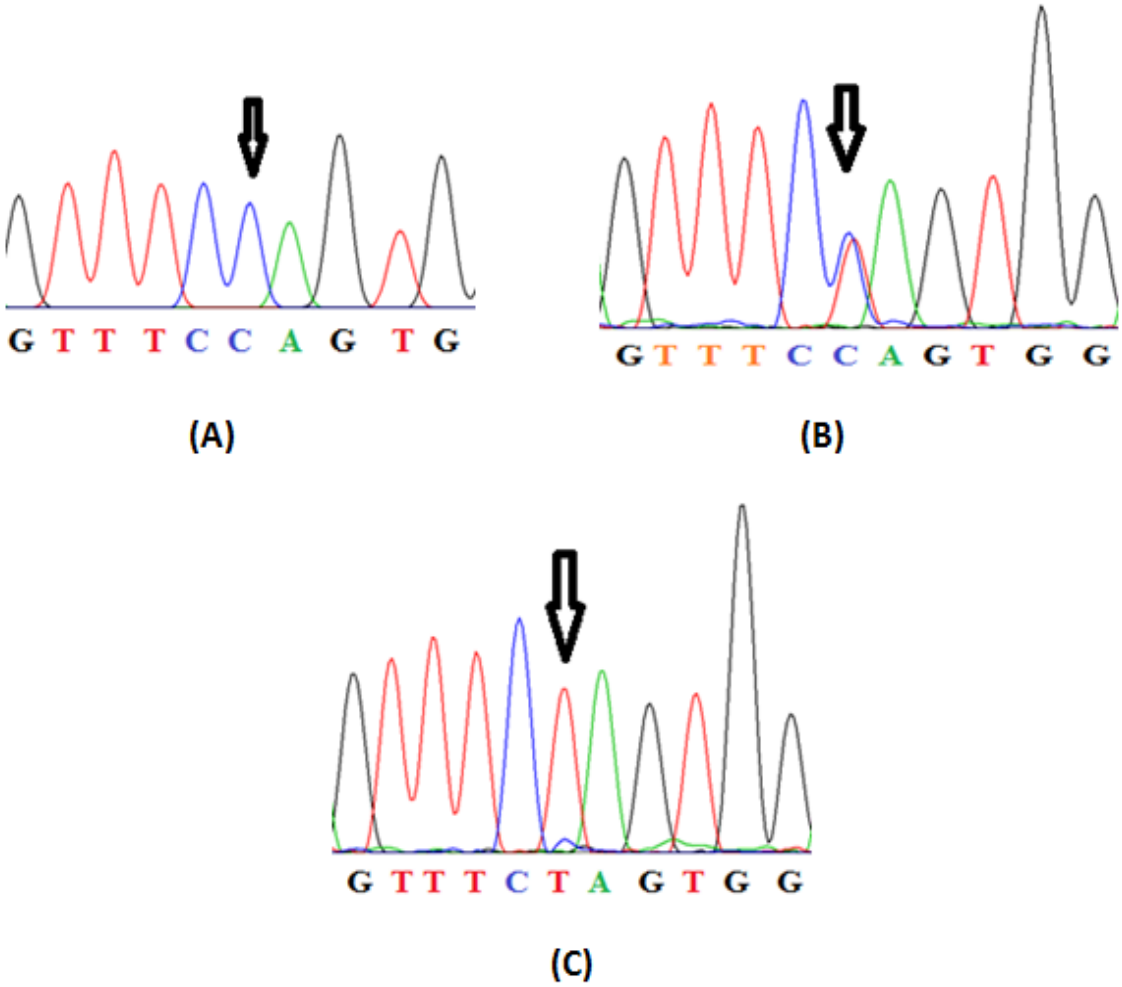

Figure 3-8: Different genotypes of the variant Pro832Leu (rs768671894), reverse strand A: homozygous wild type allele (CC), B: heterozygous allele (CT), C: homozygous mutant type allele (TT).

This variant lies in the transduction domain which converts energy from ATP hydrolysis to cation transporter. It is involves the substitution of proline with leucine. Poline is a very unusual amino acid, in that the side chain cyclizes back on to the backbone amide position. Proline's side chain has characteristic cyclic structure that bestows proline distinguishing conformational rigidity in comparison with other amino acids. It is because of this property of proline and the position of the variant, it can be postulated that the substitution of proline with leucine

reduce the ability of protein to bind ATP and eventual reduction in the ATP7B protein transport the copper outside the manufacturer's hepatocyte. This assumption was previously proposed by Raj and Stanley, (1995) who demonstrated that the ATP binding activity of viral protein was significantly weakened by the absence of proline in its ATP-binding domain.

The synonymous single nucleotide polymorphism rs1801246 (c.2973G>A (p.Thr991Thr) appeared in heterozygous form in only three patients, with the other patients and controls carrying wild type homozygous genotype (figure 3-9).

This variant was recorded by Khan *et al.* (2012) in four out of 90 Indian patients with WD. Approximately; similar frequency was recorded by Dong *et al.*, (2016) in Chinese patients. Other researchers in as many as 10 countries also reported this SNP (Gupta *et al.*, 2007).

As different forms of this variant do not cause amino acid substitution and the minor frequency allele has very low frequency, the variant likely does not have neither etiological nor diagnostic importance among Iraqi patients.

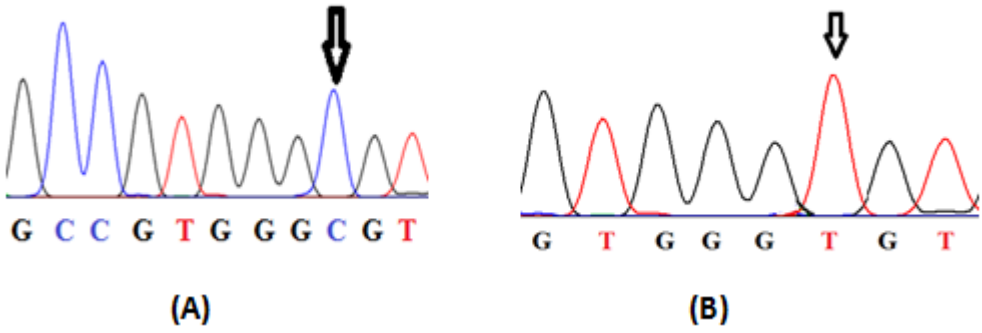

Figure 3-9: Different genotypes of the variant Thr991Thr (rs1801246), forward strand A: homozygous wild type genotype (CC), B: homozygous mutant genotype (TT).

The mutation c.3008C>T (Ala1003Val) affected three patients all of whom were homozygous (figure 3-10).

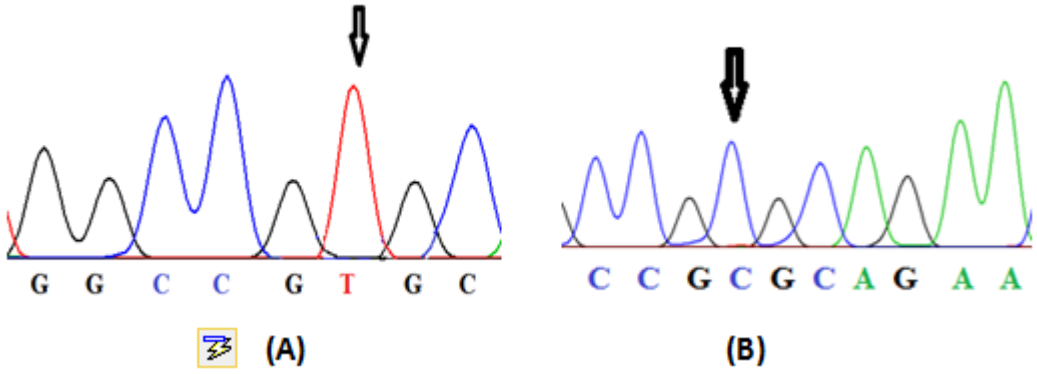

Figure 3-10:c.3008 C>T (Ala1003Val), forward strand A: homozygous mutant (TT), B: homozygous wild type(CC).

This mutation is among the most prevalent variant associated with WD world-wide. It was previously found by Laudianos *et al.*, (1999) in 1% of Turkish patients and by Santhosh *et al.* (2006) in Chinese patients. Recently Dong *et al.* (2016) found this mutation in 51 out of 632 Chinese patients with WD. The mutation lies within the ATP phosphorylation domain of ATP7B protein. The amino acid substitution implied in this mutation was predicted to be very deleterious for the protein function. Alanine may be the most sluggish amino acid. It has no particular hydrophobic character and is non-polar. Side chain of alanine id highly non-reactive and it seldom participates in protein function. However, it has a role in the recognition of substrate and in protein specificity especially in conditions that involve interaction of alanine with the other non-reactive particles like carbon (Matthew *et al*., 2003).

On the other hand, valine prefers to be embedded in hydrophobic center of the protein. While the vast majority of amino acids have just one non-hydrogen constituent linked to the Cβ carbon, valine has two .This

implies more bulkiness neighboring the protein backbone which influences the ability of the main chain of this amino acid for adoption into different conformations. Probably the most prominent effect of this is that it is very hard for this amino acid to take anα-helical conformation. Due to these differences between the two amino acids, it is reasonable to suppose some deterioration effect of valine on the activity of ATP7B protein in the phosphorylation of ATP and then the deposition of copper inside the hepatocytes.

The mutation (c.3029A>G Lys1010Arg) appeared in four WD patients all of whom were homozygote (figure 3-11).

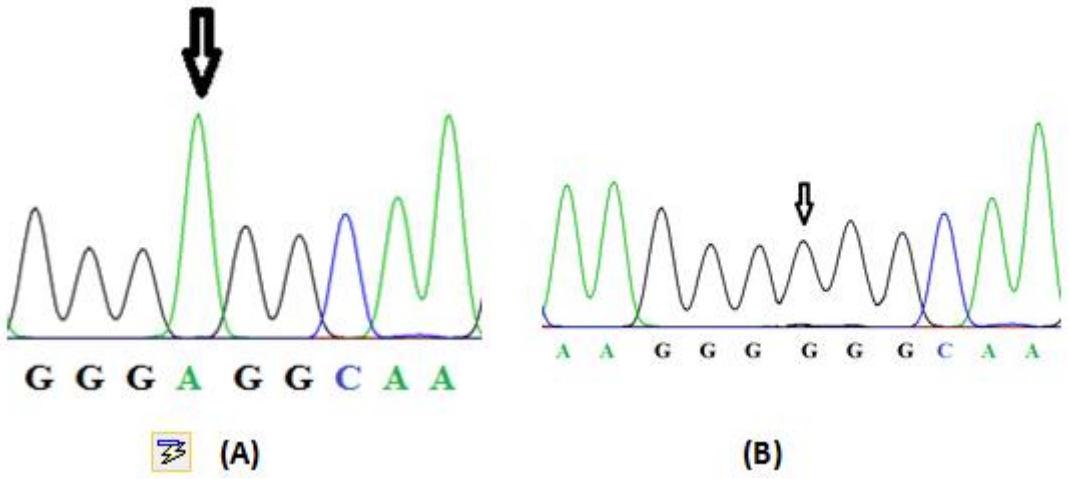

Figure 3-11: c.3029A>G, forward strand A: homozygous mutant (TT), B: homozygous wild type (CC).

This mutation was previously reported by Santhosh *et al.* (2006) and Gupta *et al.* (2007) in South Indian hepatic patients, while Dong *et al.*, found it in 16% of the Chinese patients with WD.

Under normal physiological conditions, both arginine and lysine bear positive charge (Yokota *et al.*, 2006). The two amino acids achieve crucial function in protein stability through forming hydrogen bonds and ionic interactions in the proteins and by their ability to interact with water

molecules (Strickler *et al.*, 2006).Despite their similar function as basic residue, lysine and arginine differ in supporting protein stability. Due to its geometric structure, arginine boosts protein structure more stability than lysine does. The presence of guanidinium group in arginine facilitates the interactions in three possible directions owing to its three asymmetrical nitrogen atoms. On the other hand, only one interaction direction is permitted by the basic functional group of lysine (Donald *et al*., 2011).This feature allows arginine to participate in a huge numbers of electrostatic interactions like hydrogen bonds and salt bridges compared to lysine. Thus arginine forms stronger interaction than the generated by lysine (Chan *et al*., 2011). Beside the geometric virtue, the ionic interaction formed by arginine is thought to be more stable than those formed by lysine especially in alkaline medium (Turunen *et al*., 2002).Sokalingam *et al.*, (2012) showed that the mutation of lysine to arginine could alter the electrostatic interaction in a manner that enhances the stability. Mutation of this kind can unfavorably affect the protein folding. This may explain partially the defect of ATP7B protein function in the phosphorylation of ATPase.

This study involved two novel mutations. The first one is c.2977-2978insA (figure 3-12). This frameshift mutation affected 17 patients in heterozygous pattern, while 2 healthy controls also affected in the same manner. Thus, it seems that this insertion has neither diagnostic norhasetiological effect on the disease.

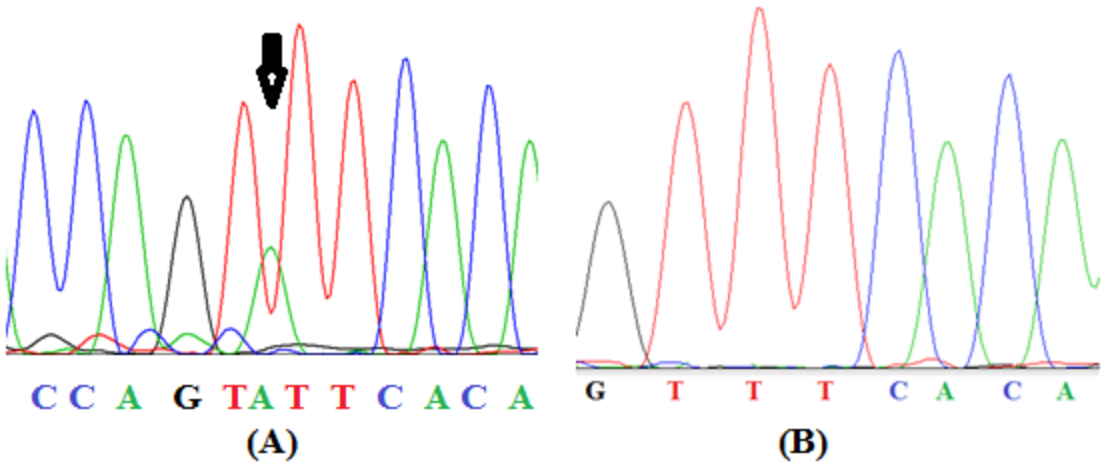

Figure 3-12: (A): c.2977-2978insA, (B): normal sequence

The other mutation is c.2456A Del (figure 3-13). The most prevalent mutation affecting 19 WD patients and absent from healthy control. It involves a deleterious effect on the transduction domain which converts energy from ATP hydrolysis to cation transporter, and therefore it may be considered as one of the important mutation associated with the disease. Furthermore, it may be used in combination with other prevalent mutation in order to establish a molecular test for diagnosis of the disease. More, test is needed to determine which mutations are the most prevalent.

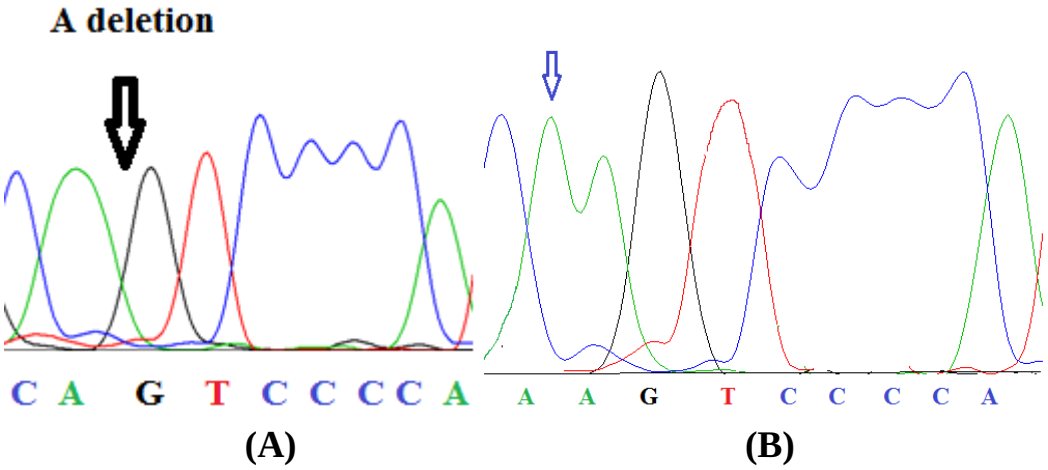

Figure 3-13: (A): c.2457delA, (B): normal sequence

Chapter Four

Conclusions and Recommendations

4.1. Conclusions

1. Neither clinical symptoms nor routine laboratory investigations could be reliable method for confirming WD.
2. The vast majority of mutations in exon 10 and exon 13 of *ATP7B*gene are heterozygous which confirmed the notion that WD is caused by compound heterozygous mutations.
3. Polymorphism and frameshift mutations and the characteristics of exon 10 mutations in Iraqi patients with WD.
4. The frameshift mutation C.2457 A Del in the most prevalent are among Iraqi patients with WD and can be utilized in combination with other most prevalent mutations for molecular diagnosis of WD.

4.2. Recommendations

1. Conducting other studies for detection of the most prevalent mutations in the other exons of *ATP7B* gene in WD patients in order to establish a molecular test for early detection of the disease.
2. It would be more interesting to establish a genetic center for genetic study in Iraq responsible for registration of results of such studies, and provides information for researchers for unification of all efforts in this field.
3. Encourage Iraqi population through different media to avoid consanguineous marriage to reduce recessive autosomal disorder.

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الخــلاصة

مرض ويلسون هو احد الامراض الوراثية النادرة والناتجة من اضطراب وراثي جسمي متنحي يؤدي الى حصول طفرات في الجين المسؤول عن نقل النحاس (*ATP7B*) وحصول علامات كبدية وعصبية نفسية . هدفت هذه الدراسة الى التقصي عن مدى اسهام الطفرات في جين (*ATP7B*) في تطور مرض ويلسون وتقيم المؤشرات المتعلقة بأيض النحاس في المرضى المصابين بهذا المرض .

جمعت عينات دم من ٣٥ مريضا (٢٠ ذكر و ١٥ انثى) وبمتوسط عمر ٩.١٢ سنة وبمدى ٥ – ٤٠ سنة فضلا عن ذلك فقد شملت الدراسة ١٥ اشخاص سليمين ظاهريا.

تم قياس المستويات المصلية لكل من (ALT, AST, ALP) وكذلك البيليروبين والتي كانت (٢٣.١٢±١٣٢.١٤ وحدة/لتر ، ١١.٢١± ٩٦.٩٣وحدة/لتر ، ٤٨.٤٥±١٨٤.٢٩وحدة/لتر و ١.١٣±٣.٦ ملغرام/ديس لتر) على التوالي. والتي اختلفت معنويا عن نظريتها في مجموعة السيطرة . تتراوح مستويات النحاس في المريض (٧٨ – ٢١٤ مايكروغرام /ديس لتر) وبمتوسط (٦٤.٤٣± ١٢٧.١٨ مايكروغرام/ديس لتر) ولم يختلف هذا المعدل عن مجموعة السيطرة .

تضمنت الدراسة فحص ٧٠ اليل لـ ٣٥ مريض بمرض ويلسون و ٢٠ اليل لافراد طبيعين للكشف عن الطفرات في exon 10 و exon 13 للجين *ATP7B* .سجلت ٧ طفرات مختلفة ثلاثة منها هي تعدد الاشكال الجينية وهي :(Lys832Arg, Pro840Leu, Thr991Thr) بنسب تكرار (٢٢.٨٦%، ٢٥.٧١ %، ٤.٢٩%) على التوالي. وكانت هناك طفرتان نقطيتان هما: (Ala1003 Val, Lys1010Arg) بتكرار (٨.٥٧% ، ١١.٤٢%) على التوالي ، فيما سجلت طفرتان ازاحيتان هما : (c.2457delA, c.2977-2978insA) وبتكرار (٢٤.٢٩% ، ٢٧.١٤) على التوالي في مجموعة المرضى .

تشير هذه النتائج الى كل من تعدد الاشكال الجيني c.2519C>T والطفرة الازاحية c.2977-2978insA يمكن استغلالهما في تطوير فحص جزيئي للكشف عن المرض ومع ذلك هنالك حاجة للمزيد من الدراسات للكشف عن الطفرات الاكثر شيوعا في الاكسونات الاخرى .

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة النهرين

كلية العلوم

قسم التقانة الاحيائية

الكشف عن الطفرت في اكسون ١٠ و ١٣ لجين *ATP7B* بين المرضى العراقيين الذين يعانون من مرض ويلسون

رسالة ماجستير

مقدمة الى مجلس كلية العلوم/جامعة النهرين كجزء من متطلبات نيل درجة الماجستير علوم/ تقانة احيائية

من قبل

اهداف عبدالرحمن جميل

بكلوريوس / علوم حياة / احياء مجهرية (٢٠٠٦)

بأشراف

الاستاذ

الدكتور صبحي جواد حمزة

محرم ١٤٣٨ تشرين الاول ٢٠١٦