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Research article

MUTATION SCREENING OF MODIFYING GENES (ATOX1 AND COMMD1) IN ASSOCIATION WITH WILSON DISEASE.

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ABSTRACT: Wilson disease (WD) results from accumulation of copper and is caused due to mutations in ATP7B, a copper transporting ATPase. Although WD is an established monogenic disorder, heterogeneity in phenotype is observed even among patients harboring mutations in ATP7B that would affect the mutant protein similarly. Such observations led to the speculation that there might be modifying loci that modulate the phenotype resulting from the aberration in the ATP7B gene. The expected genes coding for proteins that interact either directly with ATP7B or influence it indirectly might fit the role of modifier locus. ATOX1 and COMMD1 are the candidate genes that might play the role of a modifier locus having copper homeostasis pathway with such potential. To understand the role of modifying genes, we screened ATOX1 and COMMD1, a gene implicated in canine copper toxicosis, in 45 WD patients along with 50 healthy controls. This study did not yield satisfactory results concluding more patients to be analyzed.

Keywords: Wilson Disease, ATOX1, COMMD1.

Abbreviations: ATP7B-Adenosisne Triphosphate 7B, WD-Wilson Disease, COMMD1-COMM domain-containing protein 1, Metal Binding Sites/Domains-MBS/MBDs, CPC- (Cys-Pro-Cys)

INTRODUCTION

Wilson disease is a genetic disorder of copper metabolism. The disease is caused by the dysfunction of a liver enzyme or protein that transports copper (copper-transporting P-type ATPase) that has a crucial role in copper elimination from the body by excretion into bile. The gene encoding this protein, ATP7B, is located on the chromosome 13 and there are numerous gene mutations that can impair the protein's function, leading to copper accumulation mainly in the liver, but also in the brain, eye (cornea) and kidney (Medici V et al., 2006).

ATP7B contains the following functional domains: six-copper binding domains, a transduction domain, a cation channel and phosphorylation domain, a nucleotide binding domain and eight hydrophobic transmembrane sequences, in one of which (region 6) the Cys-Pro-Cys sequence is found in all P-type ATPases (Figure 1). Sequence MXGDGXNDXP connects the ATP binding domain to the transmembrane segment. The metal binding sites of ATP7B are specific for copper and stoichiometric analysis showed that six moles of copper were bound to one mole of the N-terminus containing the six metal binding regions of the protein. Each domain comprises approximately 70 amino acid residues and share 20–60% sequence identity; and each contains the highly conserved metal binding site (MBS) GMxCxxC (Fontaine LS and Mercer JFB, 2007).

The present study was aimed to evaluate the molecular factors (mutational analysis) of modifying genes (ATOX 1 and COMMD1) associated with Metal Binding Sites (MBS) of Wilson Disease gene, ATP7B.

The six N-terminal metal-binding sites(GMTCXXC) are required for trafficking and are essential for the copper transport function, however it has been suggested that the first three N-terminal motifs are not required for copper dependent intracellular tracking and cannot functionally replace sites 4-6 when placed in the same sequence position. The low level of similarity of the MBD's together with the biochemical and genetic data obtained so far make it clear that the individual MBD's are not equivalent.

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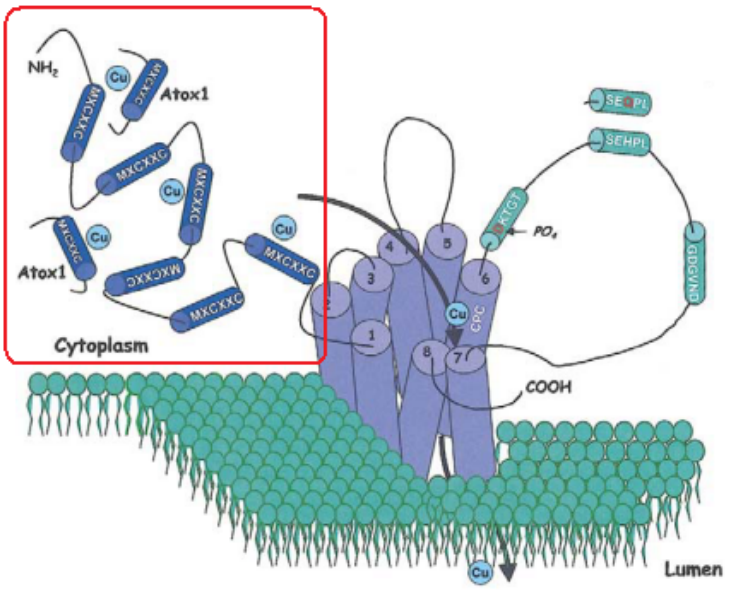

Figure 1: Structural model of the WD copper-transporting ATPase, ATP7B, illustrating ATP-dependents copper transport across the lipid bilayer. Highlighted amino acids regions include MXCXXC copper-binding regions, the DKTGT-containing site of the aspartyl phosphate, the GDGVND ATP-binding site and CPC copper-binding region in the sixth transmembrane domain. The red bordered box represents six Metal binding domains along with ATOX1 molecules. *(Source- Wilson Disease, Jonathan D Gitlin, Gastroenterology 2003;125:1868–1877).*

One study reported that MBD's 1–4 appear to be important for interaction with a modifying gene, ATOX1, and it has been proposed that these domains may serve a regulatory function. One proposal is that copper is transferred from MBD 4 to MBD5-6 which then shuttle it to the intramembrane CPC site. In contrast, mutagenesis experiments carried out for both ATP7A and ATP7B showed that while mutation of all six CxxC motifs completely abrogated any detectable copper-dependent trafficking activity, only MBS 5 or 6 were required for trafficking and they could not be replaced by any of the other sites (Fontaine LS et al., 2007).

Studies involving structural analysis of the N-terminus have revealed that both secondary and tertiary structural changes take place after the binding of copper and that copper coordination induces phosphorylation of ATP7B, which coincides with the trafficking of protein to vesicular compartments (Terada K et al., 1998, Behari M and Pardasani V, 2010).

Several motifs found in all P-type ATPases are present in ATP7B, including an invariant aspartate residue that is the site of the aspartyl phosphoryl intermediate required for adenosine triphosphate-dependent copper transport across the lipid bilayer. Copper transport requires metal transfer from the amino-terminus to a high-affinity site in the transmembrane channel, accompanied by adenosine triphosphate binding and aspartate phosphorylation. The MXCXXC motifs in the amino-terminus of ATP7B are the site of modifying genes (ATOX1,COMMD1) interaction and subsequent copper binding. The most common disease allele found in Northern European populations with WD is an H1069Q missense mutation found within a conserved SEHPL motif in the cytoplasmic loop between the fifth and sixth transmembrane domains. This mutation results in a temperature-sensitive defect in ATP7B folding and copper-dependent trafficking, suggesting a potential role for this motif in the intracellular localization of ATP7B.

Interaction of ATP7B protein with other proteins

Identification of protein–protein interactions is a powerful tool to understand protein function and how perturbation of protein function results in human disease. It is probable that molecule as complex as ATP7B will require an extensive network of tightly regulated and coordinated protein interactions to facilitate and modulate their activities. It is clear that this protein has multiple sequence and structural motifs that are critical for their copper transport and trafficking activities, but the proteins that act upon and interpret these signals have been identified.

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Although WD is an established monogenic disorder, heterogeneity in phenotype is observed even among patients harboring mutations in ATP7B that would affect the mutant protein similarly (e.g.different truncation mutations in the same region of the gene or exactly same set of mutations in two sibs). Such observations led to the speculation that there might be modifying loci that modulate the phenotype resulting from the aberration in the ATP7B gene. One would expect that genes coding for proteins that interact either directly with ATP7B or influence it indirectly might fit the role of modifier locus. The N-terminal domain is also involved in directing ATP7B to the appropriate intracellular compartments during copper-induced relocalization and is the site of interaction with various proteins, implicated in regulation of protein activity and trafficking (Bost M et al., 2012). Few of the candidate genes that might play the role of a modifier locus having copper homeostasis pathway with such potential includes copper-chaperone ATOX1, COMMD1/COMMD1, Clusterin(ApoG), Dynactin subunit p62 and Glutaredoxin (GRX1), a 45kDa isoform of the promyelocytic leukemia zinc finger (PLZF) protein, Golgi glycoprotein GP73. Although the functional consequences for most of these interactions remain to be characterized, the diversity of interacting proteins emphasizes the complexity of regulatory processes mediated by the N-terminal domain (Bartee MY and Lutensko S, 2007) and in patients with the apolipo protein E ε3/3 genotype, the onset of symptoms is sometimes delayed (Das SK and Ray K, 2006).

Recent evidence suggested that a reduction in the protein, X-linked inhibitor of apoptosis (XIAP) or the antiapoptotic protein baculoviral IAP repeat-containing protein 4 (BIRC4), induced by copper elevation, results in acceleration of caspase 3–initiated apoptosis with resultant cell death (Pfeiffer RF, 2007) So this protein is considered as a modifier gene in individuals with copper overload (Weiss KH et al., 2010).

Modifying factors may also play an additional role in the timing of disease onset, clinical phenotype and maybe even in the response to treatment in Wilson disease (Deguti MM et al, 2004). To study the molecular basis of such phenotypes, this study screened for mutations in ATOX1 and COMMD1 from genomic DNA and analyzed coding sequences in several relevant patients and healthy controls.

ATOX1

ATOX1 was the first protein shown to interact in a copper-dependent manner with the N-terminal domain of both proteins for delivery of copper to the secretory pathway. As already indicated, MBSs 1–4 seem to be most important for this interaction, in particular MBS 2 and 4. The metallochaperone ATOX1 [protein coded by the gene located on chromosome 5 (5q33.1)] interacts directly with ATP7B and can regulate its copper occupancy. This gene plays a role in the pathophysiology of Wilson Disease.

The cumulative findings of many studies so far suggest that the ATOX1 and ATP7B gene products are each integral to mammalian copper transport, and that mutations in either could produce similar clinical and biochemical phenotypes. To study the molecular basis of such phenotypes, this study screened for mutations in ATOX1 from genomic DNA, and analyzed coding sequences in several relevant patients. Characterization of the genomic structure of ATOX1 enables rapid and efficient mutation screening from genomic DNA. Identification and clinical/biochemical delineation of patients in whom alterations in ATOX1 are found will permit better understanding of the significance of this gene product in human trace metal metabolism. In addition, functional polymorphisms in ATOX1 may be relevant in terms of phenotypic modifier effects in the WD (Liu PC et al., 2003). It has also been shown that mutations that impair the interaction of ATP7B with ATOX1 are associated with Wilson disease (Hamza I et al. 2001), suggesting that ATOX1 plays an essential role in this mechanism.

COMMD1

The human COMMD1 [protein coded by the gene located on chromosome 2 (2p13-p16)] interacts with the N- terminal domain of ATP7B. COMMD1 (previously known as COMMD1) is a member of a family of COMMD proteins and is the protein product of the gene found to be defective in Bedlington terrier dogs affected by liver copper toxicosis. COMMD1 was shown to interact directly with the N-terminal domain of human ATP7B (Fontaine LS et al., 2007).

Mutations in COMMD1 are responsible for copper toxicosis in an inbred canine strain (Bedlington terriers) and an interaction between COMMD1 and the copper transporter ATP7B has been reported (Burstein E et al., 2005). The hepatic copper overload in Bedlington terriers with a homozygous deletion of the second exon of COMMD1 implies a critical role of COMMD1 in the regulation of copper excretion. In addition, knockdown of COMMD1 by RNA interference in several cell lines results in increased cellular copper levels.

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COMMD1 interacts with the copper transport protein ATP7B, which is mutated in WD, suggesting that these two proteins cooperate in the excretion of copper. Consequentially, these observations suggest that COMMD1 could play a role in the development of WD. Consistent with this notion, heterozygosity for a silent missense mutation (D164D) in COMMD1 was possibly associated with an earlier onset of the disorder in patients with known ATP7B mutations. Several WD causing mutations markedly increased the amount of ATP7B that interacted with COMMD1, suggesting that deregulation of this interaction is associated with the development of WD. In addition to previously reported effects of WD causing mutations on ATP7B-mediated copper transport, cuproenzyme biosynthesis, ATP-binding and hydrolysis, localization and copper-induced trafficking, post-translational modifications, and protein-protein interactions, this observation provides a novel molecular mechanism in the pathogenesis of WD.

By biochemical evidence, COMMD1, a protein identified through genetic studies in dogs with copper toxicosis, shows a markedly increased interaction with ATP7B affected by distinct mutations and suggests that this increased interaction contributes to the molecular basis of WD in patients harboring such mutations. It is also indicated that COMMD1 regulates ATP7B stability, rather than copper-induced trafficking or interactions with ATOX1. Interestingly, the interaction between COMMD1 and ATP7B is markedly increased when WD associated mutations of ATP7B are present. These mutations are associated with misfolding, mislocalization of ATP7B to the endoplasmic reticulum, and decreased protein expression due to increased proteasomal degradation.

These observations initially led to the suggestion of COMMD1 as a candidate gene for human copper overload disorders. However, no disease-causing mutations in COMMD1 have been detected in several cohorts of patients with WD, Indian Childhood Cirrhosis (ICC), Endemic Tyrolean Infantile Cirrhosis (ETIC) or Idiopathic Copper Toxicosis (ICT) (de Bie et al, in press).

PATIENTS AND METHODS

Patients: We examined 45unrelated WD patients. In the absence of a Kayser–Fleisher ring, hepatic or neurological symptom, the diagnosis of WD was based on the presence of at least two abnormal biochemical tests among which: raised urinary copper excretion (>15-50µgs/dL), decreased serum ceruloplasmin (<20 mg/dL) and serum copper levels (>65-165µgs/dL). Patients were classified according to the WD form: liver form (37 patients) neurologic form (1 patient) and combined form (7 patients).

The patients were registered at the Institute of Genetics and Hospital for genetic Diseases, Asian Institute of Gastroenterology, NIMS and Gandhi Hospital. All patients and a control group of 50 healthy individuals (without any WD symptoms) were requested to sign an informed consent form.

Methods

DNA extraction: Genomic DNA was extracted by Phenol-Chloroform-Ethanol extraction according to the Blin and Stafford method. The quantification of each sample was measured at 260nm in a UV-Visible spectrophotometer.

Mutation detection in ATOX1 and COMMD1 genes:

The three exons each of the ATOX1 gene and COMMD1 gene including the intron–exon boundaries were amplified by PCR, using the oligonucleotide primers and PCR conditions described by Liu P-C et al., 2003 and Gupta A et al., 2010 respectively (Table 1). PCR was carried out for each fragment in a total volume of 25 µL using 1.5 mM of $MgCl_2$ with 35 cycles of denaturation at 94°C (30s), appropriate annealing temperature (varying between 55°C and 62°C) for 30s and an extension temperature of 72°C (30s).

Table 1: The PCR primer sequences for scanning ATOX1 gene (Po-Ching Liu, 2003) and COMMD1 gene in WD patients (Gupta A, 2010).

Exon	Forward primer (5'- 3')	Reverse primer (5'- 3')	Size
ATOX1			
1	AGGCGCTGCTGACACCGCCG	TTCAAGATCAGCATCCGGT	151
2	AGGCTTCTGATGAGTCTGAT	TCTGCATGCATCTGAACAT	273
3	TGAGTAGTAATTTAGAGCCT	AGGTGTTCGCTCTGATGAG	327
COMMD1			
1	GTGGTGGTTTTGCACAGGC	TCCAAGCCGGAGACTACAG	301
2	TTCAGTGATTTAAGAGTCA	GAATAGACAAGCTAACATG	456
3	GAGTTTGGTCATGCCAGAT	GTGAGAACCTCTGCACTGG	379

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The primers were procured from Eurofins, Bangalore. The Dream Taq Green DNA Polymerase and $MgCl_2$ were provided by Fermentas, New Delhi. The PCR products, free of contaminating bands due to non-specific amplification, were Silica gel purified using Bangalore genei PCR-purification kits.
Direct bi-directional sequencing was performed on an AB 3730 Genetic Analyzer, using Gene Mapper software (Applied Biosystems). Sequences obtained by sample sequencing were compared with the normal ATOX1 gene sequence NM 004045.3 and normal COMMD1 gene sequence NM 152516.2 (http://www.ncbi.nlm.nih.gov/BLAST).

RESULTS

The study group consisting of 67(60.9%) males and 43(30%) females were divided into three sub-groups, Heaptic, Hepato-Neuro and one Neuro case depending upon the type of disease proliferation. 45 Wilson disease patient samples and 50 healthy controls samples were subjected to PCR and bidirectional sequencing. This study has not yielded any variations in both the genes. The chromatogram in exon 3 of COMMD1 gene has shown the normal sequence for a non-synonymous mutation c.521 ACG>ATG (Figure 2) as detected by Gupta A et al in 2010.

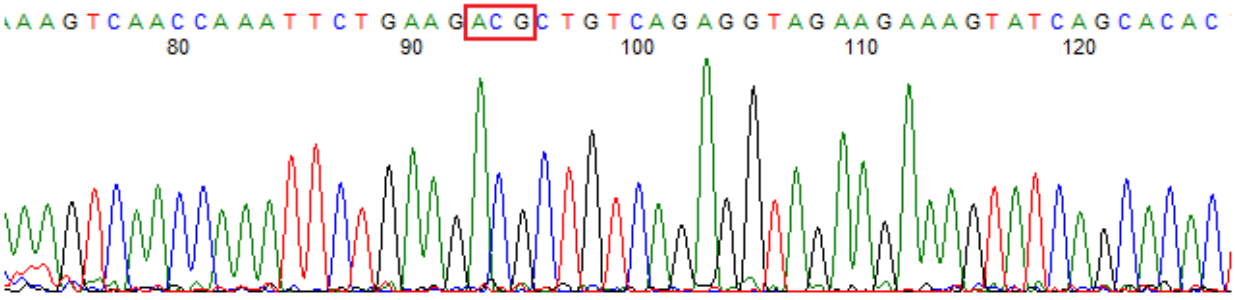

Figure 2: Chromatogram of exon 3 of COMMD1 gene showing the normal sequence, ACG at the location c.521. The non-synonymous mutation detected in COMMD1 in Indian patients is located at this site c.521 ACG>ATG (p.Thr174Met).

DISCUSSION

This would be the second study in India analyzing modifying factors, ATOX1 and COMMD1. The other study center studying the modifying gene mutations in India was by Gupta A et al, 2010, India.
Studies involving structural analysis of the N-terminus have revealed that both secondary and tertiary structural changes take place after the binding of copper and that copper coordination induces phosphorylation of ATP7B, which coincides with the trafficking of protein to vesicular compartments (Terada K et al., 2008, Behari M and Pardasani V, 2010).
Po-Ching Liu et al., in 2003 by their cumulative findings suggest that the ATOX1 and ATP7B gene products are each integral to mammalian copper transport, and that mutations in either could produce similar clinical and biochemical phenotypes. Previous studies indicated that the G85V and G591D mutations markedly impair the interaction of ATP7B with ATOX1 (de Bie P, 2007).
Recently Ferenci P et al found no mutation of the ATOX1 gene in 80 patients with WD in whom no mutation of ATP7B was detected (Ferenci P et al, unpublished observations). Simon I et al in 2008 analyzed ATOX1 gene in 63 Wilson disease patients by direct sequencing. They did not find any alterations in the ATOX1 coding exon sequence or splice junction sequence be detected in any of the individuals. Direct sequencing of the ATOX1 gene within the 5'-UTR region located before exon 1 examined revealed one known polymorphism within the ATOX1 gene in 31 (49%) of the Wilson patients. The change detected was a heterozygous T/C at 5'UTR -99. In few patients, previously undescribed 5'UTR -68 C>T (heterozygote) genetic variation was detected.
Gupta A et al screening COMMD1 in 109 Indian patients failed to establish a causal relationship of the COMMD1 variant with specific disease phenotype. They identified three nucleotide changes. A synonymous change which has been reported in European population i.e., c.492 GAT>GAC (Asp164Asp) and a novel variant c.170+122C>T (IVS1+122C>T) which is a SNP and previously reported were detected. The third reported was a non-synonymous change (c.521 ACG>ATG; Thr174Met). This variant represents a novel change that occurred in exon 3 of COMMD1.

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This change resides in the region identified as the second 'Nuclear Export Signal (NES)' i.e., amino acids 168-179. This is the first mutation to be reported in COMMD1 which appears to influence pathogenesis of the patient involving elevated copper accumulation. It is likely that, mutation in COMMD1, by itself is not sufficient to cause a disease with a clinical manifestation. Alternatively, since COMMD1 is involved in multiple physiological processes, mutant COMMD1 on both the alleles might cause pre-natal fatality (Gupta A et al., 2010).

Recently, the GAT/GAC heterozygous state at codon Asp164 of the COMMD1 gene has been detected in 15 of 63 analyzed WD patients of European origin and suggested to be associated with an earlier onset of neurological symptoms. Wu ZY et al working on 218 Chinese patients could not distinguish any correlation between COMMD1 and WD patients. They found a rare polymorphism, heterozygous nucleotide change, 3′+119T→A was found in the 3′UTR. The previously reported IVS2+63C→G was also found but the Asn164 was not detected (Wu Z-Y et al., 2006).

Lovicu M et al. (2006) detected six nucleotide substitutions of COMMD1, of which four (180+4 C→T, S117S, R120W, and D164D) were present with similar frequencies in one or both of the control groups, indicating that they are most likely polymorphisms that do not affect the function of the protein and therefore lack any pathogenetic role. In addition, RNA studies showed that the consensus sequence 180+4 C→T and D164D (GAT→GAC) variations had no effect on the splicing process. Furthermore, D164D was also detected in the homozygous state in normal subjects, thereby definitively excluding the possibility of a pathogenic role for this substitution. This silent nucleotide substitution was previously reported to be associated with an earlier manifestation of WD.

But from their study D164D substitution showed no phenotypic variation between WD patients. The two remaining substitutions, G4R and F163F, were found in the heterozygous state in two different patients, one of which was affected by WD with only one detected mutation in the ATP7B gene and the other affected by undefined abnormal copper metabolism with no detected mutation in the ATP7B gene. Both mutations were absent in the control groups. The F163F is a silent substitution that does not lie in a DNA sequence suspected to influence the splicing process and therefore is most likely a very rare polymorphism not affecting gene function. The G4R mutation, in which a glycine is replaced by an arginine, is a nonconservative substitution that may affect the function of the COMMD1 protein. However, since in the Bedlington terrier the copper toxicosis state has an autosomal recessive transmission pattern, heterozygosity for the G4R mutation should not have any effect in humans, which can be clarified by functional assays for affecting the ATP7B-COMMD1 interaction, thereby negatively influencing copper metabolism. A silent G9G substitution and a nonconservative T87I missense substitution were also detected.

Stuehler B et al. 2004, found a correlation of early age of onset in patients harboring H1069Q homozygous mutation in ATP7B with the silent nucleotide change c.492T>C (D164D) in COMMD1. However in a study involving a heterogeneous cohort consisting of Eastern European and Mediterranean WD patients, no correlation was found between the change c.492T>C and the age of disease onset (Lovicu M et al,2006). Gupta et al, 2010 who reported first mutation in COMMD1 suggests that the COMMD1 variants do not contribute to great phenotypic heterogeneity observed in WD.

In conclusion for the mutational analysis done on COMMD1, the frequencies of the various substitutions were very low and similar in the analyzed categories, and no apparent association with Wilson disease was observed. These data suggest that the COMMD1 and its protein product are unlikely to play a primary role in the pathogenesis of Wilson disease. More extensive studies with larger and homogeneous DNA samples in combination with extensive clinical studies of positive cases as compared to control groups should be carried out to establish whether nucleotide alterations in the COMMD1 gene may have a role as modifying factors in explaining phenotype variability in WD. Furthermore, functional assays of the missense substitutions should help clarify their role in ATP7B-COMMD1 protein interaction (Lovicu M et al., 2006).

This study revealed no mutations of the ATOX1 and COMMD1 gene analyzed in patients with WD. It is worth mentioning here that in 45 patients studied, this study did not find any nucleotide variant in COMMD1 and ATOX1 worthy not to be mentioned as putative modifying genes. Study in more patients in order to analyze ATOX1 and COMMD1 variants can be done which may have major contribution towards phenotypic heterogeneity observed in WD.

All this information would greatly help genetic counseling for WD families, carrier detection and prevent the clinical manifestations of the disease in asymptomatic individuals.

CONCLUSION

In this study, we did not find any variations in the exons of ATOX1 and COMMD1 genes. More number of patients are needed to be screened.

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