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Mutation Profile in Wilson's Disease from North Indian Patients

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ABSTRACT Wilson disease (WD) is an autosomal recessive disorder caused by defects in the ATPase, Cu2transporting, polypeptide gene (*ATP7B*) resulting in accumulation of copper in liver and brain. The study was conducted in the Department of Neurology, of a tertiary care center in India from 2004 to 2009. DNA samples of 90 WD patients, their unaffected first degree relatives and 90 unrelated healthy controls were analyzed for mutations in ATP7B gene *(ATP7B)*. The researchers screened the entire coding region of *ATP7B*. The desired sequence of the gene was amplified using PCR followed by SSCP. Samples showing shifts in the banding pattern on SSCP were sequenced commercially. Out of 90 WD patients 41 variations in 55 WD patients were observed in DNA samples for *ATP7B*. Of these 24 patients were homozygous, 6 were compound heterozygous and 25 were heterozygous. Of these 6 were known mutations and the rest 33 was novel. The researchers observed exon 2, 13 and 18 as hot spot exons of *ATP7B* with large number of variations. Lack of common dominant mutations prevented correlation of individual mutations with WD phenotype. The researchers did not observe common mutations reported in *ATP7B* in other countries.

INTRODUCTION

Wilson's Disease (WD), a progressive hepatolenticular degeneration was first described by Dr. Samuel Alexander Kinnier Wilson in 1912 (Wilson 1912) as familial, lethal neurological disease accompanied by chronic liver disease leading to cirrhosis (Diane and Eve Roberts 1995). Untreated, it is invariably fatal, however, all the clinical manifestation can be resolved if treated early (Sternlieb and Scheinberg 1968; Cartwright 1978). The disease is diagnosed on the basis of clinical symptoms and conventional biochemical parameters, which include low serum concentrations of ceruloplasmin, increased excretion of urinary copper, and presence of the Kayser–Fleischer (KF) ring (Sternlieb et al. 1990). It has been documented that the biochemical defects related to copper disturbances in WD are present from birth but the symptoms of WD may not become apparent until late childhood or adolescence. For this reason many patients usually remain untreated until they manifest the symptoms. The available treatment regime makes WD different from other genetic disorders in that it can be completely cured. WD occurs in populations of every geographic and ethnic origin with demographic difference related to the consanguinity rate (Figus et al. 1985; Hoogenraad et al. 1996). Prevalence of WD has been estimated to be approximately 30 per million and frequency of heterozygous carriers one per 90 persons (Scheinberg and Sternlieb 1984). However, lower prevalence rates have been suggested between 1: 30,000 and 1 in 100,000 individuals in certain regions (Park et al. 1991). Heterozygous carriers neither develop the disease nor require specific treatment; they may exhibit mild abnormalities of copper metabolism, which can result in diagnostic confusion. Mutation in ATP7B gene can be detected in 90% of WD patients. 60% are homozygous for ATP7B mutations, 30% have only one abnormal copy while 10% have no detectable mutations (Merle et al. 2007). Over a period of 100 years, biochemical assays are well established for the diagnosis of WD, but the overlap in these parameters among the carriers and the non-carriers of the mutated allele requires a molecular diagnostic test to avoid any misdiagnosis. Identification of genetic mutations/variations in siblings of WD patient will help in identification of these patients before symptoms appear to enable us to start preventive and therapeutic medications. Also, carrier detection can be used for genetic counseling for those groups who practice consanguinity (Arnab et al. 2007).

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The gene for WD (*ATP7B*) was mapped to chromosome 13 on its long arm (Bull et al. 1993; Tanzi et al. 1993) and encodes a 165 kDa membrane protein. Prevalent mutations in *ATP7B* have been identified in many population groups (http: //www.medicalgenetics. med.ualberta.ca/wilson/index.php). Most WD mutations are rare, with a very low frequency that differs greatly from population to population.

The researchers report mutational profile among the north Indian WD patients, irrespective of age of onset, type of clinical manifestation and gender and observed a heterogeneous mixture of variations.

MATERIAL AND METHODS

The study was conducted in the Department of Neurology, All India Institute of Medical Sciences (AIIMS) from 2004 to 2009. Wilson's disease (WD) patients fulfilling the diagnostic criteria (Morrison et al. 2000; Sternlieb 2000) were recruited in the study after obtaining informed consent from the patients and/or their legal representatives. The study was approved by institute ethics committee.

Broadly, the diagnostic criteria suggestive of WD are clinical history and examination suggestive of chronic or acute liver disease and neurologic examinations suggesting progressive extrapyramidal syndrome, ataxia and neuropsychiatric features along with presence of KF ring, low serum ceruloplasmin levels (< 0.2 OD units) and high 24 hours urinary copper(> 50 µg/day) levels. Neuro-radiological investigations were performed in all WD cases

The researchers collected blood samples from 84 index cases. On screening of families, 6 cases were identified from 5 families of index cases making a total of 90 WD patients. Blood samples were also collected from 90 age and gender matched unrelated healthy controls for the study.

Collection of Blood Samples and Genomic DNA Preparation

The researchers collected 10 ml of venous blood samples in ethylenediamine tetra – acetic acid (EDTA) tube for DNA extraction and plain tube for biochemical investigations from all the subjects. The researchers prepared genomic DNA from fresh whole blood using the conventional phenol-chloroform method, followed by ethanol precipitation, after which the DNA was dissolved in TE buffer (10 mmol/L Tris-HCl, 0.1 mmol/L EDTA, pH 8.0) (Sambrook et al. 1989).

Amplification of Exons and Flanking Regions, DNA Sequencing, and Genotype

Exons and the flanking regions of WD gene from isolated DNA were amplified using Polymerase Chain Reaction (PCR). 100 µl reaction mixture contained 10Xconcentration of ready reaction mix, 2 µl of 5mM dNTPs, 1.5 µl primers (20pM), 1 µl of Taq polymerase. This reaction master mix was aliquoted into 25 µl each and 1.5 µl of genomic DNA (100ng) was mixed in each reaction tube respectively. The researchers used Single Strand Conformation polymorphism (SSCP) as a mutation screening tool (Orita et al. 1989). In brief equal volume of PCR products were mixed with the loading buffer and the mixture was denatured at 94ºC for 5-6 minutes and was snap- chilled on ice to quench reannealing of the ssDNA. The gel was preloaded with 2 µl of the sample prior to the denaturation. This step ensured that there was sufficient dsDNA to visualize heteroduplexes when they were present. The wells were re-loaded with 10 µL of each sample on a 12% Acrylamide-bisacrylamide gel (49: 1 ratio of cross-linking) having a length of 30-50 centimeters to visually differentiate the mobility difference between heteroduplex and homoduplex DNA. Electrophoresis was carried out at a constant voltage of 200 V, 50 mA and 15W for 20-22 hours and PCR products with band pattern different from that of healthy control were sequenced commercially after purifying them using PEG precipitation method (Rosenthal et al. 1993).

Statistical Analysis

The researchers detected novel nucleotide changes by comparing the sequence obtained in the chromatogram with the normal gene sequence (NCBI Reference Sequence: NC_000013.10; *Homo sapiens* chromosome 13 genomic contig) using pairwise Basic Local Alignment Search Tool (BLAST) which is a sequence similarity search program.

RESULTS

The study group consisting of 66 (73.33%) males and 24 (26.66%) females, were divided

Table 1: Clinical features of WD patients with respect to subtypes

S. No.	*Clinical features*	*WD patients n=90 (%)*	*Neurologic n=66 (%)*	*Hepatic n=19 (%)*	*Neuro-hepatic n=05 (%)*
1	Tremors	70(77.77)	66(100)	0 (0)	4 (80)
2	Dystonia	68(75.55)	66(100)	0 (0)	2 (40)
3	Dysarthria	52(57.77)	49 (74.24)	0 (0)	3 (60)
4	Dysphagia	58(64.44)	56 (84.84)	0 (0)	2 (40)
5	Drooling	71(78.88)	66(100)	0 (0)	5(100)
6	Seizures	12(13.33)	11 (16.66)	0 (0)	1 (20)
7	Psychiatric	2 (2.22)	2 (3.03)	0 (0)	0 (0)
8	Behavioral changes	28(31.11)	28 (42.42)	0 (0)	0 (0)
9	History of jaundice	58(64.44)	34 (51.51)	19(100)	5(100)
10	Pallor	0 (0)	0 (0)	0 (0)	0 (0)
11	Ascites	7 (7.77)	0 (0)	6 (31.57)	1 (20)
12	Cirrhosis	5 (5.55)	0 (0)	3 (15.78)	2 (40)
13	KF ring	86(95.55)	66(100)	15 (78.94)	5(100)

n = Number of Patients

into three sub-groups, Group A (neurologic n= 66), Group B (hepatic n=19) and Group C (neuro-hepatic n=05) depending upon the type of disease presentation. Table 1 shows the clinical profile of all WD patients with respect to the subgroups/types.

DNA analysis of the WD gene using SSCP and direct sequencing of the shifted bands enabled us to characterize 41 mutations, out of which six mutations have already been reported from different regions of the world (Table 2).

A very heterogeneous mutation profile was observed and the detail of all the mutations detected is summarized in (Table 2). The researchers observed 18 missense mutations, 8 silent mutations, 3 splice sites, duplication, 4 deletions and one nonsense mutation in the exons 1, 2, 8, 11, 13, 14, 15, 16 and 18, rest of the 4 mutations were observed in the nucleotide sequence before the start codon of the ATP7B gene in the exon 1 region (Table 2).

The researchers also observed ten homozygous mutations and five compound heterozygous mutations summarized in Tables 3.1 and 3.2. All the mutations observed were not present in any of the control samples

Genotype-phenotype Correlations

Out of the 41 mutations observed, 28 mutations were in the neurologic WD patients, two mutations were seen in neuro-hepatic WD patients and none of these mutations were found in any patient with hepatic WD. The rest of the 11 mutations were distributed in all the three WD subgroups. Of these, 3 mutations were present both in the neurologic and hepatic WD patients, 7 in both neurologic and neuro-hepatic WD patients and only 1 mutation was present in hepatic and neuro-hepatic WD patient. From this we see that certain mutations were observed in neurologic patients, either alone or in combination with hepatic patients. Only four mutations were observed in WD patients with hepatic manifestations either alone or in combination with neurologic manifestations. There were no mutations which were solely present in hepatic WD patients.

In this study all the mutations were observed in WD patients with neurologic involvement (alone or in combination with hepatic involvement). The reason for this could be that the majority of cohort in our study consisted of neurologic patients (78%). Although the hepatic group consisted of 22% (19 WD patients), still this number is rather small to observe any mutation which was confined only to the hepatic group of WD patients.

Other than the type of mutations observed, even the presentation of the mutation such as homozygous, heterozygous and compound heterozygous was more in neurologic WD patients. Again all the compound heterozygous mutations observed in our study were present only in the neuro-WD patients (Table 3.2).

DISCUSSION

In this communication, the researchers report the molecular analysis of the WD gene in Wilson's disease patients from north India. Using SSCP analysis followed by direct sequenc-

Table 2: Mutational profile

Nucleotide change	*Amino acid change*	*E*	*Type*	*Functional domain*	*Reference*	*Country*
c.-49C>G	-	1	-	-	Present Study	-
c.43A>G/	p.S14G	1	Missense	-	Present Study	-
c.- 75C>A	-	1	-	-	Present Study	-
c.- 75C>A	-	1	-	-	Present Study	-
c.- 128A>C	-	1	-	-	Present Study	-
c.108-27C>T fs	-	1	Splice site	-	Present Study	-
c.1252G>A	p.E417K	2	Missense	Cu4	Present Study	-
c.1255 G>A	p.D418N	2	Missense	Cu4	Present Study	-
c.1038T>C	(p.= P)	2	Silent	Cu4	Present Study	-
c.1186G> C	p.E395Q	2	Missense	Cu4	Present Study	-
c.1194T>C	(p.=T)	2	Silent	Cu4	Present Study	-
c.1195G>C	p.A398P	2	Missense	Cu4	Present Study	-
c.1216 T>G	p.S405A	2	Missense	Cu4	Present Study	-
c.37582_83insCA fs	p.C582_H583dup	2	Duplication	Cu4	Present Study	-
c.1163_64 insA	(p.=Q)	2	Silent	Cu4	Present Study	-
c.1216 T>G	p.S405A	2	Missense	Cu4	Present Study	-
c.1132 G>T	p.E377X	2	Nonsense	Cu4	Present Study	-
c.2267C>T	p.A756V	**8**	Missense	Tm3/4	Caprai et al. 2006	Italy
c. 2697-2723 del 27	-	11	Deletion	Td domain	Present Study	-
c.2730-32A>T	-	11	Splice site	Td domain	Present Study	-
c.2679_80insT	p.T893Y	11	Missense	Td domain	Present Study	-
c.2682_83insT	p.T894L	11	Missense	Td domain	Present Study	-
c.2973 G>A	(p.=T)	13	Silent	Tm6	Present Study	-
c.3012G>A	(p.=Q)	13	Silent	Tm6	Present Study	-
c.2930C>T	p.T976M	13	Missense	Tm6	Present Study	-
c.2976C>A	(p.=P)	13	Silent	Tm6	Present Study	-
c.2973 G>A	(p.=T)	13	Silent	Tm6	Present Study	-
c.3053C>T	p.A1017V	13	Missense	Tm6	Present Study	-
c.3008 C>T	**p.A1003V**	**13**	Missense	Tm6	Loudianos et al (1999)	Turkey
c.3026delTCA	-	13	Deletion	Tm6	Present Study	-
c. 3146del C fs	-	14	Deletion	Ph	Present Study	-
c.3182G>A	**p.G1061E**	**14**	Missense	ATP loop	Santhosh et al (2006)	India
c.3402C>T	(p.=P)	15	Silent	ATP loop	Present Study	-
c.3305 T>C	**p.I1102T**	**15**	Missense	ATP loop	Kumar et al (2006), Pandit et al (2002)	India
c.3301G>A	**p.G1101R**	**15**	Missense	ATP loop	Thomas et al (1995c), Eisen-bach et al (2007), Firneisz et al (2002)	Hungary Germany Hungary
c.3461T>C	p.L1154P	16	Missense	ATP loop	Present Study	-
c. 3903-6C>T	-	18	Splice site	ATP hinge	Present Study	-
c.3809A>G	**p.N1270S**	**18**	Missense	ATP hinge	Shah et al (1997), Santhosh et al (2006), Tanzi et al (1993)	USA (Russian, Swedish & North American WD patients) India USA (New York)
c.3894 delC	-	18	**Deletion**	ATP hinge	Present Study	-

E = Exon

Table 3.1: Homozygous mutations observed in relation to clinical subtypes of WD patients

Mutations	*Exon*	*Number of samples*	*N*	*H*	*NH*	*Control*
c.- 75C>A/ c.- 75C>A	1	1	1	0	0	0
c.- 128A>C/ c.- 128A>C	1	1	0	0	1	0
c.108+27C>T fs/ c.108+27C>T fs	1	1	1	0	0	0
p.S405A/ p.S405A	2	2	2	0	0	0
c. 2697-2723 del 27/ c. 2697-2723 del 27	11	1	1	0	0	0
p.G1061E/ p.G1061E	14	3	2	0	1	0
p.I1102T/ p.I1102T	15	2	2	0	0	0
c. 3903-6C>T/ c. 3903-6C>T	18	10	8	0	2	0
p.N1270S/ p.N1270S	18	2	2	0	0	0
c.3894 delC/ c.3894 delC	18	1	1	0	0	0
Total	**-**	**24**	**20**	**0**	**4**	**0**

N= neurologic, H= hepatic, NH= neuro-hepatic

Table 3.2: Compound heterozygous mutations and their relation to clinical subtypes of WD patients

Nucleotide change	*Amino acid change*	*E*	*n*	*N*	*H*	*NH*	*C*
c.[1252G>A ; 1255 G>A]	p.[E417K ; D418N]	2	2	2	0	0	0
c.[1186G> C;1194T>C;1195G>C]/	p.[E395Q ; (p.=T) ; A398P]	2	1	1	0	0	0
c.[1186G> C;1194T>C;1195G>C]/	p.[E395Q ; (p.=T) ; A398P]	2	1	1	0	0	0
c.[2973 G>A ; 3053C>T]	p.[(p.=T) ; A1017V]	13	1	1	0	0	0
c.[3008 C>T ; 3026delTCA]	p.A1003V	13	1	1	0	0	0
Total	**-**	**-**	**6**	**6**	**0**	**0**	**0**

E = exon, n = number, N = Neurologic, H = Hepatic, NH = Neuro-hepatic, C= control

ing, the researchers characterized 41 mutations in 55 WD patients; six of which have already been reported and the rest were novel mutations. It is of interest that the mutations described in WD patients worldwide are rather heterogeneous.

In the current study the researchers also tried to establish genotype-phenotype correlation in WD patients from northern regions of India. Most of the patients in our study were neurologic WD (78.8%) as it was done primarily in neurology department. The proportion of neurologic vs. hepatic patients in any series depends on the primary facility conducting the study. This type of bias is seen in other reports as well. Prominent hepatic presentations were reported from Caprai et al. (2006) (100% of hepatic WD cases), Lui et al. (2004) (88% of hepatic WD cases), Pandit et al. (2002) (54% of hepatic WD cases), Nanji et al. 1999 (81% of patients with liver WD). Similarly, Asadi-Pooya et al. (2005) studied 111 patients from Turkey, of which 83% had hepatic, 24.3% neurologic and 23.4% had psychological manifestations. On the other hand, prominent neurological cases were reported by Butler et al. 2001 (84% neurologic WD cases) and Taly et al. 2007 (69.1% WD patients with neurological manifestations).

The role of mutations in *ATP7B* responsible for WD is noncontroversial but none of the studies done so far have been able to identify any single common mutation consistently associated with WD. Several exonic, intronic and splice site mutations and polymorphisms have shown homozygous, heterozygous and compound heterozygous patterns. Previous studies have shown presence of some of these sequence variants among WD patients as well as control subjects (Forbes and Cox 2000). The significance of these variations remains uncertain. The present study is a preliminary attempt to describe the mutation spectrum of *ATP7B* among the north Indian population.

The spectra of *ATP7B* mutations in India from four study centers, that is, Chandigarh, in north (Kumar et al. 2005), Kolkata in east (Gupta et al. 2005), Vellore in south (Santhosh et al. 2006) and Lucknow in central India (Kalita et al. 2010) are different: (a) The group from Chandigarh have shown T3305C, C2975A, 2977insA and 3031insC in 6% of WD cases each; (b) whereas the group from Kolkata have shown C813A in 19% of WD cases. (c) On the other hand Vellore group have shown G3182A in 16% and C813A in 12% of WD cases and, finally, (d) the group from Lucknow reported

absence of H1069Q and R778L, mutations, commonly reported in European and Asian (Oriental) populations respectively.

So far, a total of 51 mutations of ATP7B have been documented in India including 34 novel mutations (Chandigarh-18, Kolkata-5, and Vellore-11). To this the researchers can add 35 novel mutations observed in the present study. The commonest mutation observed in this study group was I1102T observed in 3.6% of WD patients. This mutation was the commonest mutation in only one Indian WD genetics study (Kumar et al. 2005). In another study in WD patients from India this mutation was found in only four patients (Pandit et al. 2002). None of our WD cases had C813A mutation reported by other authors from India.

Systematic screening for mutations in all exons, exon-intron boundaries of *ATP7B* revealed a heterogeneous spectrum of mutations in 55 WD patients, where exons 2, 13 and 18 of *ATP7B* gene were 'hotspots' while exon 5 was the hotspot in the study from Kolkata, exons 8, 12, 13, 15, 16 and 18 in the study from Chandigarh and exons 13 and 18 were hotspot exons in the study from Vellore.

In the Indian WD population, although prevalent mutations account for approximately 41% of the total mutations, rare mutations are numerous and do not cluster to any specific region of the gene (Gupta et al. 2005). Hence, it is an arduous job to identify the mutations in WD patients and screen those mutations in siblings. The problem is even more difficult in populations in whom prevalent mutations are either not identified or account for a very low percentage of the total WD mutations.

Of the 41 mutations observed 18 were missense mutations, 8 silent mutations, three splice site, one duplication leading to frame-shift, 4 deletions and one nonsense mutation. Six out of 41 mutations have already been reported. Of these six known mutations, p.A756V missense mutation was reported in Italian WD population (Caprai et al. 2006). p.A1003V, missense mutation was reported in Turkish WD population (Loudianos et al.1999), p.G1061E, missense mutation was reported in Indian WD population from southern part of India (Santhosh et al 2006), p.I1102T missense mutation was reported in Indian (Pandit et al. 2002; Kumar et al. 2005)WD population from Kolkata and Chandigarh respectively. p.G1101R missense mutation was reported in Hungarian and German populations (Thomas et al. 1995c; Firneisz et al. 2002; Eisenbach et al. 2007) and p.N1270S missense mutation was reported from USA (Tanzi et al. 1993; Shah et al. 2006; Santhosh et al. 2006).

Irrespective of the type of phenotype exhibited by the affected individuals common mutations were not specially seen in any of the clinical subtypes. Specific genotype-phenotype correlations were not possible in the current study.

The population of India is composed of many thousands of subpopulations, divided by geography, language, religion and caste or *biraderi* (patrilineage) boundaries, with endogamous marriage the norm. The net effect is the creation of multiple genetic isolates with individual mutation profiles, but to date the clinical consequences of this highly complex differentiation have been largely ignored (Bittles 2002).

Although in the present India, broadly, the Indians are divided in to Austro–Asiatic (AA) which are the ancient tribal inhabitants of India localized mostly in the Central and Eastern parts of the country, Tibeto–Burman (TB) supposedly immigrants to India from Tibet and Burma (now, Myanmar), Indo–European (IE) are inhabitants of the northern and Dravidian (DR) language families to the southern parts of the subcontinent respectively. Apart from the linguistic distribution, even the major four morphological types-Caucasoid, Mongoloid, Australoid and Negrito are also present in the Indian population (Malhotra 1978). A study (Bhasin 2009) on morphological and molecular traits observed among the population groups inhabiting different geographical zones of India *viz.* North, West, East, Central, South and Islands. The variations in the frequencies of genetic markers and mean values of morphological traits distribution in the Himalayan region may be due to contacts between the various population groups of Western and Central Himalayas with population groups of Central Asia, and that of Eastern Himalayas with the Northern Mongoloid populations. In Central India admixture with the population groups of West India can be assumed. In South India, a different pattern of allele/haplotype frequencies and mean values is generally observed among the tribal population groups, for which one of the main causes might be seen in small population sizes. Inbreeding is prevalence among cer-

tain communities like Muslims, Parsis etc. and in most of the different population groups particularly from South India, which might have also resulted in the marked variation in distribution of frequencies and mean values of different genetic markers and morphological traits. The variations observed for the various morphogenetic traits in the distribution of allele/haplotype frequencies and mean values among the Indian population are due to racial elements present among them in varying degrees, migrations and admixture from time to time and other factors of evolutionary changes like mating patterns, genetic drift, mutation and selection under different environments (Bhasin 2009).

In the present study the mutation profile that emerged at the end of the investigation was very heterogeneous. Although the common mutations reported in the world literature were not detected in any of the patients in our study and the same has been reported from the other parts of India. Studies from other parts of India reflect the genetic heterogeneity among the Indian population as well; as is evident from the spectrum of mutations observed in WD population. The absence of any common mutation among the unrelated WD population might be due to the existence of broad gene pool leading to genetic heterogeneity because of regular admixture of genes in different centuries.

Therefore, the present structure of the genetic diversity among Indian population leaves us with a note that such type of mutation detection studies among large group of patients are needed to define the spectrum of the mutations and to frame the absolute genetic makeup among the patients in the Indian subcontinent.

CONCLUSION

No clear genotype phenotype correlation could be determined, as the sample size was small in hepatic and neuro-hepatic groups. However, the researchers observed most of the mutations were seen in either neurologic WD or neuro-hepatic WD patients. There may be some modifying variations which lead to more severe and prominent hepatic involvement. The present study also brings to forth that the mutation profile of Indian patients is clearly different from that of the other populations from different parts of the world and the mutation profile among Indian WD patients is also varied. The researchers also found several novel mutations in Indian WD patients.

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