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DNA linkage based diagnosis of Wilson disease in asymptomatic siblings

A. Gupta, R. Neogi, M. Mukherjea*, A. Mukhopadhyay, S. Roychoudhury
A. Senapati†, P.K. Gangopadhyay† & Kunal Ray

Human Genetics & Genomics Division, Indian Institute of Chemical Biology & †Bangur Institute of Neurology, Kolkata, India

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***Background & objectives*: Wilson disease (WD) is an autosomal recessive disorder caused by defects in *ATP7B* gene located in chromosome 13q14, and manifested as hepatolenticular degeneration as a result of accumulation of copper. No information on the mutation in the *ATP7B* gene and haplotypes using linked markers is available for WD patients in India. Hence, the present study was undetaken to identify, by a PCR-based molecular diagnostic test, presymptomatic siblings of WD affected individuals in families with multiple offspring.**

***Methods*: Genomic DNA was prepared from the peripheral blood of the patients, siblings and his/her first degree relatives. The repeat-markers flanking WD locus were amplified by PCR using fluorescent labeled primers. Amplified DNA fragments were analyzed by polyacrylamide gel electrophoresis in ABI 377 DNA sequencing system. Genotypes of the samples were determined using Genescan software. Haplotypes were determined based on segregation of the alleles in the families under study.**

***Results*: Among 15 WD affected families with multiple children, 4 cases were identified where younger siblings shared same genotype as the patient at all three markers analyzed. Further, eight different haplotypes were detected in the four patients.**

***Interpretation & conclusion*: The siblings of the WD patients carrying the same genotype at the markers linked to WD locus were presymptomatically diagnosed individuals. Presence of eight different haplotypes in the four patients suggested mutational heterogeneity at the WD locus. The test helps clinicians for therapeutic intervention in suspect WD cases by copper chelating agents prior to manifestation of overt clinical symptoms.**

Key words *ATP7B* - genotype - haplotype - microsatellite - Wilson disease

Wilson disease (WD) is a genetic disorder, which manifests as hepatolenticular degeneration as a result of accumulation of copper in the brain, liver, kidney and cornea due to its deranged biliary excretion[1]. In 1912, a WD was described as a familial syndrome of progressive lenticular degeneration associated with cirrhosis of the liver[2]. The etiological role of copper in the pathogenesis of WD was recognized much later in 1948[3], and the autosomal recessive mode of inheritance was established in 1953[4]. In India WD was first reported in 1963[5]. Though the manifestations of WD appear at a median age of 12 to 23 yr, the biochemical defects are present from the birth. The diagnosis is usually based on clinical and biochemical observations. Traditional screening includes slit-lamp examination of the eyes and

**Present address*: Tufts University, Boston, Massachusetts, USA

serum ceruloplasmin level measurement. As the disease does not usually manifest earlier than the second decade of life, the patients usually go untreated till they show the symptoms though it can be thwarted by the use of chelating agents such as penicillamine and zinc acetate[6]. Recent reviews[7-10] describe the advances made in the molecular genetic studies in WD, which have brought new hope to the management of the disease. Till date, genetic analysis is the most successful method for presymptomatic identification of the patients [11,12]. Progress in diagnosis of WD has been made by locating the causal gene (*ATP7B*) on the long arm of chromosome 13, enabling the use of flanking microsatellite markers to study the transmission of WD in siblings of affected individuals by linkage analysis[13,14]. Specifically, three highly polymorphic dinucleotide-repeat markers D13S314 (CA repeat), D13S133 ($GA_{14}GT_{17}$ repeat) and D13S316 (CA repeat) are used for this purpose. The reported numbers of alleles are 12, 17, and 9 in the size-ranges 137-163 for D13S314, 134-187 for D13S133 and 138-154 for D13S316 loci, respectively[14]. Presence of mutation spanning the entire length of the *ATP7B* gene and the large size (80 kb) of the gene make mutation screening an arduous and time consuming effort.

No information regarding the mutation in the *ATP7B* gene and haplotypes using linked markers is available for WD patients in India. As part of a broader study to identify prevalent mutations among Indian WD patients based on haplotype analyses and mutation screening, we have been genotyping the parents and all the siblings of WD patients. Here we report identification, by a PCR-based molecular diagnostic technique, of younger presymptomatic siblings of WD patients in the affected families.

Material & Methods

Clinical diagnosis of index cases: Wilson disease patients mostly with neurological problems were examined at the Bangur Institute of Neurology, Kolkata. Diagnosis was made according to the Sternlieb's criteria[1] which include overt neurological disorders, low serum ceruloplasmin (<20 mg/dl), 24 h urine copper excretion (>100µg) and Kayser-Fleischer (K-F) ring in the cornea by slit-lamp examination. Other relevant tests were conducted according to the need of the patients and for the diagnosis of the disease.

Collection of blood samples and genomic DNA preparation: About 5 ml peripheral blood samples were collected in EDTA vials from 136 individuals who belonged to 38 WD affected families with one patient in each family

Among 38 families, 23 had single child per family, and 15 had multiple children (2-4 siblings). Prior to sample collection, written consent was taken from all participants in this study. The internal review committee on research using human subject cleared the protocol of this study. The blood samples from the patients and their family members were collected from 1998 onwards and the molecular studies were undertaken from 1999. Genomic DNA was prepared from fresh whole blood using the conventional phenol-chloroform method[15] followed by ethanol precipitation and the DNA was dissolved in TE buffer (10 mM Tris-HCl, 1mM EDTA, *p*H 8.0).

Polymerase chain reaction (PCR) and genotyping: PCR was carried out in a total volume of 25.0 µl containing 100 ng genomic DNA, 0.4 µM of each primer (Invitrogen, Carlsbad, California), 0.2 mM of each dNTP (Invitrogen, Carlsbed, California), 2.5 mM of $MgCl_2$ (Sigma-Aldrich Fine Chemicals, St. Louis, Missouri) and 0.5 unit of Taq polymerase (Invitrogen, Carlsbad, California) in a thermocycler (GeneAmp-9700, Applied Biosystems, California, USA). Dinucleotide-repeats (D13S133,

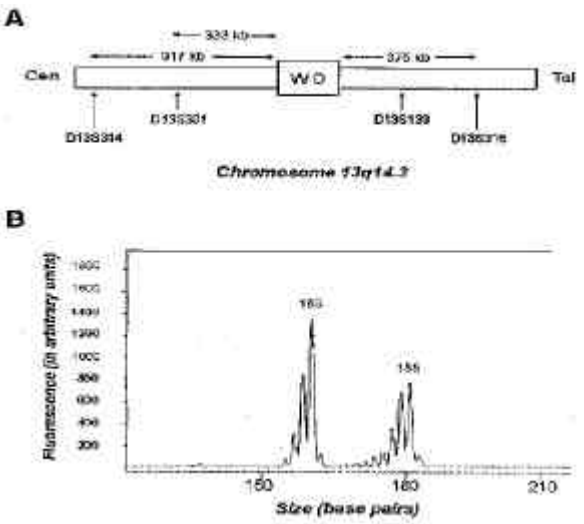

Fig. 1. WD locus map and chromatogram for typical marker analysis. Panel **A**, location of markers used for genotyping relative to WD locus. Among four markers shown, D13S301 could not be amplified consistently despite multiple attempts under various conditions. Marker D13S314 is located on the centromeric (Cen) end, and markers D13S133 and D13S316 on the telomeric (Tel) end relative to location WD locus on the chromosome 13. Panel **B**, a typical chromatogram obtained for dinucleotide repeat marker is shown with the size of the alleles. Smaller peaks preceding both the alleles are due to 'stutter bands' typically seen for PCR-amplified dinucleotide repeat markers irrespective of the method of analysis using. The two peaks represent allele sizes of 163 and 185 base pairs bp.

D13S314 and D13S316) flanking WD locus (Fig.1, panel A) were amplified using fluorescent labeled primers as described previously[13,14]. The sequence of the primers used and the fluorescent labels are shown in Table I. Amplified PCR products were separated by 6 per cent native polyacrylamide gel analysis in ABI 377 DNA sequencing system using Genescan - 500 TAMRA size Standard (Applied Biosystems, California, USA). The size of the alleles represent the length of the amplified DNA fragment for each marker locus in bp as determined by the Genescan software (Applied Biosystems, California, USA). The output chromatogram, typical for dinucleotide repeat markers, is illustrated in Fig. 1 (panel B).

Results & Discussion

All 38 families included in this study had one child affected with WD but neither of the parents in any family was affected with the disease. Fifteen of the 38 couples had multiple offspring, who were genotyped using markers flanking WD locus for molecular diagnosis of presymptomatic children. Genotypes of all the children of these 15 families are shown in Table II.

Clinical history of the patients in families where presymptomatic individuals were diagnosed: A young female (18 yr) student (Fig. 2A,II-1) was presented in April 1999 with complaint of yellow discolouration of the eyes and urine, and swelling of legs and abdomen for the last 3 months. She had a past history of tuberculosis adenitis, which was confirmed by fine needle aspiration cytology, and was on antitubercular drugs since October 1997 but had developed drug induced jaundice during the treatment. On examination in 1999, she was diagnosed to have hepatitis with ascitis and K-F ring in her eyes. Neurological examination was uneventful. Laboratory investigations revealed a picture of hepatitis and copper overload confirming it to be due to WD. The patient improved with penicillamine and later put on oral zinc therapy.

A 10 yr old boy (Fig. 2B II-1) was presented in the clinic in February 2002 with behavioural abnormalities like aloofness, apathy, drooling of saliva and sustained abnormal posturing of limbs as well for the past 3 months. There was an acceleration of the disease process for the past 2 months from when dysarthria, dysphagia,

Table I . Primers used for amplification of microsatellite markers flanking WD locus

Locus name	Nature of repeat	Sequence of primers (5' to3') Forward*	Reverse
D13S316	CA repeat	Fam-GCA GCA ATG CTT TGT GCA TAA	TGT TTC CCA CCA ATC TTA CCG
D13S133	$(GA)_{14}$ $(GT)_{17}$	Fam-GCT AGG ACT ACA GGT GCA AAC C	GGC AAC ATA GGG AAA CCC TAGC
D13S314	CA repeat	Tet-GAG TGG AGG AGG AGA AAA GA	GTG TGA CTG GAT GGA TGT GA

*forward primers were fluorescent tagged as indicated

Table II . Families affected with Wilson disease

Family no.	Child(ren) per family	Genotypes of children (+,+)	(+,WD)	(WD,WD)
1	2	-	-	1+1*
2-7	2	-	1	1
8	2	1	-	1
9	2	nd	nd	1
10-11	3	0	2	1
12	3	1	0	1+1*
13	3	2	0	1
14	3	0	1	1+1*
15	4	0	2	1+1*

nd, not determined due to identified cross over between D13S314 and D13S133 markers; (+,+), homozygous normal; (+,WD), heterozygous who are phenotypically normal carriers; (WD,WD), homozygous for mutation who are affected with the disease excepting four individuals in four families marked with asterisk. In 23 out of 38 families, the proband did not have any sib, hence not included in the Table.

choking, nasal regurgitation and abnormal straightening of the legs and twisting of the hands started resulting in rapid incapacitation of the patient to carry out his daily activities. KF ring was detected in his eyes. His blood tests revealed a decrease of serum ceruloplasmin (2.5 mg%) and increased 24 h urinary copper excretion (160 µg) indicating copper over-load. Magnetic resonance imaging (MRI) suggested WD.

Another 14 yr old boy (Fig. 2C, II-1) started behavioural abnormality from mid 1998 in the form of sense of fear, restlessness, incoherent speech, destructive attitude, running amok and visual hallucination. He was brought to the clinic in October 2001 and put on antipsychotic medication without any relief. Gradually, the patient developed dystonic posturing of limbs and trunks, had difficulty in walking, dysarthria, drooling of saliva with tremor of limbs. Later, the patient developed focal convulsion. He did not have past history of jaundice or any family history of such a presentation. Examination revealed that the patient was anarthric, generalized cogwheel rigidity with dystonic posturing without any pyramidal sign. Cerebellar functions were difficult to assess during admission but later cerebellar test revealed abnormality in coordination. Ophthalmic examination revealed K-F ring. Serum ceruloplasmin levels were 3.0 mg%; 24 h urine copper 320 µg; liver function tests and routine haematological examinations were under normal limits. MRI suggested WD.

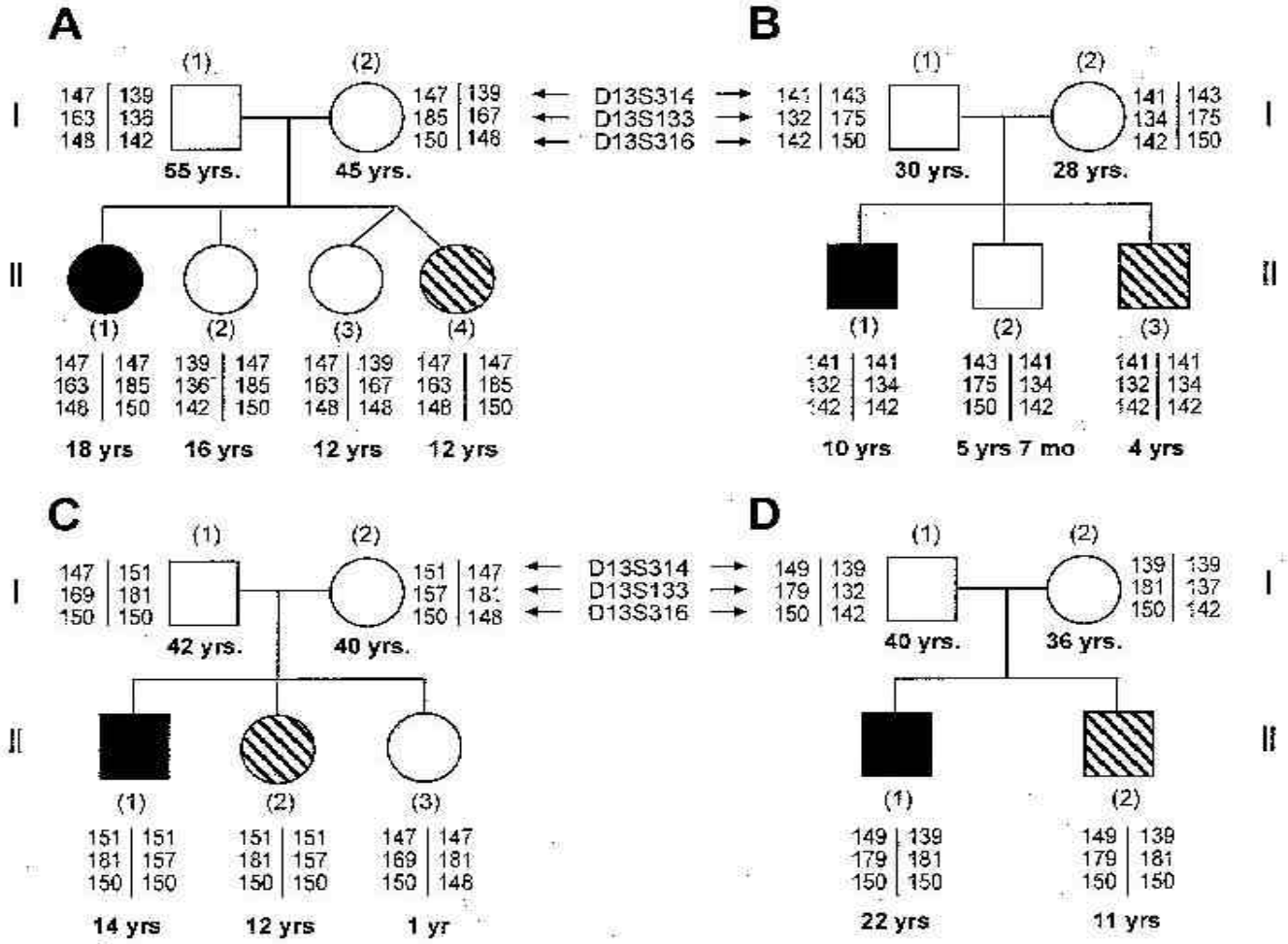

Fig. 2. Genotype and haplotype analysis in Wilson disease affected families. Squares and circles represent males an females, respectively. The probands and his/her clinically normal siblings sharing the same genotypes are denoted b shaded and stripped symbols, respectively while all other clinically normal individuals in the family (both homozygou normal and WD-carrier) are shown by open symbols. In each pedigree, unique numbers identifies all individuals in th generation (I or II) they belong to, and their ages are also shown in each case. Haplotypes as determined from th genotypes of 3 marker loci (D13S1316, D13S133 and D13S314) are shown for all the individuals included in the study Unlike other di-nucleotide repeats, even and odd size alleles observed for D13S133 *(e.g.* panel A, I-1; panel B, I-1 & I- has been previously reported[14] and might result from presence of, yet uncharacterized, single nucleotide insertion in th region amplified by PCR.

A 22 yr old male (Fig. 2D, II-1) complained of dribbling of saliva, sleep disturbance, inability to move limbs fluently, trembling of the extremities and difficulty of swallowing for the past 18 months when he was presented to the clinic in June 1999. He also gave the history of cognitive decline. Before that he was entirely well and his development as described by his parents was normal. On examination he showed evidence of slurred speech, tremor, incoordination in movements, spastic - ataxic features with presence of K-F ring in the eye. Jerks were brisk and planter flexor. Biochemical examination showed serum ceruloplasmin 8 mg per cent, serum glucose and urea were in normal range, urinary copper excretion for 24 h was 160 µg indicating copper overload. Routine CSF examination was non-contributory. The patient was treated with penicillamine.

Haplotype analysis: All the three CA-repeat markers (D13S314, D13S133 and D13S316) were informative in the families included in this study. The total number of alleles identified for D13S314, D13S133 and D13S316 markers were 11, 18 and 9, respectively. These values are comparable to the reported numbers of alleles, that is, 12, 17, and 9 in the size-ranges 137-163 for D13S314, 134-187 for D13S133 and 138-154 for D13S316 loci, respectively[14]. Genotypes were determined at each of the three dinucleotide repeat markers for each member of the core family (both parents and siblings) of the index patients. In each case the haplotype was constructed using the genotype data, following the Mendelian pattern of inheritance of the markers in the family, to identify whether any sibling of the index patient shared both set of haplotypes to identify the individual in a preclinical stage of WD. Theoretically, predictive value for diagnosis using any one of the three multi-allelic markers used would be equally good. Additional marker analyses were done for reproducibility and confidence in the predictive diagnosis. Also, since the distance of the flanking markers vary from 917 to 375 kb from WD locus, one has to take note of the rare but real possibility of recombination between the marker and WD loci. The reported number of crossover for the most distant marker D13S314 (917 kb upstream from *ATP7B*) is1 in 68 meosis[14]. We also observed one crossover in 68 meosis. To prevent such diagnostic error, the diagnostic determination was based on the haplotype constructed using all three chosen markers in the families (Fig. 2).

In the first family (Fig. 2A), the index patient had three younger sisters including a pair of non-identical twin with no one having any obvious sign of clinical abnormality. By haplotype analysis it was revealed that both the haplotypes (147-163-148 and 147- 185 - 150) of the patient (Fig. 2A, II-1) were shared by one of the twin sisters (Fig. 2A, II-4) and was thus predicted to harbor both the mutated copies of the *ATP7B* present in the index patient. Between two other sisters detected to be carrier, the older one (Fig. 2A, II-2) inherited the mutant chromosome from her mother and the younger one (Fig. 2A, II-3) inherited the mutant chromosome from her father.

In the second family, between two brothers of the patient (Fig. 2B II-1) who were phenotypically normal, 4 yr old younger one (II-3) shared the same pair of haplotypes (141- 132- 142 and 141- 134- 142) with his WD affected brother, and is under observation. The other sibling, II-2 inherited a mutant chromosome (141- 134- 142) from his mother and the normal chromosome (143- 175- 150) from his father and hence was diagnosed as a carrier for WD.

In the third family (Fig. 2C), among two younger sisters of the patient one (12 yr old; II-2) shared the same genetic materials (151- 157- 150 and 151- 181- 150) around WD locus as her affected brother. Though she showed no immediate symptoms of WD, she is under regular observation. The other sister (Fig. 2C, II-3) inherited both normal copies of the gene (*ATP7B*) from the parents as evident from the haplotypes (147- 169- 150 and 147 - 181- 148).

In the fourth family, the younger brother (11 yr old; Fig. 2D; II-2) of the index patient had identical haplotypes (149-179-150 and 139-181-150) as the patient (Fig. 2B, II-1). In this case, however, the mother (Fig. 2D 1-2) of the patient was homozygous (139, 139) for the D13S314 marker, which meant that the haplotype 139-181-150 in the younger son (Fig. 2D, II-2) could be a recombinant, and might not carry the mutation at the WD locus. Identification of heterozygosity of the marker (D13S301) located in between D13S314 and WD locus (Fig. 1, panel A), which could not be amplified by PCR, might have resolved the issue. The suspected boy in this family showed no clinically overt neurological abnormality though a low normal serum ceruloplasmin was observed.

One of the twin sisters of the first family having mutated copies of the *ATP7B* presented after a gap of

2 yr with prominent abdomen, occasional blackening, loss of weight and failure to concentrate on her studies and remember things. She had minimal neurological abnormalities in the form of tremor and dysarthria; her liver and spleen were enlarged with the presence of abdominal fluid. Laboratory investigations revealed a picture of hepatitis and copper overload confirming it to be Wilson disease. This patient responded well by treatment with penicillamine.

In the four cases described, DNA analysis was very useful for early diagnosis of the presymptomatic siblings of WD patients that would have remained undetected without the molecular diagnosis, thus helping the clinicians to prevent this crippling disease by timely medical intervention. The PCR-based diagnostic test can be performed at any age including *in feto*.To reduce the cost of the diagnostic test, one could use only D13S133- a complex repeat ($GA_{14}GT_{17}$) and most heterozygous among the 3 markers analyzed. Identification and use of markers proximal to or within the WD locus, including intragenic single nucleotide polymorphism (SNP) in the *ATP7B*, would allay any confusion related to the potential occurrence of recombination of the distant markers.

In this study, the younger siblings of the probands identified as presymptomatic for WD had the opportunity for appropriate therapeutic intervention prior to the onset of the disease. Without the genetic study, their predisposition for the disease would have remained unnoticed, till they manifested the signs for the disease. In WD changes in the biochemical parameters in the blood and urine precede the clinical symptoms[1]. Hence a patient attending the clinic with complaints of early WD symptoms, actually have a modest to high derangement of biochemical parameters. Thus individuals identified as presymtomatics by molecular diagnostic method can be monitored for progression of the disease by regular checkups of the biochemical parameters for therapeutic intervention at appropriate time.

At present studies are in progress to identify the haplotype(s), which are over-represented among WD affected individuals in the population, as a strategy to identify the prevalent mutation(s) in the relatively large (80 kb) *ATP7B* gene causal to Wilson Disease. Presence of eight different haplotypes in the four patients suggests mutational heterogeneity at the WD locus. Two of these haplotypes differ in 1-2 repeat units in only one of the three markers used. It is, thus, possible that some of the haplotypes might have resulted from same mutant chromosome by acquiring allelic variation later in one of the 3 repeat-marker loci by DNA slippage during replication.

In developing countries like India, where large families are common occurrence, multiple members of the family have potential to be affected with inherited disorders. The negative impacts on health care cost and quality of life are particularly high for such debilitating neurological genetic disorder as WD. The molecular diagnostic test needs to be used for better clinical management of the potential patients and genetic counseling to the family harboring the mutant copies of the gene for proper family planning.

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Reprint requests : Dr Kunal Ray, Human Genetics and Genomics Division, Indian Institute of Chemical Biology
4 Raja S.C. Mullick Rood, Jadavpur, Kolkata 700032, India
e-mail: kunalray@vsnl.com