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Genetic defects in Indian Wilson disease patients and genotype–phenotype correlation

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ABSTRACT

Wilson disease (WD) is caused by defects in *ATP7B* gene due to impairment of normal function of the copper transporting P-type ATPase. This study describes a comprehensive genetic analysis of 199 Indian WD patients including mutations detected in our previous studies, undertakes functional assessment of the nucleotide variants in *ATP7B* promoter and correlates genotype with disease phenotype. The patient cohort harbors a total of 10 common and 48 rare mutations in the coding region of *ATP7B* including 21 novel changes. The common mutations represent 74% of characterized coding mutant alleles with p.C271X (63/260) and p.G1101R (7/31) being the most prevalent in eastern and western Indian patients, respectively. The mutation spectrum between east and west is mostly different with only three mutations (p.G1061E, p.N1270S and p.A1049A-fs) being shared between both the groups. Eight novel and 10 reported variants have been detected in the promoter and non-coding regions (5′ and 3′UTRs) of *ATP7B*. Promoter reporter assay demonstrated that 3 novel variants and 5 reported polymorphisms alter the gene expression to a considerable extent; hence might play important role in *ATP7B* gene regulation. We devised the neurological involvement score to capture the spectrum of neurological involvement in WD patients. By utilizing the age at onset, neurological involvement score and *ATP7B* mutation background, we generated a genotype–phenotype matrix that could be effectively used to depict the phenotypic spectra of WD affected individuals and serve as a platform to identify prospective "outliers" to be investigated for their remarkable phenotypic divergence.

1. Introduction

Wilson Disease (WD, MIM# 277900) is an inborn error of copper metabolism inherited in an autosomal recessive manner. The disease is caused due to mutations in the P-type ATPase gene (*ATP7B*) [1]. Impaired function of ATP7B results in copper accumulation and toxicity leading to hepatic and neuropsychiatric manifestations of WD. Studies on the biochemical properties and regulation of ATP7B have been described in a number of reviews [2,3]. WD is one of the most widely studied diseases of copper accumulation.

Mutation screening has revealed a large number of defects covering almost the entire *ATP7B* (http://www.wilsondisease.med.ualberta.ca/database.asp). But efforts to correlate a specific mutation to a particular phenotype has been complicated by factors such as compound heterozygotes, large number of different mutations, limited number of patients and clinical heterogeneity even between affected sibs bearing the same set of *ATP7B* mutations [4,5]. Also, sequence variations in modifier loci have been postulated to influence the WD phenotype [6–8].

Although *ATP7B* promoter is well characterized [9], only a few reports have focused on screening the promoter region [10,11] but not 3′-untranslated region (UTR) which might be potentially

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regulated by miRNA. Since a considerable number of patients (10–20%) have been reported to have uncharacterized mutations even after screening much of the coding region [12,13], it is possible that the regulatory regions harbor mutations that might affect *ATP7B* expression.

Thus, this study deals with mutation screening of *ATP7B* in eastern and western Indian WD patients to find out the complete variation profile of *ATP7B*, explores the effect of mutations by genotype–phenotype correlation, undertakes functional assessment of nucleotide variants identified in the promoter and 5′UTR and finally draws a comprehensive picture of Wilson disease genetics from eastern and western parts of India. While identification of common and rare mutations in a population can be useful for rapid preclinical diagnosis, functional analysis of genetic variants might help to elucidate the underlying mechanism of disease pathogenesis and correlate genotype with the molecular and clinical phenotype.

2. Patients and methods

2.1. Study subjects

A total of 199 WD patients (174 eastern Indian and 25 western Indian) mostly with neurological problems from 178 unrelated families comprising of 687 individuals have been included in this study prospectively. This group is an extension from a previous report where 25 eastern Indian patients had been characterized with both *ATP7B* mutations and single mutations found in a few [14]. In the present study, mutation screening has been undertaken in 174 patients (eastern Indian: 149, western Indian: 25). Finally, a comprehensive mutation profile of all 199 patients is described (Fig. 1A). WD patients were examined at the Bangur Institute of Neurosciences, Kolkata, National Medical College, Kolkata, West Bengal and King Edward Memorial hospital, Pune, Maharashtra by respective neurologists and clinical details were filled up in a semi structured proforma. WD diagnosis was based on Sternlieb's criteria [15] characterized by suggestive clinical features with evidence of positive KF ring, low serum ceruloplasmin, high urinary copper excretion. KF ring negative hepatic cases had no clinical or lab evidence of the etiology of other disease. Additional details are furnished in the supplemental material. Ethnically matched healthy control samples were selected from the general population. The study protocols adhered to the Declaration of Helsinki. The internal review board on research using human subjects reviewed and cleared the project as per Indian Council of Medical Research regulations. All participants gave informed written consent (unless the patient was a minor in which case their parents signed the consent form).

2.2. Detection of sequence variants

PCR-Sanger sequencing was performed to screen 20 *ATP7B* exons (2–21) and flanking regions from genomic DNA samples as described previously [14]. The promoter, 5′UTR, exon 1 (1832bp) and 3′UTR (2478bp) were screened in patients (Fig. 1A) where either single ($n = 63$) or no mutation ($n = 22$) could be identified in the coding region. Patient DNA samples which could not be characterized for both *ATP7B* mutations were subjected to MLPA (*Multiplex Ligation-dependent Probe Amplification*) assay (Salsa P098 kit, MRC-Holland, the Netherlands). NCBI NM_000053.2 and ENSG00000123191 were used as a reference sequence. *In silico* analysis tools PolyPhen-2 v2.2.2 (http://genetics.bwh.harvard.edu/pph2/), TFSEARCH (http://mbs.cbrc.jp/research/db/TFSEARCH. html), and miRBase (http://www.mirbase.org/search.shtml) were used to predict the putative effect of relevant nucleotide variants, as appropriate. One hundred ethnically matched control chromosomes were screened to check for the absence of each novel mutation and confirm their genetic status. Thirty four parent DNA samples, corresponding to 17 patients (14 patients bearing both promoter and coding mutations and 3 patients carrying multiple promoter variants but no coding mutations) were genotyped by sequencing to find out the haplotypes indicated by allele segregation pattern in the analyzed pedigrees (Supplemental Table 3).

2.3. Subcloning of promoter variants and reporter gene assay

The *ATP7B* promoter and 5′UTR (1595bp, ENSG00000123191 chromosome: GRCh37: 13: 52585474-52587068) was amplified from the genomic DNA of normal individual with a specific pair of primers: *AAGCTTTATATTTAAGTGACGTGTT* and *CGTCCCGCACGGACACCGAA*. The fragment was subcloned via TOPO-TA vector mediated cloning (Invitrogen, CA, USA) into pGL3 basic vector (Promega, WI, USA) after *KpnI-XhoI* digestion (NEB, MA, USA). Site directed mutagenesis was performed using Quick change XL kit (Agilent, TX, USA) as per manufacturer's instructions. The sequences of the clones generated were confirmed by automated DNA sequencing of all constructs.

HEK293 cells were grown in 35 mm dishes up to approximately 70% confluency. A 30:1 ratio of experimental vector (Promoter-pGL3) to co-reporter vector (pRL-TK) (Promega, WI, USA) was used for cotransfection experiments using 4 μl Turbofect (Fermentas, MD, USA) per dish. Dual luciferase assay was carried out with Dual Luciferase Reporter Assay System (Promega, WI, USA) following manufacturer's recommendations. All experiments were done in triplicate and the average value obtained from 3 independent assays was considered for analysis.

2.4. Genotype–phenotype (G2P) correlation

The Neurological Involvement Score (NIS) was designed to capture the spectrum of neurological involvement of WD patients across cognition, behaviour and motor domains. NIS has been devised following the Tier 2 of the Global Assessment Scale (GAS) for WD as proposed by Aggarwal et al. [16]; further details are provided in the supplemental material. Finally, the age at onset and NIS for individual patients were

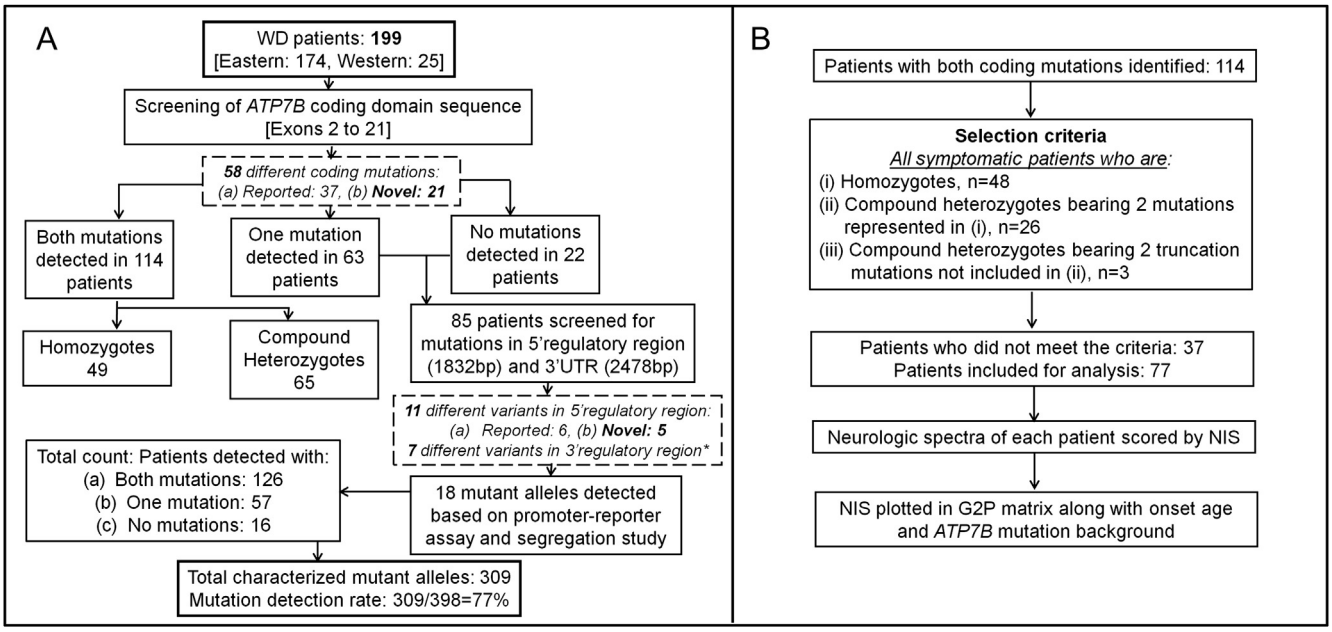

Fig. 1. Flow diagram for (A) Genetic analysis of *ATP7B* in WD patient cohort, and (B) Patient samples selected for genotype–phenotype (G2P) correlation. *Refer to Supplemental Table 2 for details.