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A genetic study of Wilson's disease in the United Kingdom

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Previous studies have failed to identify mutations in the Wilson's disease gene *ATP7B* in a significant number of clinically diagnosed cases. This has led to concerns about genetic heterogeneity for this condition but also suggested the presence of unusual mutational mechanisms. We now present our findings in 181 patients from the United Kingdom with clinically and biochemically confirmed Wilson's disease. A total of 116 different *ATP7B* mutations were detected, 32 of which are novel. The overall mutation detection frequency was 98%. The likelihood of mutations in genes other than *ATP7B* causing a Wilson's disease phenotype is therefore very low. We report the first cases with Wilson's disease due to segmental uniparental isodisomy as well as three patients with three *ATP7B* mutations and three families with Wilson's disease in two consecutive generations. We determined the genetic prevalence of Wilson's disease in the United Kingdom by sequencing the entire coding region and adjacent splice sites of *ATP7B* in 1000 control subjects. The frequency of all single nucleotide variants with *in silico* evidence of pathogenicity (Class 1 variant) was 0.056 or 0.040 if only those single nucleotide variants that had previously been reported as

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mutations in patients with Wilson's disease were included in the analysis (Class 2 variant). The frequency of heterozygote, putative or definite disease-associated *ATP7B* mutations was therefore considerably higher than the previously reported occurrence of 1:90 (or 0.011) for heterozygote *ATP7B* mutation carriers in the general population ($P < 2.2 \times 10^{-16}$ for Class 1 variants or $P < 5 \times 10^{-11}$ for Class 2 variants only). Subsequent exclusion of four Class 2 variants without additional *in silico* evidence of pathogenicity led to a further reduction of the mutation frequency to 0.024. Using this most conservative approach, the calculated frequency of individuals predicted to carry two mutant pathogenic *ATP7B* alleles is 1:7026 and thus still considerably higher than the typically reported prevalence of Wilson's disease of 1:30 000 ($P = 0.00093$). Our study provides strong evidence for monogenic inheritance of Wilson's disease. It also has major implications for *ATP7B* analysis in clinical practice, namely the need to consider unusual genetic mechanisms such as uniparental disomy or the possible presence of three *ATP7B* mutations. The marked discrepancy between the genetic prevalence and the number of clinically diagnosed cases of Wilson's disease may be due to both reduced penetrance of *ATP7B* mutations and failure to diagnose patients with this eminently treatable disorder.

Keywords: Wilson's disease; *ATP7B*; genetic prevalence

Abbreviations: SNV = single nucleotide variant

Introduction

Wilson's disease (OMIM #277900) is an autosomal recessive disorder of copper metabolism, characterized by decreased biliary excretion of copper and reduced incorporation of copper into apoceruloplasmin (Ala *et al.*, 2007). This leads to an accumulation of copper in the liver and extrahepatic organs such as the brain and cornea, resulting in oxidative stress and eventually cell death. Patients with Wilson's disease typically present with hepatic or neurological/psychiatric symptoms but other presentations such as haemolytic crisis are well recognized. There is a significant phenotypic variation between individuals with the same mutation and even within families and in monozygotic twins (Czlonkowska *et al.*, 2008, 2009; Kegley *et al.*, 2010). There is also a wide range in the age of onset including early-onset hepatic disease in a 3-year-old child and late onset disease in two siblings in their 70s (Ala *et al.*, 2007) with the mean age of onset of 15.9 years (Taly *et al*., 2007). If Wilson's disease is diagnosed at an early stage of the illness and the patient is subsequently put on appropriate lifelong treatment such as the copper chelating agent D-penicillamine, patients improve and are then often largely asymptomatic for the rest of their life. However, typical features such as corneal Kayser-Fleischer rings or low ceruloplasmin levels in serum can be absent and thus delay the diagnosis. Indeed, failure to diagnose Wilson's disease is the principle cause of death for this condition (Czlonkowska *et al.*, 2005; Walshe, 2007).

Wilson's disease is typically caused by homozygous or compound heterozygous mutations in the *ATP7B* gene, which encodes a copper-transporting P-type ATPase. Over 500 mutations have now been reported in the *ATP7B* gene (Wilson's disease mutation database http://www.wilsondisease.med.ualberta.ca/database.asp, access date: 1 October 2012). However, previous studies have repeatedly reported cases with a confirmed clinical and biochemical diagnosis of Wilson's disease in whom two *ATP7B* mutations could not be identified (Kenney and Cox, 2007; Mak *et al.*, 2008; Nicastro *et al.*, 2010; Park *et al*.,2010). The possibility of a second Wilson's disease gene has been discussed, but causative mutations in other genes involved in copper homeostasis have not yet been identified (Lovicu *et al.*, 2006).

Sheffield Diagnostic Genetics Service (SDGS) is the only UK diagnostic laboratory providing direct sequence analysis of *ATP7B* as a national service for patients with clinically suspected Wilson's disease and has been operational since 1995. This provided a unique opportunity to undertake a comprehensive service evaluation of diagnostic referrals from the entire UK to determine the spectrum, detection rate and distribution of *ATP7B* mutations in patients with the clinical diagnosis of Wilson's disease.

There is an ongoing debate about the prevalence of Wilson's disease. The widely cited prevalence figure of 1:30 000 with a carrier frequency of 1:90 pre-dates the discovery of *ATP7B* as the disease-causing gene defect and has been questioned (Scheinberg and Sternlieb, 1984; Park *et al.*, 1991). In the UK, with its population of ~60 million inhabitants, one would expect ~2000 cases of Wilson's disease based on this prevalence estimate. This number vastly exceeds the number of patients with the clinical diagnosis of Wilson's disease in the UK. Mass screening for Wilson's disease in East Asian populations, based on ceruloplasmin level measurements, suggested a significantly higher frequency of Wilson's disease, ranging from ~1:1500 (Ohura *et al.*, 1999) to 1: 3000 (Hahn *et al.*, 2002). Similar studies have not yet been carried out in the UK or other diverse populations of European ancestry. Furthermore, only limited research has been undertaken so far to determine the genetic prevalence of Wilson's disease in typical, non-isolated populations (Olivarez *et al.*, 2001). As mentioned above, >500 different mutations have now been described in the *ATP7B* gene, which spans 21 exons (Kenney and Cox, 2007). Any studies aiming to establish the genetic prevalence of Wilson's disease by investigating the frequency of a single or only a limited number of *ATP7B* mutations in a control population are therefore likely to be of limited value. As the second part of our study, we therefore investigated the genetic prevalence of Wilson's disease in the UK by sequencing the entire *ATP7B* gene in 1000 control subjects and putative mutation hot spots in a further 5000 control subjects.

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Materials and methods

ATP7B diagnostic testing

All UK referrals to the Sheffield Diagnostic Genetics Service for *ATP7B* testing between January 1995 and April 2009 were reviewed. Inclusion criteria for the study cohort were either a confirmed diagnosis of Wilson's disease by identification of two mutations or confirmation of diagnosis by clinician on follow-up, applying standard diagnostic criteria (EASL, 2012). Seventy-seven cases were excluded from this study after the referring clinicians confirmed that a different diagnosis had been reached in these patients subsequent to the initial request for *ATP7B* testing or in whom clinical details could not be obtained. Genomic DNA was extracted from whole blood according to standard protocols. Mutation analysis was carried out in two stages. From 1995–99 mutational hot spots exons 8, 14 and 18 were screened using pre-screening methods such as single stranded conformational polymorphism analysis and restriction enzyme analysis followed by DNA Sanger sequencing. In 1999 the first stage was extended to seven exons (exons 2, 8, 13–15, 18 and 19) by direct sequencing (Curtis *et al*., 1999). If the first stage analysis failed to identify two *ATP7B* mutations, the remainder of the gene was screened by direct screening of all 21 exons of *ATP7B* plus 25–50 bp of adjacent intronic sequence in 23 fragments using M13-tailed primers (primer sequences available on request). This methodology was applied retrospectively to all confirmed referrals. The presence of any single nucleotide polymorphism within primer annealing sites was excluded (http://ngrl.manchester.ac.uk/SNPCheckV2/snpcheck.htm). Fragments were sequenced using an ABI Prism Dye Terminator v1.1 kit and run on an ABI3730 (Applied Biosystems). Sequence traces were analysed visually using the Staden package (Sanger) by two trained individuals and compared to the complementary DNA sequence NM_000053.3. If two mutations had not been identified at this point, the *ATP7B* promoter from the ATG initiation codon (c.1) to 600 bp upstream (c.-600) was analysed, including the region containing the previously described c.-441_-427del15 mutation (Loudianos *et al*., 1999). This was then followed by multiplex ligation-dependent probe amplification (MLPA) in all those samples that still only had one or no *ATP7B* mutation in the coding region, adjacent splice sites or the promoter region. The multiplex ligation-dependent probe amplification experiments were carried out according to the manufacturer's instructions (MLPA kit P013, MRC-Holland, The Netherlands) and results were analysed using automated Excel macros (http://www.ngrl.org.uk/Manchester/mlpapubs.html). Any novel missense variants or atypical splice variants outside the ±2 GT and AG donor and acceptor site dinucleotides were assessed for pathogenicity according to the Clinical Molecular Genetics Society best practice guidelines for unclassified variants (http://cmgsweb.shared.hosting.zen.co.uk/BPGs/pdfs%20current%20bpgs/UV%20GUIDELINES%20ratified.pdf). Alamut Mutation Interpretation software (Interactive Biosoftware) v2.1 was used to access SIFT aligned sequences (http://sift.jcvi.org/www/SIFT_aligned_seqs_submit.html using sequences from *Homo sapiens, Pan troglodytes, Macaca mulatta, Rattus norvegicus, Mus musculus, Canis familiaris, Felis catus, Gallus gallus, Xenopus tropicalis, Tetraodon nigroviridis* and *Saccharomyces cerevisiae* for comparison); PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/); dbSNP (http://www.ncbi.nlm.nih.gov/snp/); HGMD professional (https://portal.biobase-international.com/cgi-bin/portal/login.cgi); Google (www.google.com) plus Grantham distance (integrated in Alamut 2.1); and splice prediction software SpliceSiteFinder-like, MaxEntScan, NNSplice, GeneSplicer and Human Splicing Finder (all integrated in Alamut v2.1). In addition to Alamut 2.1, Mutation taster (http://mutationtaster.org/MutationTaster/index.html), Panther (http://www.pantherdb.org/tools/csnpScoreForm.jsp), and Russell amino acid properties (http://www.russelllab.org/aas/) were assessed.

Some of the findings in 52 of the 181 patients included in this study have been published previously (Curtis *et al*., 1999).

Determination of genetic prevalence

The study was reviewed by the Ethics Committee of the Sheffield Children's National Health Service (NHS) Foundation Trust. DNA was extracted from sequential dried blood spot cards supplied by the Regional Newborn Screening laboratory at Sheffield Children's Hospital. This laboratory screens all babies born in the East Midlands, South Yorkshire and North East Lincolnshire portion of Yorkshire and Humber, Derbyshire, Leicestershire, Lincolnshire, Northamptonshire, Nottinghamshire and Rutland (~75 000 per year). Punches from blood spot cards were placed in individual wells of a 96-well plate to which 100 μl per well of 50 mM NaOH were added. The plate and its contents were heated to 95°C for 5 min. Twenty microlitres of 1 mM Tris-HCl (pH 8) were added to each well and the samples were stored at -20°C until required. Before PCR, DNA samples were diluted 1:25 in 0.1 M TE. Each DNA sample was subjected to quality control testing by PCR with a standard sequence-tagged sites (primer sequences are available on request) to check performance prior to use.

Primer design, polymerase chain reaction and sequencing

Manual primer design was used to design a total of 29 amplicons with an average size of 550 bp to cover each of the 21 exons of the *ATP7B* gene and at least 50 bp of intronic flanking sequence where possible (see Supplementary Table 1 for primer sequences and annealing temperatures). Overlapping amplicons were designed to cover the larger exons where necessary (exons 2 and 21). Universal M13 tails were added to the unique primer sequences for ease of sequencing for some of the exons. Primer pairs were pre-screened as described to determine the optimum conditions for amplification (http://www.sanger.ac.uk/resources/downloads/human/exoseq.html). PCR and capillary sequencing were carried out as described (http://www.sanger.ac.uk/resources/downloads/human/exoseq.html). The majority of exons were amplified at 60°C with the addition of 7-deaza-GTP if necessary. Bi-directional sequencing of amplicons was carried out using Big Dye® chemistry.

Sequence analysis

Sequences were aligned to the reference genome and variants called using ExoTrace, a novel algorithm developed in-house at the Wellcome Trust Sanger Institute (for the detection of heterozygotes in sequence traces). ExoTrace processes the sense and antisense sequence reads separately and subsequently combines the results to allow single nucleotide variant (SNV) scoring of base substitutions. Each SNV is assigned a status according to a set of predefined rules. All SNVs below a certain threshold were subjected to manual review using a modified version of GAP4, part of the Staden Sequence Analysis Package software, created for the ExoSeq project. Further details can be found at http://www.sanger.ac.uk/resources/downloads/human/exoseq.html.

All SNVs were assessed for pathogenicity as described above. All those SNVs with *in silico* evidence for pathogenicity were then also compared to the Wilson's disease Mutation Database (WDMD) at the

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University of Alberta, Canada (http://www.wilsondisease.med.ualberta.ca/database.asp) to determine which of these SNVs with putative pathogenicity had been previously described in patients with Wilson's disease and classified as a 'Disease causing variant' or mutation.

Statistical analysis

The 1K and 5K data sets were analysed independently. The occurrence of SNVs was assumed to follow a Poisson distribution and exact 95% confidence intervals were computed. The null hypothesis that the SNV frequency is 1:90 or less was tested using a one-sided test for a Poisson variable.

Results

ATP7B sequence analysis in patients with Wilson's disease

A cohort of 181 index cases were identified who had either two *ATP7B* mutations or who fulfilled the clinical diagnostic criteria for Wilson's disease with a score of >4 in the scoring system developed at the 8th International Meeting on Wilson's disease, Leipzig 2001 (EASL, 2012). One hundred and sixteen different mutations, including 32 novel mutations, were identified on 356 alleles from 179 individuals in the coding region or adjacent splice sites of *ATP7B* (Supplementary Table 2). Two *ATP7B* mutations were found in 177 cases and three mutations in three cases giving a mutation detection frequency of 98%. Only two individuals were found with one *ATP7B* mutation each (1%). No mutations were found in a further two individuals (1%). Neither the two cases with one mutation only nor the two cases with no mutation were related to each other. This detection rate is thus significantly higher than previously reported (Schmidt, 2009).

Mutations were distributed throughout the gene in all exons except exon 21. The mutation frequency for each exon is shown in Fig. 1. The exons harbouring the highest percentage of mutations were exon 14 (24%), exon 8 (20%) and exon 2 (12%). Analysis of these three exons would detect 56% of mutations from our cohort. Analysis of eight exons (exons 2, 5, 8, 13, 14, 18, 19 and 20) would detect 82% of the mutations found in this population. The most frequent mutation identified was c.3207C>A, p.(His1069Gln), in exon 14, at a frequency of 19%, which is similar to the frequency previously reported in the UK but significantly lower than that found in other European countries (Ferenci, 2006). The next most common mutation was c.2305A>G, p. (Met769Val), in exon 8 found at a frequency of 6%, similar to that previously reported (Curtis *et al.*, 1999). A total of 40 homozygotes were found, of which 11 were homozygous for p. (His1069Gln). One hundred and three out of the 116 mutations (89%) were found at a frequency of ≤1%.

Routine family investigations in two patients apparently homozygous for different mutations showed no paternal contribution in one case and no maternal contribution in the other. Alternative primers and sequencing showed no evidence of a single nucleotide

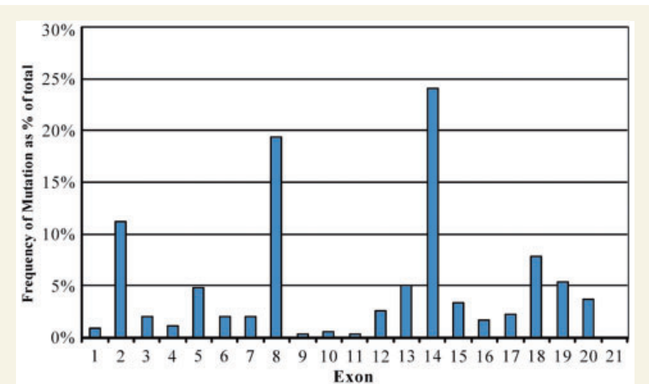

Figure 1 Distribution of mutations in the *ATP7B* coding region in patients with Wilson's disease. The frequency of mutations found in the cohort of 181 Wilson's disease index cases is given per exon as a percentage of the total. Mutations were detected in all exons except for exon 21. Three exons contained >10% of all mutations, namely exons 2, 8 and 14.

polymorphism under primer binding sites and multiplex ligation-dependent probe amplification analysis gave no indication of the presence of a large deletion. Use of multiple microsatellite markers representing the entire chromosome 13 demonstrated segmental paternal isodisomy giving rise to autozygosity for c.3207C>A, p.(His1069Gln) in one patient and maternal segmental isodisomy resulting in autozygosity for c.3955C>T, p.(Arg1319*) in the other. There was no concordance with the size or extent of the isodisomic segment (Fig. 2).

In three cases, three different missense mutations were detected. In the first case, analysis of other family members confirmed the genotype as p. [(Ile381Ser;Ile1184Thr)];[(Leu722fs)]. The novel p.(Ile381Ser) and p.(Ile1184Thr) putative mutations are inherited in *cis*. *In silico* analysis supports their classification as likely pathogenic. In the second case, no family members were available for testing but two of the mutations present, p.(Gln1142His) and p.(Ile1148Thr), both in exon 16, had been previously reported *in cis* (Mak *et al.*, 2008). Therefore the assumed genotype is p.[(Gln1142His);(Ile1148Thr)];[(Met769 Val)]. For the final case, analysis of family members confirmed the genotype as p.[(Asn41Ser);(Ile1021Val)];[(Asn1270Ser)]. The novel p.(Ile1021Val) mutation affects the first base of exon 14 and is predicted to disrupt splicing by creating a new cryptic donor site at c.3061. However the exact effect on RNA is unknown and further samples for RNA analysis were unavailable.

Included in this study were two non-consanguineous families of European ancestry with confirmed Wilson's disease in two generations (Fig. 3, Pedigrees 1 and 2). In Pedigree 1, mutations p.(Arg778Trp), c.2731-2A>G and p.(Met665Ile) were segregating, with compound heterozygotes for p.(Arg778Trp) and p.(Met665Ile) in the proband and p.(Arg778Trp) and c.2731-2A>G in a paternal aunt. In a second family (Pedigree 2), the affected adult proband was compound heterozygous for p.(Arg778Trp) and p.(Ile582Argfs*25), c.1745_1746del. He and his partner expressed concern regarding the genetic risk to their

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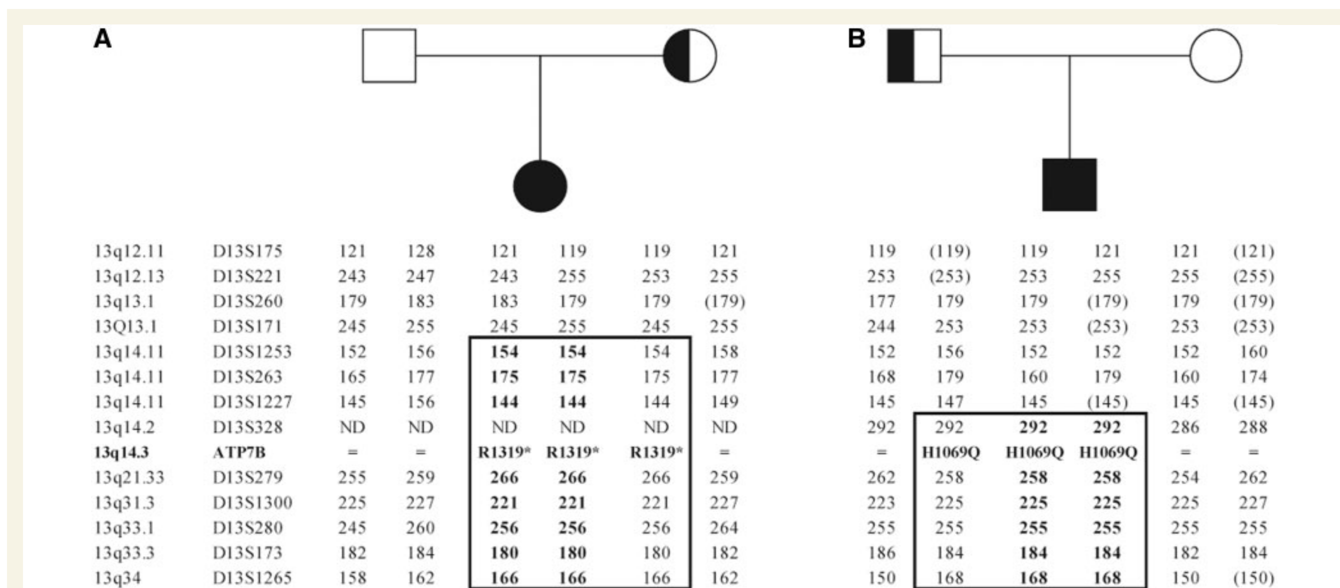

Figure 2 Uniparental segmental isodisomy as a novel disease-causing mechanism for Wilson's disease. Uniparental disomy results in the patient inheriting the identical *ATP7B* mutation carrying allele twice from the same parent. This was due to a duplication of the boxed region from 13q14.11 to 13q34 in Family A with maternal isodisomy and from 13q14.2 to 13q34 in Family B with paternal isodisomy. ND = not done; '=' = no mutation; R1319* = p.Arg1319*, (c.3955C > T); H1069Q = p.His1069Gln, (c.3207C > A). Microsatellite markers with allele sizes as marked.

two children. Despite being given a very low risk and there being no clinical symptoms in either child, they remained concerned so *ATP7B* sequence analysis was also carried out in his wife. A novel variant p.(Val1234Phe), c.3700G > T was identified which *in silico* analysis indicated was likely to be pathogenic. Following extensive genetic counselling, both children were tested for the presence of these mutations. The daughter was found to be a compound heterozygote p.[(Val1234Phe)];[(Arg778Trp)]. A subsequent liver biopsy revealed marked micro- and macrosteatosis, mild to moderate portal inflammation and focal lobular hepatitis. There was also excess of copper in periportal hepatocytes and she was started on penicillamine at the age of 5 years. The other child is a heterozygote carrier for p.(Ile582Argfs*25). A third family of Sikh origin showed affected individuals in two generations with the same genotype in both affected father and son (Fig. 3, Pedigree 3). Consanguinity was denied. Nevertheless, one of the detected variants p.(Val995Ala) was not only present in the affected father and son, but also in the mother who was a clinically unaffected heterozygote carrier of this mutation. This either suggests that the parents were distantly related or that the p.Val995Ala variant may be particularly common in the Sikh community.

The clinical manifestation of Wilson's disease was typical in all these patients with unusual genetic aspects of their condition (uniparental disomy, the presence of three *ATP7B* mutations or apparent pseudo-dominant inheritance, see Supplementary material).

Promoter analysis was carried out on a small subset of five patients with only one ($n = 3$) or no ($n = 2$) mutation in the *ATP7B* coding region or the adjacent splice sites. One variant c.-442G > A was identified in a subject with one previously identified mutation [p.Met769Val, c.2305G > A]. This c.-442G > A variant was not detected on 188 UK normal control chromosomes and is directly adjacent to the common Sardinian founder mutation (c.-441-427del) (Loudianos *et al*., 1999). *In silico* analyses, Supershift Electrophoretic Mobility Shift assays and competition assays support pathogenicity for this variant (to be reported in detail elsewhere).

Multiplex ligation-dependent probe amplification analysis was carried out in both individuals with only one *ATP7B* mutation as well as both individuals with no mutation. No exonic deletions or duplications were detected. Two mutations c.1745_1746delT, p.Ile582Argfs*25 and c.2930C > T, p.(Thr977Met) caused false positive results. This emphasizes the requirement to sequence all single exon deletions to confirm that there are no mutations or single nucleotide polymorphisms under probe-binding sites.

Genetic prevalence

In order to determine the genetic prevalence of Wilson's disease in the UK population, all 21 exons of the *ATP7B* gene were amplified and sequenced in 1008 DNA samples (referred to as the 1K data set) with exons 8, 14 and 18 being sequenced in an additional 5376 samples (referred to as the 5K data set). At the time these three exons had been identified as mutation hot spots, containing > 50% of all mutations in UK patients with Wilson's disease (Curtis *et al*., 1999). Analysis was focused on SNVs only. The functional consequences of all variants found in both the 1K and 5K data sets are summarized in Table 1.

A total of 144 SNVs were detected in the 1K data set with sequence covering each variant in an average of 941 individuals (Supplementary Table 3). Following *in silico* analyses as described above, 20 out of the144 variants were predicted to be pathogenic (referred to as 'Class 1 SNV', Table 2). Eight of these 20 putative pathogenic SNVs had previously been identified in patients with

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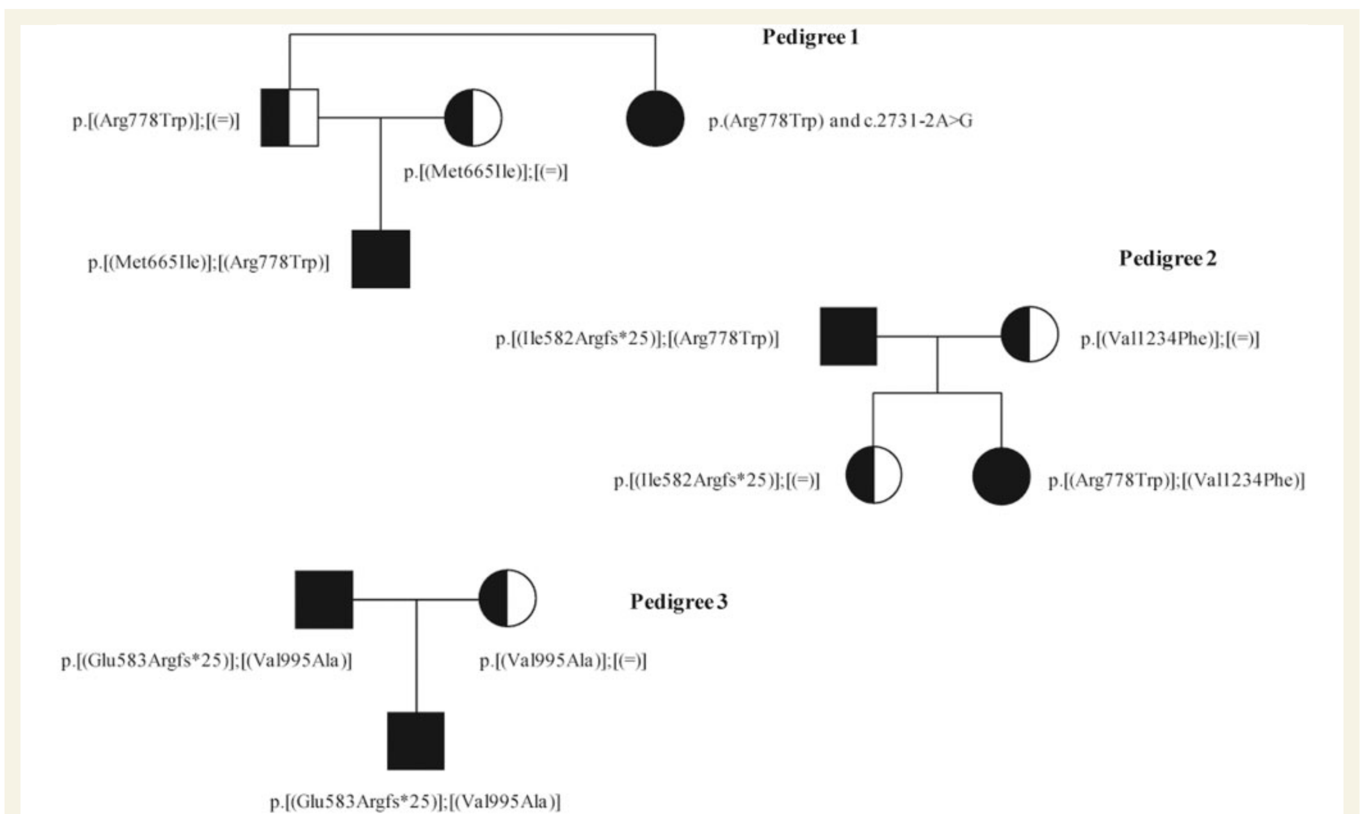

Figure 3 'Pseudodominant' Wilson's disease families. Pedigrees of three families with members affected by Wilson's disease in two consecutive generations. In Pedigree 1, both parents of the affected child were unaffected but a two paternal aunt also has Wilson's disease. In Pedigrees 2 and 3, Wilson's disease is present in father and daughter, or father and son, respectively. Circles = female family members; squares = male family members; half-shaded symbols = heterozygote mutation carriers; fully shaded symbols = homozygous mutation carriers.

Table 1 Consequences of all variants and the subset of possibly pathogenic variants found in the 1K and 5K sample sets

Consequence	Number found in 1K sample set		Number found in 5K sample set	
	All variants	Possibly pathogenic variants	All variants	Possibly pathogenic variants
Upstream	2	0	N/A	N/A
5′ UTR	4	0	N/A	N/A
Non-synonymous	36	22	15	13
Synonymous	16	0	11	0
Stop codon	0	0	1	1
Intronic	48	2	19	1
3′ UTR	36	0	N/A	N/A
Downstream	2	0	N/A	N/A

UTR = untranslated region; N/A = non-applicable.

Wilson's disease and have been classified as 'disease-causing variant' or mutation in the Wilson's disease database or seen by in patients with Wilson's disease by Sheffield Diagnostic Genetics Service (from herewith referred to as 'Class 2 SNV'). An additional four SNVs predicted to be neutral and tolerated (p.Val536Ala, p.Glu541Lys, p.Met645Arg, p.Met665Ile) were also included as Class 2 SNVs on the basis that they had been reported as disease-causing variants in the Wilson's disease database or seen in patients with Wilson's disease by the Sheffield Diagnostic Genetics Service. This brings the total of Class 2 SNVs to 12. Class 2 SNVs were detected in exons 2, 4, 5, 6, 7, 11-14, 18, 19 and 21, but not in exons 1, 8, 9, 10, 15, 16, 17 and 20 (Fig. 4). Some of these SNVs were detected in more than one sample with Class 1 SNVs being detected 54 times and Class 2 SNVs 39 times. Two individuals were identified with two variants, both with a Class 1 variant and a Class 2 variant each [first

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Table 2 Putative pathogenic variants identified in the 1K dataset

Exon	Variant coordinate	Nucleotide change	Consequence in OTTHUMT00000045981	Amino acid change	Seen by SDGS*	In WDMD DB**	Frequency (%)	*In silico* analysis	Effect on splice site
2	52548950	c.406A > T	NON_SYNONYMOUS	p.Arg136Trp	No	No	0.12	Deleterious	n/a
2	52548914	c.442C > T	NON_SYNONYMOUS	p.Arg148Trp	No	No	0.42	Deleterious	n/a
2	52548211	c.1145C > G	NON_SYNONYMOUS	p.Ser382Cys	No	No	0.11	Deleterious	n/a
4	52542680	c.1607T > C	NON_SYNONYMOUS	p.Val536Ala	No	Yes	1.05	Tolerated	n/a
4	52542666	c.1621G > A	NON_SYNONYMOUS	p.Glu541Lys	No	Yes	0.10	Tolerated	n/a
5	52539088	c.1789G > A	NON_SYNONYMOUS	p.Val597Ile	No	No	0.11	Deleterious	n/a
5	52539037	c.1840G > T	NON_SYNONYMOUS	p.Gly614Cys	No	No	0.11	Deleterious	n/a
6	52535997	c.1922T > C	NON_SYNONYMOUS	p.Leu641Ser	No	Yes	0.42	Deleterious	n/a
6	52535985	c.1934T > G	NON_SYNONYMOUS	p.Met645Arg	Yes	Yes	0.11	Tolerated	n/a
7	52534410	c.1995G > A	NON_SYNONYMOUS	p.Met665Ile	Yes	Yes	0.33	Tolerated	n/a
11	52524268	c.2605G > A	NON_SYNONYMOUS	p.Gly869Arg	No	Yes	0.11	Deleterious	n/a
12	52523908	c.2755C > T	NON_SYNONYMOUS	p.Arg919Trp	No	Yes	0.10	Deleterious	n/a
12	52523857	c.2806T > G	NON_SYNONYMOUS	p.Leu936Val	No	No	0.20	Deleterious	n/a
13	52520550	c.2930C > T	NON_SYNONYMOUS	p.Thr977Met	Yes	Yes	0.10	Deleterious	n/a
13	52520508	c.2972C > T	NON_SYNONYMOUS	p.Thr991Met	No	Yes	1.15	Deleterious	n/a
13	52520429	c.3051G > A	NON_SYNONYMOUS	p.Met1017Ile	No	No	0.11	Deleterious	n/a
14	52518281	c.3207C > A	NON_SYNONYMOUS	p.His1069Gln	Yes	Yes	0.10	Deleterious	n/a
14	52518280	c.3208C > T	NON_SYNONYMOUS	p.Pro1070Ser	No	No	0.10	Deleterious	n/a
intron 14	52518240	c.3243 + 5G > A	SPLICE_SITE, INTRONIC	n/a	No	No	0.10	No analysis	Decreases efficiency of donor splice site by between 7–34%#
18	525117766	c.3749C > G	NON_SYNONYMOUS	p.Ala1250Gly	No	No	0.10	Deleterious	n/a
18	52511706	c.3809A > G	NON_SYNONYMOUS	p.Asn1270Ser	Yes	Yes	0.31	Deleterious	n/a
intron 19	52511409	c.4021 + 3A > G	SPLICE_SITE, INTRONIC	n/a	No	Yes	0.10	No analysis	Decreases efficiency of donor splice site by between 4 and 48%#
21	52508998	c.4292C > A	NON_SYNONYMOUS	p.Ser1431Tyr	No	No	0.10	Deleterious	n/a
21	52508995	c.4295C > T	NON_SYNONYMOUS	p.Ser1432Phe	No	No	0.10	Deleterious	n/a

*denotes that the variant has been seen by the Sheffield Diagnostic Genetic Service.
**denotes that the variant has been previously identified in patients with Wilson's disease and classified as 'disease-causing variant' in the Wilson Disease Mutation Database (WMDM DB, http://www.wilsondisease.med.ualberta.ca/database.asp).
#denotes the result was seen in 5/5 splice programs used.
n/a = not applicable.

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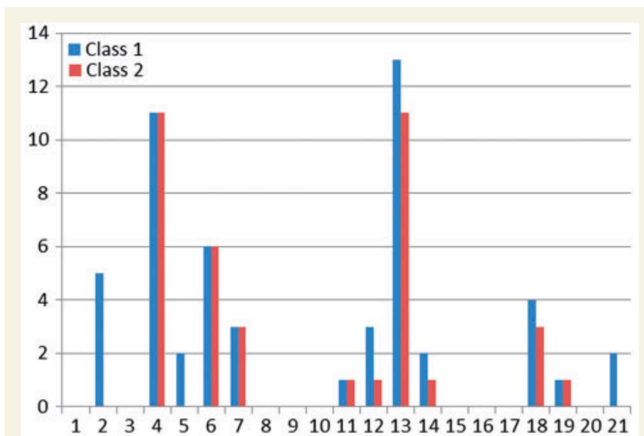

Figure 4 Exonic frequency and distribution in the *ATP7B* gene of all SNVs in the 1K data set with *in silico* evidence of pathogenicity (blue bars). The red bars denote those SNVs that have previously been identified in patients with Wilson's disease and classified as 'pathogenic' in the Wilson's disease database, University of Alberta or by the Sheffield Diagnostic Genetics Service. Exons are numbered along the bottom axis with the numbers of SNVs per exon on the left-hand axis.

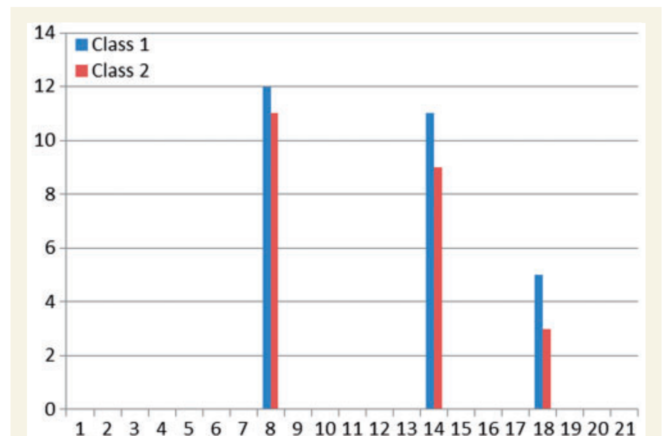

Figure 5 Exonic frequency and distribution of all ATP7B SNVs in the 5K data set with *in silico* evidence of pathogenicity (blue bars). The red bars denote those SNVs that have been previously identified in patients with Wilson's disease and classified as 'pathogenic' in the Wilson's disease database, University of Alberta or by the Sheffield Diagnostic Genetics Service. Exons are numbered along the bottom axis with the numbers of SNVs per exon on the left-hand axis.

individual: p.Ser382Cys (Class 1) and p.Leu641Ser (Class 2); second individual: p.Arg136Trp (Class 1) and p.Asn1270Ser (Class 2)]. However, it is not known if these variants are in *cis* or in *trans* and thus no further conclusions can be drawn.

The resulting variant frequency in the 1K data set for Class 1 SNVs is 0.056 [confidence interval (CI): 0.042–0.074]. For Class 2 SNVs, this reduces to a frequency of 0.040 (CI: 0.028–0.055). This frequency of heterozygote mutation carriers is considerably higher than the previously reported occurrence of 1:90 or 0.011 ($P < 2.2 \times 10^{-16}$ for Class 1 single nucleotide polymorphisms, $P < 5 \times 10^{-11}$ Class 2 single nucleotide polymorphisms only). Accordingly, the calculated frequency of individuals predicted to carry two mutant pathogenic *ATP7B* alleles (based on the frequency of Class 2 SNV only) is ~1:2500 and thus considerably higher than the frequently quoted prevalence of 1:30 000 for Wilson's disease.

One of the four Class 2 SNVs predicted to be neutral, p.Val536Ala, was found to be present at a particularly high frequency of 1.05% in our cohort. This raised questions concerning its pathogenicity and the introduction of possible skewing to our frequency calculations. The calculations were therefore repeated excluding these four Class 2 SNVs which had been reported as disease-causing but failed to have *in silico* evidence of pathogenicity (p. Val536Ala, p.Glu541Lys, p.Met645Arg, p.Met665Ile). This reduced the SNV frequency for the 20 remaining SNV to 0.024 (CI: 0.015–0.036). The calculated frequency of individuals predicted to carry two mutant pathogenic *ATP7B* alleles is then lower at ~1:7026 but still considerably higher than 1:30 000 ($P = 0.00093$).

For the 5K dataset (only exons 8, 14 and 18 were amplified), a total of 46 SNVs were detected with sequence covering each SNV in an average of 5241 individuals (Supplementary Table 3). Variants were found in all three exons but fewer in exon 18 compared with exons 8 and 14 (Fig. 5). Following *in silico* analyses as described above, 15 out of 46 SNVs were predicted to be pathogenic (Class 1 SNV, Table 3); nine of which had previously been identified in patients with Wilson's disease and classified as 'disease-causing variant' or mutation or had been seen by the Sheffield Diagnostic Genetics Service (Class 2 SNV). In total, Class 1 SNVs were detected 30 times in the 5K data set, 23 out of the 30 were also defined as Class 2 SNVs. The resulting overall frequency for Class 1 SNVs is 0.0057 (CI: 0.0039–0.0082). For Class 2 SNVs, this reduces to a frequency of 0.0044 (CI: 0.0028–0.0076). The observation of a <50% frequency of the Class 2 SNV in the 5K cohort compared to the overall Class 2 SNV frequency in the 1K cohort may be due to reduced penetrance of mutations outside these mutation hot spots.

Discussion

The phenotypic spectrum of Wilson's disease is considerably wider than previously thought with a range in age of onset from early infancy to septuagenarians (Ala *et al.*, 2005; Beyersdorff and Findeisen, 2006). *ATP7B* mutation analysis can be particularly useful in patients with an atypical presentation (Seto *et al.*, 2009). However, direct genetic analysis of *ATP7B* was previously reported to lead to the discovery of pathogenic mutations in only 80% of all patients with Wilson's disease with a confirmed clinical diagnosis (Schmidt, 2009). We now report a very high, near complete detection rate (98%) of two (or more) *ATP7B* mutations in our large UK Wilson's disease patient cohort. Typically, the previously used automated high-throughput technology such as microarrays or the 'Wilson chip' detected only a limited number of specific mutations (Gojova *et al.*, 2008). Our data would suggest that the mutation detection rate of these techniques can be

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Table 3 Putative pathogenic variants identified in the 5K dataset

Exon	Variant coordinate	Nucleotide change	Consequence in OTTHUMT00000045981	Amino acid change	Seen by SDGS*	In WD MD DB**	Frequency (%)	*In silico* analysis	Effect on splice site
8	52532610	c.2192T > C	NON_SYNONYMOUS	p.Val731Ala	No	No	0.02	Deleterious	n/a
8	52532568	c.2234T > C	NON_SYNONYMOUS	p.Leu745Pro	Yes	No	0.02	Deleterious	n/a
8	52532497	c.2305A > G	NON_SYNONYMOUS	p.Met769Val	Yes	Yes	0.14	Deleterious	n/a
8	52532470	c.2332C > T	NON_SYNONYMOUS	p.Arg778Trp	Yes	Yes	0.04	Deleterious	n/a
8	52532466	c.2336G > A	STOP_GAINED	p.Arg779X	No	Yes	0.02	No analysis	n/a
14	52518427	c.3061A > G	SPLICE_SITE NON_SYNONYMOUS	p.Ile1021Val	Yes	No	0.02	Deleterious	[#]Creates new donor cryptic splice site at c.3061
14	52518367	c.3121C > T	NON_SYNONYMOUS	p.Arg1041Trp	No	Yes	0.02	Deleterious	n/a
14	52518315	c.3173C > T	NON_SYNONYMOUS	p.Ala1058Val	No	No	0.04	Deleterious	n/a
14	52518281	c.3207C > A	NON_SYNONYMOUS	p.His1069Gln	Yes	Yes	0.14	Deleterious	n/a
14	52518267	c.3221C > T	NON_SYNONYMOUS	p.Ala1074Val	No	No	0.02	Deleterious	n/a
n/a	52518240	c.3243 + 5G > A	SPLICE_SITE,INTRONIC	n/a	No	No	0.02	No analysis	Decreases the efficiency of donor splice site by 7–34%[#]
18	52511719	c.3796G > A	NON_SYNONYMOUS	p.Gly1266Arg	Yes	Yes	0.02	Deleterious	n/a
18	52511706	c.3809A > G	NON_SYNONYMOUS	p.Asn1270Ser	Yes	Yes	0.04	Deleterious	n/a
18	52511623	c.3892G > C	NON_SYNONYMOUS	p.Val1298Leu	No	No	0.02	Deleterious	n/a
18	52511623	c.3892G > A	NON_SYNONYMOUS	p.Val1298Ile	No	No	0.02	Deleterious	n/a

*denotes that the variant has been seen by the Sheffield Diagnostic Genetic Service.
**denotes that the variant has been previously identified in patients with Wilson's disease and classified as 'disease-causing variant' in the Wilson Disease Mutation Database (WDMD DB, http://www.wilsondisease.med.ualberta.ca/database.asp).
[#]denotes the result was seen in 5/5 splice programs used.
n/a = not applicable.

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greatly improved upon by undertaking direct sequence analysis of the entire *ATP7B* coding region.

Our study highlights the need to consider unusual genetic mechanisms in the pathogenesis of this condition. The detection of three *ATP7B* mutations had previously only been reported in a single patient with Wilson's disease from a remote, isolated community in Crete with a very high incidence of Wilson's disease (Dedoussis *et al.*, 2005). The identification of a further three patients with Wilson's disease with three *ATP7B* mutations in our more diverse UK population raises important issues. In particular, laboratories not undertaking full sequencing of the entire *ATP7B* gene need to be aware of this potential pitfall and confirm that any two mutations identified are in *trans* before offering predictive or carrier testing.

The occurrence of Wilson's disease in two consecutive generations had initially only been reported in a consanguineous family and a family from the Roma sub-isolate 'Feredjelli' (Firneisz *et al.*, 2001; Mihaylova *et al.*, 2007). More recently it has also been reported in a family of Polish origin in whom all family members originated from the same small village, suggesting distant consanguinity (Dziezyc *et al.*, 2011). The identification of three families with Wilson's disease in subsequent generations in our study emphasizes the need to consider the diagnosis of Wilson's disease even in those patients in whom the family history suggests autosomal dominant inheritance. 'Pseudodominant' inheritance has been described for other recessive conditions such as Friedreich ataxia and suggests that heterozygote carrier status for *ATP7B* mutations may be more common in the UK than previously thought (Lamont *et al.*, 1997).

In uniparental disomy, both homologues of a chromosome or chromosomal region originate from only one parent. It has an estimated incidence of ~1:3500 live births (Robinson, 2000). The identification of two cases of segmental uniparental disomy in our study suggests that uniparental disomy should also be taken into consideration for genetic counselling for Wilson's disease. In addition, it highlights further the importance of confirming the *ATP7B* genotype in clinically unaffected parents of patients with Wilson's disease, whenever possible.

A 15 bp deletion in the *ATP7B* promoter region is the single most common Wilson's disease mutation in Sardinians (Loudianos et al., 1999). Subsequent studies have failed to identify this deletion in other, non-Sardinian patients with Wilson's disease, or any other *ATP7B* promoter mutations in patients with Wilson's disease in whom sequencing of the coding region and intron/exon boundaries had not identified one or both disease-causing mutations (Cullen *et al.*, 2003). The c.-442G>A promoter sequence change directly adjacent to the Sardinian mutation was the only promoter mutation detected in our study confirming that mutations in the *ATP7B* promoter region are very rare.

A gross partial *ATP7B* gene deletion covering exon 20 and major parts of the adjacent introns has previously been reported and it was suggested that large deletions of *ATP7B* may be an overlooked cause of Wilson's disease (Moller *et al.*, 2005). However, the results of our study and additional service data (unpublished) indicate that large whole exon deletions/duplications of the *ATP7B* gene are only a rare event, at least in the UK population. Thus, time consuming analysis of *ATP7B* using multiplex ligation-dependent probe amplification or other methods may only be of limited value in the molecular diagnosis of Wilson's disease.

Clustering of mutations in distinct subregions of *ATP7B* in different populations is well recognized, but has to be newly established for every population (Shah *et al.*, 1997). In our study of patients with Wilson's disease referred to the Sheffield Diagnostic Genetics Service, 82% (272/359) of all mutations could be detected by a limited screen of eight exons. This is considerably cheaper than sequencing the entire 21 exons of the *ATP7B* gene coding region. However, 24% (85/359) of all mutations were outside these 'mutation hot spots' with an additional 13% (46/359) being novel mutations. Two putative mutations (p.Val995Ala, p.Ala1063Val) were detected, the pathogenic nature of which has been a matter of debate (Thomas *et al.*, 1995; Forbes and Cox, 1998). p.(Val995Ala) was found in the compound heterozygous state in a family where both father and son had Wilson's disease (Fig. 3, Pedigree 3). In these cases, the diagnosis of Wilson's disease had been confirmed clinically and biochemically and the entire *ATP7B* coding region sequenced. All *in silico* analysis supports pathogenicity.

Only one mutation was identified in two individuals after thorough analysis of the entire *ATP7B* gene and in two individuals no mutations were detected. Possible explanations for non-detection of mutations are that the mutations are present deeper in the introns or in a regulatory region not currently analysed, a rare structural variation such as an inversion, or non-amplification of the mutated allele due to rare single nucleotide polymorphisms in primer binding sites.

High genetic prevalence of Wilson's disease

The estimated prevalence of Wilson's disease varies between 1:30 000 and 1:100 000 but can be considerably higher in isolated populations (Shah *et al.*, 1997; Ala *et al.*, 2007) with a carrier frequency of 1/11 in a remote, mountainous area of Crete (Dedoussis *et al.*, 2005). Previous attempts to determine the genetic prevalence of Wilson's disease in a more diverse typical, outbred population have been limited to the analysis of a single (His1069Gln) mutation or a limited number of *ATP7B* mutations (Olsson *et al.*, 2000; Olivarez *et al.*, 2001). The frequently quoted prevalence estimate of 1:30 000 is based on three sources (Saito, 1981; Scheinberg and Sternlieb, 1984; Park *et al.*, 1991). USA mortality figures for Wilson's disease were 13.21 per million for the years 1968–78, which Scheinberg and Sternlieb (1984) assumed was half the true number. An epidemiological study carried out in former East Germany reported an annual incidence of 2.9 per 100 000, and a further study of 162 families with Wilson's disease conducted in the Japanese population estimated a Wilson's disease population frequency of 1 in 30 000 frequency for homozygotes and 1 in 91 for heterozygotes (Bachmann *et al.*, 1979; Saito, 1981). Some of the subsequent epidemiological studies largely supported these assumptions with a resulting gene carrier frequency of 0.36–0.5% (1 in 277 to 1 in 200) (Tschumi *et al.*, 1973; Reilly *et al.*, 1993). In contrast, a study conducted in

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Scotland concluded that the prevalence rate was considerably lower (Park *et al*., 1991). Computational prediction of pathogenicity is less informative than direct experimental evidence and the accuracy of prediction is at best 75–80% (Sunyaev, 2012). However, our study suggests a high genetic prevalence of Wilson's disease even if only those SNVs are included that have previously been identified in patients with Wilson's disease and classified as pathogenic (Class 2 SNV). In addition, 17% of alleles and 22% of all mutations in Wilson's disease in the UK are small insertions or deletions (indels) that cannot reliably be detected by the high throughput sequencing and analysis strategy used for the control cohorts in our study. It is therefore likely that the frequency of Wilson's disease in the population studied is ~20% higher than either the 1K or the 5K data set indicates.

The discrepancy between the calculated genetic prevalence and the number of patients diagnosed with Wilson's disease in the UK raises profound questions. Scheinberg (1982) estimated that the correct diagnosis is only made in a quarter of all patients with the disease. Others have stated that 'perhaps 1000 cases' remain undiagnosed in the UK (Saito, 1981; Parkes, 1984). Delayed diagnosis is the most common cause of death in Wilson's disease (Walshe, 2007). Thus, perhaps the most worrying conclusion to be drawn from our data is that a large number of patients with Wilson's disease die due to lack of diagnosis or ineffective treatment.

A further possible explanation for the discrepancy between the genetic prevalence and the number of patients diagnosed with Wilson's disease in the UK is reduced penetrance of *ATP7B* mutations (Czlonkowska *et al*., 2008, 2009). The higher relative frequency of SNVs in the entire *ATP7B* coding region (1K data set) compared with the SNV frequency in the mutation hot spots (5K data set) may reflect reduced penetrance of those SNVs outside the mutation hot spots.

The identification of mechanisms determining the penetrance of *ATP7B* mutations may influence current clinical practice to categorize any individual (typically clinically unaffected siblings) with abnormal biochemical test results in keeping with Wilson's disease and/or two *ATP7B* mutations as 'presymptomatic Wilson's disease' and start them on life-long treatment.

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Supplementary material

Supplementary material is available at *Brain* online.

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