Chinese Medical Journal 2000; 113(2): 172-174

Identification of a mutation hotspot in exon 8 of Wilson disease gene by cycle sequencing

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Keywords: *Wilson disease · WD gene · cycle sequencing · mutation detection*

Objective To screen for mutation hotspot of Wilson disease (WD) gene in Chinese population.

Methods Cycle sequencing was used to detect mutation in exon 8 of WD gene in 30 patients with Wilson disease.

Results The same missense mutation, Arg779Leu, was identified in 14 WD patients, four of whom were homozygous and the other heterozygous for this mutation. The frequency of this mutation in Chinese patients was 30%.

Conclusion The codon 779 (CCG→CTG) of exon 8 of WD gene was one of mutation hotspots in Chinese.

Chin Med J 2000; 113 (2): 172-174

Wilson disease (WD) is an autosomal recessive disorder of copper metabolism first reported by Kinnier-Wilson in 1912 as hepatolenticular degeneration.[1] The disorder has an early onset in children and adolescence with the symptoms of cirrhosis, neuronal degeneration of the brain especially in the basal ganglia, Kaiser-Fleischer ring of cornea and kidney damage.

In 1985, Frydman demonstrated the linkage of Wilson disease locus (WND) to the erthrocyte esterase D (ESD) on chromosome 13[2] and at the end of 1993 the WD gene, which encodes a copper transporting P-type ATPase (ATP7B), was cloned.[3-5] In 1995, Thomas et al[6] investigated 58 WD families and detected a missense mutation (Arg779Leu) on exon 8 from two Chinese patients from Hong Kong, one of whom is homozygous and the other heterozygous. But this mutation was not detected in non-Chinese patients, which suggested that this site might be a mutation hotspot in Chinese.

METHODS

Patients and extraction of human genomic DNA

A total of 30 sporadic patients (19 males and 11 females) with a mean onset age of 16.10±6.83 years, all were patients in the WD-Ward of the Institute of Neurology, Anhui College of Traditional Chinese Medicine (TCM) from March 1995 to April 1996. They came from all over the country, 6 from Anhui, 5 from Zhejiang, 4 from Jiangsu, 4 from Shandong, 2 from Heilongjiang, 2 from Beijing, 2 from Hubei, 1 from Hebei, 1 from Jilin, 1 from Liaoning, 1 from Henan, and 1 from Jiangxi. Diagnosis of these patients was based on the WD standard and biochemical tests of serum copper, copper in 24-hour urine and ceruloplasmin. The five controls were healthy volunteers without WD family history from Fudan University. Genomic DNA was extracted by salting-out as previously reported[7] and examined by agarose gel electrophoresis and proven to be larger than 23 kb.

PCR amplification and purification of PCR products

Oligonucleotide primers were designed according to Thomas et al.[6] The primers for amplifying the exon 8 of WD gene were 5'-AACCCTTCACTGTCCTTGTC-3' and 5'-AGGCAGCTCTTTTCTGAAC-3' with the 296 bp amplified fragment. The reaction was carried out in 50 μl of solution containing 800 ng of genomic DNA, 0.5 μmol/L of each primer, 200 μmol/L of each dNTP, 20 mmol/L of Tris. HCl (pH 8.5), 50 mmol/L of KCl, 3 mmol/L of $MgCl_2$ and 1.5 U of Taq polymerase (Promega). The amplification was performed on thermocycler (PTC-200, MJ Research Inc., U S A), with a predenaturing procedure for 4 minutes at 94 ℃, for 35 cycles (denaturing at 94 ℃ for 1 minute,

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This work was supported by National Science Foundation of China (No. 39740017)

annealing at 60 ℃ for 1 minute and extension at 72 ℃ for 1 minute) followed by an additional 10-minute incubation at 72 ℃. The PCR products were examined by 1.5% agarose gel electrophoresis and then purified by PCR Product Purification Kit (Boehringer Mannhem), according to the protocol.

DNA sequence analysis

The purified PCR products were directly used as templates for cycle sequencing (Sequi Therm™ Cycle Sequencing Kit, Epicentre Technologies) according to the manufacturer's instruction. (1) End labeling of the primer: 8 sequencing reactions contain 0.5 μl of [γ-^{32}P] ATP (80 μCi; 12 pmols), 1 μl of primer (12 pmols), 2.5 μl of 10× PNK Buffer, 3 μl of T4 PNK (1 U/μl). Add deionized sterile water to an ultimate volume of 25 μl. Incubate at 37 ℃ for 30 minutes for labeling and then 10 minutes at 65 ℃ to inactivate the PNK. (2) Sequencing reaction: one reaction consists of 3 μl of labeled primer (1.5 pmols), 2.5 μl of 10× Sequencing Buffer, 1 μl of template (50 fmols). Add 1 μl Sequi Therm EXCEL Ⅱ DNA polymerase (5 U/μl) and water to 17 μl and mix well. Add 4 μl of this mixture to four tubes each containing 2 μl of A/T/G/C Sequi Therm EXCEL Ⅱ Termination Mix and overlaying with mineral oil. PCR was performed under the conditions as previously mentioned. After reaction, 4.5 μl of Stop Buffer was added to each tube and 2 μl of sample was loaded in each well of sequencing gel containing 8% polyacrylamide in 8 mol/L urea for electrophoresis, and finally autoradiograph was performed.

RESULTS

The PCR products of exon 8 of Wilson disease (WD) gene from 5 controls and 30 patients were sequenced and the same missense mutation Arg779Leu (CGG → CTG) was detected in 14 patients (Table 1). The clinical phenotype and the point mutation in the 14 patients were listed in Table 2. The sequence with the mutation was shown in the Fig.

Table 1. Mutation Arg779Leu frequency in WD patients

Groups	Number of cases	Homozygous mutation	Heterozygous mutation	Frequency of mutation
Control	5	0	0	0
WD	30	4	10	30%(18/60)

WD: Wilson disease.

Table 2. Clinical phenotype and mutations detected in 14 WD patients

No. of patient	Sex	Onset age(years)	Clinical phenotype	Clinical classification (Goldstein)	Mutation	Mutation type	Exon	Domain	Predicted effect
1-85	F	15	Cerebral	Ⅰ	Arg779Leu	Homozygous	8	Tm4	Disrupt Tm4
3-12	M	10	Cerebral	Ⅰ	Arg779Leu	Homozygous	8	Tm4	Disrupt Tm4
4-75	M	20	Cerebral	Ⅱ	Arg779Leu	Homozygous	8	Tm4	Disrupt Tm4
5-61	M	19	Cerebral	Ⅰ	Arg779Leu	Homozygous	8	Tm4	Disrupt Tm4
1-100	F	11	Cerebral	Ⅳ	Arg779Leu	Heterozygous	8	Tm4	Disrupt Tm4
2-11	F	14	Cerebral	Ⅰ	Arg779Leu	Heterozygous	8	Tm4	Disrupt Tm4
2-17	M	23	Cerebral	Ⅱ	Arg779Leu	Heterozygous	8	Tm4	Disrupt Tm4
2-22	F	25	erebral	Ⅱ	Arg779Leu	Heterozygous	8	Tm4	Disrupt Tm4
2-48	M	14	Cerebro-visceral	Ⅲ	Arg779Leu	Heterozygous	8	Tm4	Disrupt Tm4
3-13	M	18	Cerebral	Ⅱ	Arg779Leu	Heterozygous	8	Tm4	Disrupt Tm4
3-15	M	21	Cerebral	Ⅰ	Arg779Leu	Heterozygous	8	Tm4	Disrupt Tm4
4-11	F	8	Cerebral	Ⅰ	Arg779Leu	Heterozygous	8	Tm4	Disrupt Tm4
5-12	M	34	Cerebral	Ⅱ	Arg779Leu	Heterozygous	8	Tm4	Disrupt Tm4
5-54	M	10	Cerebro-visceral	Ⅳ	Arg779Leu	Heterozygous	8	Tm4	Disrupt Tm4

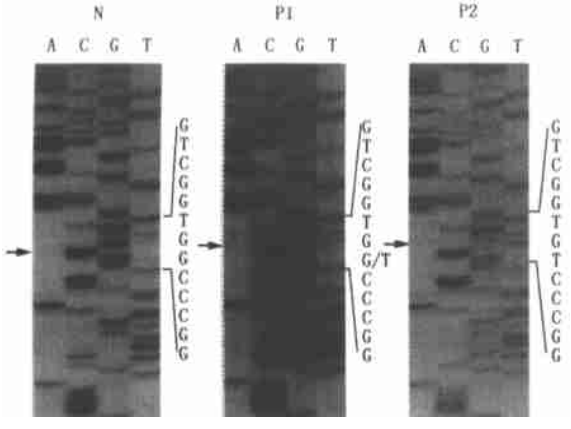

Fig. Sequence analysis of exon 8 in Wilson's disease gene. The star shows mutant site. N: control CGG/CGG, (Arg/Arg); P1: Heterozygous CGG/CGG → CTG, (Arg778/Arg778Leu); P2: Homozygous CGG → CTG/CGG → CTG, (Arg778Leu/Arg778Leu).

DISCUSSION

The identification of mutation hotspots and the early diagnosis of WD

Wilson disease (WD) is a common hereditary disease of the nervous system and is one of the rare hereditary diseases that could be cured with good effects at present. The earlier the treatment, the better the result. But the prenatal and early diagnosis cannot be carried out by the traditional methods based on biochemical data.

Since the cloning of WD gene at the end of 1993, it is possible to take out prenatal and early diagnosis. Presently, there are two major methods, linkage analysis based on RFLPs and/or STR markers and direct detection of gene

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mutations. The direct detection of mutations, which we regard as the first choice of the gene diagnosis of WD, is the most reliable and has no limitation of the family history and other conditions. However, the WD gene contains a 4.1kb encoding region in 22 exons spreading over 80 kb genomic DNA so that it is almost impossible to screen the whole gene for mutation and the selection of exon for detection will greatly affect the efficiency of diagnosis. A total of 25 mutations have been identified in exons 2, 5, 7, 8, 10, 12, 14-20 which include 13 insertions and deletions, 7 missense mutations, 3 splicing site mutations and 2 nonsense mutations.[3,4,6] Two missense mutations are relatively the most frequent, representing 38% of mutations in 34 European origins, 28% for a His1070Gln mutation in exon 14 and 10% for a Gly1267Lys mutation in exon 18 which implies that these sites might be the mutation hotspots in Europeans. To identify whether these sites are also mutation hotspots in Chinese, we took out a mutation detection in 3 exons including exon 14 in a total of 141 WD patients. A new missense mutation (Ser662Cys) was found but we can not draw a consistent conclusion about the mutation hotspot between Chinese and European.[8] Wang et al[9] detected exon 5, 14 of ATP7B gene using PCR-SSCP in 36 patients with WD. The possible mutation in exon 5 was found in 10 out of 36 patients, which suggested that exon 5 may be one of mutation hot regions in Chinese patient. However, this result from PCR-SSCP was not been confirmed by sequencing. As the WD patients have a diversity of clinical phenotypes, which strongly suggest that there are hereditary heterogeneity in WD. In the present study, 30 WD patients were screened on exon 8 using PCR-cycle sequencing and the same missense mutation (Arg779Leu) was surprisingly found in 14 patients including 4 homozygotes and 10 heterozygotes. The frequency is as high as 30%, which shows that this site is definitely a mutation hotspot in Chinese.

The relationship between WD gene mutation (Arg779Leu) and the clinical phenotype

The P-type ATPase ATP7B encoded by WD gene consists of three main domains. The first is the metal iron binding region (Cu1 ~ Cu6) situated at the N-terminal, the second one is the functional region of P-type ATPase (phosphatase domain, phosphorylation domain and ATP binding domain) and the last one has 7 transmembrane helixes (Tm1 ~ Tm7) that interspersed in the whole peptide. The mutation Arg779Leu situated in Tm4. Thomas et al[6] reported this mutation in two Chinese patients. One is homozygotes with the cerebral type and onset age of 18 and the other is a presymptomatic sib of age 15. Among the 14 patients carrying Arg779Leu mutation, 4 patients are homozygotes and others are heterozygotes. The homozygous patients showed a phenotype of cerebral while the heterozygous patients can be divided into two groups, i.e., 8 cerebral-type and 2 cerebro-visceral type. The mutation Arg779Leu is supposed to disrupt Tm4 so that the transport of copper is impaired but not abolished,[6] which is identical to the light state of illness (Goldstein Ⅰ-Ⅱ). Correlation of mutations with phenotype is still difficult because of the limitation of patients and the need to further study of exons.

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(*Received May 29, 1998*)
本文编辑: 王