Molecular and Cellular Probes 25 (2011) 195–198

Contents lists available at SciVerse ScienceDirect

Molecular and Cellular Probes

journal homepage: www.elsevier.com/locate/ymcpr

DNA and RNA studies for molecular characterization of a gross deletion detected in homozygosity in the NH_2-terminal region of the ATP7B gene in a Wilson disease patient

Simona Incollu[a], Maria Barbara Lepori[a], Antonietta Zappu[a], Valentina Dessì[a], Maria Cristina Noli[a], Eva Mameli[a], Raffaele Iorio[b], Giusy Ranucci[b], Antonio Cao[c], Georgios Loudianos[d,*]

[a] Dipartimento di Scienze Biomediche e Biotecnologie, USC, Cagliari, Italy
[b] Dipartimento di Pediatria, Università Federico II, Naples, Italy
[c] Istituto di Neurogenetica e Neurofarmacologia, CNR, Cagliari, Italy
[d] Ospedale Regionale per le Microcitemie, ASL 8, Cagliari, Via Jenner s/n, 09121 Cagliari, Italy

ARTICLE INFO

Article history:
Received 1 June 2011
Received in revised form
8 July 2011
Accepted 27 July 2011
Available online 7 September 2011

Keywords:
DNA
RNA
ATP7B
Wilson disease
Molecular diagnosis

ABSTRACT

Wilson disease is an autosomal recessive disorder caused by defective function of the copper transporting protein ATP7B. Approximately 520 Wilson disease-causing mutations have been described to date. In this study we report the use of DNA and RNA analysis for molecular characterization of a gross deletion of the ATP7B gene detected in homozygosity in a Wilson disease patient. The c.51+384_1708-953del mutation spans an 8798 bp region of the ATP7B gene from exon 2 to intron 4. The results obtained suggest that the combination of DNA and RNA analyses can be used for molecular characterization of gross ATP7B deletions, thus improving genetic counselling and diagnosis of Wilson disease. Moreover these studies, help to better establish the molecular mechanisms producing Wilson disease.

Published by Elsevier Ltd.

1. Introduction

Wilson disease (WD) is an autosomal recessive disorder caused by defective function of the copper transporting ATP7B protein [1]. This defect leads to progressive copper accumulation in the liver and brain, and organ damage. According to the HUGO database (http://www.wilsondisease.med.ualberta.ca/database.aspdatabase), approximately 520 mutations have been found in WD patients, many of which were identified in populations of Mediterranean origin. Missense mutations are largely prevalent, while approximately 80 are deletion mutations. Almost all of the latter are small deletions involving a limited number of nucleotides with the exception of an interstitial deletion encompassing the Retinoblastoma (RB) and Wilson disease loci, [46,XY,del(13)(q14q22).ish del(13)(q14q14)(rb-)] and a 2144 bp deletion removing exon 20 and large parts of the flanking introns [2,3]. In this study we report the use of DNA and RNA analysis for molecular characterization of a gross partial gene deletion detected in the homozygous state in the 5′ end of the ATP7B gene in one Wilson disease patient.

2. Materials and methods

2.1. Case description

The patient was born at term after uncomplicated pregnancy. The parents, both from the Reggio Calabria region in Southern Italy, were healthy and consanguineous. At the age of 6 months the patient was diagnosed as affected with Kinsbourne myoclonic encephalopathy and treated with anti-epileptic drugs (in a first phase ACTH, then carbamazepine and phenobarbital) until 8 years of age. At 18 months the subject showed spastic paraplegia with no morphological alterations visible by brain computed tomography; neurological impairment remained unchanged during the following observational period. At the age of 4 years, during a check-up while under phenobarbital therapy, increased serum levels of amino-transferases (AST 78 UI/L; ALT 154 UI/L) and gamma-glutamyl transferase (γGT 70 UI/L) were found. A drug-induced liver disease was suspected. At the age of 8 years, phenobarbital therapy was

* Corresponding author. Tel.: +39 070 6095504; fax: +39 070 503696.
E-mail addresses: gloudian@mcweb.unica.it, gloudian@yahoo.com (G. Loudianos).

0890-8508/$ – see front matter Published by Elsevier Ltd.
doi:10.1016/j.mcp.2011.07.003

stopped without occurrence of seizures. At the age of 13 years, the patient was hospitalized because of persistently increased serum aminotransferase and γGT levels. Wilson disease was diagnosed on the basis of low serum levels of ceruloplasmin, increased urinary copper excretion and high liver copper content (Table 2). The patient was initially treated with penicillamine which showed a poor control of Wilson's disease, in terms of aminotransferase serum levels and urinary copper excretion after 6 months of therapy. When therapy was shifted to zinc, a progressive improvement in copper metabolism parameters (urinary copper excretion $<$ 75 mcg/24 h; urinary zinc excretion $>$ 2 g/24 h) and complete normalization of liver enzymes was observed after 10 months of therapy.

2.2. DNA analysis

DNA samples were extracted from peripheral blood using salt extraction [4]. Mutation detection was carried out as previously described [5]. Mutation nomenclature was according to the Human Genome Variation Society guidelines (www.hgvs.org). Selective amplification of the deleted ATP7B allele was performed using W-2 and W-5 primers flanking the deleted DNA fragment (Table 1).

Multiplex PCR analysis for direct detection of 8798 bp deletion was carried out using 12.5 pmol of primers W-2/W-5 flanking the exon 2/intron 4 deletion breakpoints and 7.5 pmol of primers W-6/W-7 amplifying exon 20 as internal control (Table 1). Amplification reaction conditions were identical to those previously described [5].

2.3. RNA analysis

Total RNA was extracted from peripheral lymphocytes of propositus and both parents as previously described [6]. RT and nested-PCR were carried out using appropriate pairs of primers as previously described (Table 1). Sequencing was performed as described above [5]. The research procedures for both DNA and RNA analyses were in accordance with the institutional guidelines and the Declaration of Helsinki.

3. Results

Using the SSCP method for mutation analysis of the ATP7B gene we observed in the propositus, that part of exon 2 and two consecutive exons, 3 and 4, could not be amplified, suggesting the presence of a gross deletion spanning this genomic region. Using different combinations of primers for different positions in exon 2 and intron 4, we obtained a 300 bp PCR product. The combination of sense primer W-2 in exon 2 with the W-5 reverse primer localized in intron 4 spanned the deletion breakpoint (data not shown). Sequencing of the amplified fragment showed that the deletion covered a DNA region starting from nucleotide position 435 in exon 2 to nucleotide position −953, 5′ to the acceptor splice site of intron 4, for a total of 8798 bp. This deletion was named c.51+384_1708-953del (Figs. 1A, 3). The same deletion was detected in heterozygosity in both parents (data not shown).

Table 1
DNA primers used in DNA and RNA studies.

Name	Sequence (5′–3′)	Direction	Location
W-1	ATGCCTGAGCAGGAGAGACA	Forward	1-20 Exon 1
W-2	TGTGAAGTCCATTGAGGACAG	Forward	216-236 Exon 2
W-3	TGGTCCAAGTGATGAGCGTTG	Reverse	1928-1908 Exon 6
W-4	TCTATGTTGTGGACACAGGA	Reverse	1748-1729 Exon 5
W-5	GAGCCAAGGGTCCCATCT	Reverse	(1708-891)(1708-873) Intron 4
W-6	GACCTAGGTGTGAGTGCAGTT	Forward	(4022-35)(4022-13) Intron 19
W-7	CAAGTTCCACTGTGCTAAGC	Reverse	(4124+80)(4124+61) Intron 20

Table 2
Patient's clinical and laboratory data.

	Patient
Sex	M
Year of birth	1986
Age at onset (years)	14
Age at diagnosis (years)	14
Epileptogenic encephalopathy	+
Spastic quadriplegia	+
Dysarthria	−
Dyslalia	−
Tremor	−
Hepatomegaly	+
Splenomegaly	+
Kayser–Fleischer (KF) rings	−
Laboratory	
Ceruloplasmin (mg/dL)	7
Aspartate aminotransferase (AST) UI/L	90
Alanine aminotransferase (ALT) UI/L	130
Gamma-glutamyl transferase (GGT) UI/L	52
Serum copper (mcg/dl)	15
Urine Cu basal (mcg/24 h)	140
Urine Cu after penicillamine (mcg/24 h)	Not done
Liver biopsy	
Steatosis	+
Liver cirrhosis	+
Chronic hepatitis	+
Inflammation	−
Fibrosis	+
Cu content (mcg/g dry tissue)	670
Penicillamine	7 months non responder
Zinc	Responder

Normal values: Cp: 20–60 mg/dL; AST: 9–45 U/L; ALT: 8–38 U/L; Serum copper: 100–150 γ/dL; Urine copper: <100 γ/24 h; Liver copper content: <250 γ/g dry tissue.

To better understand the impact of the 8798 bp deletion on RNA structure we performed RNA studies using total RNA from peripheral lymphoblasts from propositus and both parents. Agarose gel electrophoresis of the nested-PCR products of the W-2 and the W-4 antisense primer in exon 5 showed a 159 bp band in all the analyzed samples (data not shown). Sequence analysis of the 159 bp PCR product revealed a different 5′ breakpoint in nt 333 due to the deletion of the canonical donor splice site of intron 2 that activates a cryptic GT donor splice site in nucleotide positions 334–335 (Fig. 1B and C). This 1374 bp in-frame deletion removes part of exon 2 and all of exons 3 and 4 (Figs. 1B, 3).

Screening for direct detection of deletion using multiplex PCR analysis in a group of 46 WD patients from Southern Italy who were incompletely characterized for ATP7B using SSCP and sequencing methods failed to detect the c.51+384_1708-953del (Fig. 2, data not shown).

4. Discussion

In this study we report the results of DNA and RNA studies for the molecular characterization of a large deletion detected in homozygosity in a Wilson disease patient. The c.51+384_1708-953del mutation removes part of exon 2 and exons 3 and 4 in their entirety (Fig. 1A and B). The resulting protein is predicted to lack amino acids 111–569 (458aa), theoretically resulting in the removal of part of MBD1 and the entire MBD2–MBD4 copper binding domains (Fig. 1B). Copper doesn't bind to the N-terminal portion of ATP7B as a free ion but is transferred from the Atox1 metallochaperone. The transfer of copper from Atox1 to the N-terminal domain of ATP7B occurs by a selective labelling firstly of MBD2, that results in changes of protein folding, allowing access to the other MBDs [7,8]. Since the N-terminal domain controls the