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CASE REPORT

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First application of next-generation sequencing in four families with Wilson disease in Morocco

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Abstract

Background Wilson disease is a rare autosomal recessive disorder characterized by toxic accumulation of copper in various organs, principally in the liver and brain. The disease can be manifested with hepatic, neurologic and ophthalmic signs and in a rare case with psychiatric, hematological, renal and skeletal signs; symptoms vary among and within families. Traditionally, Wilson disease was diagnosed on the basis of biochemical markers which include low ceruloplasmin levels and elevated urinary and hepatic copper. However, theses parameters are not specific and can been seen in other disorders. Genetic testing is now considering the most specific test allowing a precise diagnosis. In this study, we report the results of molecular analysis of four unrelated patients with Wilson disease from Morocco; we used a next-generation sequencing customized multigene panel to investigate the *ATP7B* gene for the four unrelated patients with Wilson disease.

Results Genetic tests based on next-generation sequencing allow to the identification of four previously described variants. One in compound heterozygous state and three at homozygous state.

Conclusions Our results confirm the clinical diagnosis of Wilson disease in these reported families and have implications for their genetic counselling and clinical management. Diagnosis of Wilson disease is a major challenge in clinical practice, and Genetic testing of *ATP7B* gene should be recommended in patients with suspected Wilson disease.

Keywords Wilson disease, Morocco, *ATP7B*, Next-generation sequencing

Background

Wilson's Disease (WD; OMIM #277900), also known as progressive hepatolenticular degeneration, is a rare autosomal recessive disorder caused by pathogenic variants of the copper transporting *P*-type ATPase, *ATP7B* leading to toxic accumulation of copper in various systems, principally in the liver and brain [1]. WD is a rare autosomal recessive disorder of copper metabolism, affecting approximately 1 individual from 30,000 of the general population [2]. WD is a potentially fatal genetic disorder with diverse phenotypic manifestations; some patients may not have any symptoms during their whole life; whereas in other cases, WD can cause severe symptoms

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such as acute liver failure and severe neurologic dysfunction [1, 2].

Traditionally, diagnosis of Wilson disease is usually made on the basis of clinical findings and biochemical parameters which include low ceruloplasmin concentration, elevated urinary copper excretion and liver copper concentration [3, 4]. However, these parameters are often not specific for WD and can be seen in others Chronic liver diseases and some healthy peoples [5].

The gene responsible for WD was first cloned in 1993, and it encodes a copper-transporting P-type ATPase that play an important role in maintaining cellular copper levels (*ATP7B*; OMIM *606882) [6, 7]. The *ATP7B* gene is located in chromosome 13 (13q14.3-q21.1) and consisting of 21 exons [8].

To date, more than 800 variants distributed throughout the coding regions and flanking intronic sequences are reported in the Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff (HGMD, http://www.hgmd.cf.ac.uk/ac/gene.php?gene=ATP7B). Because of the high degree of heterogeneity of WD compared to other genetic disorders, diagnosis poses a major challenge in clinical practice, and genetic testing is now considering the most specific test allowing a precise diagnosis [1, 3].

The present study reports the molecular results of a series of four unrelated Moroccan patients with WD using for the first time in Morocco a next-generation sequencing (NGS) customized multigene panel to investigate *ATP7B*, the WD gene. Genetic testing allows to the identification of four previously described variants. One in compound heterozygous state and three at homozygous state.

Case presentation

Patients

Four Moroccan patients belonging to four families were referred from Pediatrics services of Children's Hospital of Rabat to the department of medical genetics in National Institute of Health at Rabat, for genetic diagnosis. The age at onset, type of symptoms, presence of Kayser–Fleischer (KF) rings and biochemical parameters were collected for each patient. Table 1 summarizes the main patient characteristics and the last laboratory tests, including liver function, serum and urine copper tests.

Written informed consent was obtained from all parents of the four WD patients prior to implementation of the genetic study reported here.

Clinical data

WD-P1: Patient IV.5 of family 1 (F1) is a 15 years old girl (Patient IV.5, Fig. 1, F1), born to consanguineous parents, presented with jaundice of the bulbar conjunctiva, distension of abdomen and general tiredness. Her older and little brother died from end stage hepatic failure of unknown causes at the age of 13. Physical examination

Table 1 Clinical details, laboratory data and pathogenic variants of the four patients reported in this paper

	Patient 1	Patient 2	Patient 3	Patient 4
Age at diagnosis (years)	15	15	8	7
Male/Female	Female	Male	Male	Male
Consanguineous	+	+	–	+
Jaundice	+	+	–	+
Hepatomegaly	+	+	+	+
cirrhosis	+	+	–	–
Extrapyramidal signs	–	–	–	+
ALT (< 36U/L)	127	135	132	168
AST (< 45U/L)	109	120	91	84
Serum ceruloplasmin (0.15–0.3 g/L)	< 0.05 g/l	< 0.03 g/l	< 0.03 g/l	< 0.03 g/l
Serum copper (11–20 µmol/L)	1.13	1.25	2.5	1.7
Urine copper basal (< 50 µg/24 h)	1266	1106	960	1323
Kayser–Fleischer rings	absent	absent	absent	absent
ATP7B mutation NM_000053	c.3244-2A>G in exon 15 and c.3664delG (p.Asp1222fs) in exon 17	c.2507G>A; p.Gly836Glu	c.865C>T (p.Gln289Ter)	c.3059A>G p.Lys1020Arg
Previous reports	c.3244-2A >G in Italian patient [20] and c.3664delG in Chinese patient [21]	Moroccan patient [20]	High prevalence in Crete	French patient

AST aspartate aminotransferase, *ALT* alanine aminotransferase

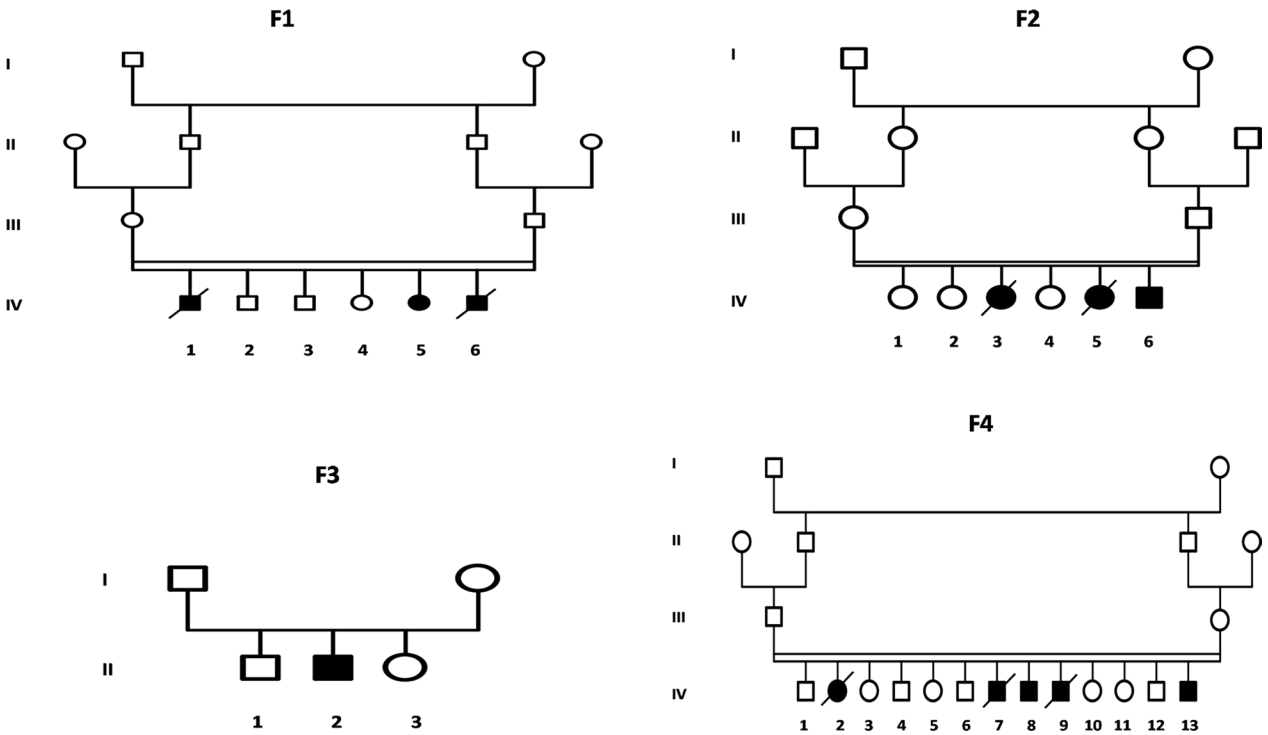

Fig. 1 Pedigrees of affected families (**F**1, **F**2, **F**3, and **F**4). Filled symbols represent affected individuals, and open symbols represent unaffected individuals

reveals the presence of jaundice, pedal edema, ascites and huge hepatomegaly with smooth surface, sharp border and non-tender on palpation. Initial investigations showed ascites and appearance of a cirrhotic liver in abdominal ultrasound scan. No KF rings were seen on ophthalmoscopy examination. The initial prescribed dose of d-penicillamine was 250 mg orally twice daily.

WD-P2: Patient IV.6 of family 2 (F2) is a 15-year-old boy (Patient IV.6, Fig. 1, F2), born to consanguineous parents, presented with fulminant hepatic failure. Two of his elder siblings died from end stage hepatic failure. Physical examination showed scleral icterus, a massive enlargement of the spleen, but liver was not palpable. KF ring was not detected on ophthalmoscopy examination. A combination of zinc and d-penicillamine was used as first-line therapy.

WD-P3: Patient II.2 of family 3 (F3) is an 8-year-old boy (Patient II.2, Fig. 1, F3) investigated for hypertransaminasemia of unknown origin that was diagnosed during the work-up to determine the cause of recurrent fever. On physical examination, the abdomen was soft and non-distended. Diagnosis of WD was based on low ceruloplasmin and serum copper levels and increased 24-h urine copper and liver copper content. No KF ring was seen on ophthalmoscopic examination. Abdominal ultrasound scan revealed hepatomegaly. Based on these findings, he was considered to be an asymptomatic WD patient with a very mild clinical picture and zinc therapy was proposed to prevent disease symptoms.

WD-P4: Patient IV.13 of family 4 (F4) is a 7-year-old boy (Patient IV.13, Fig. 1, F4), born to consanguineous parents, presented with mixed hepatic and neurological symptoms including jaundice of the bulbar conjunctiva, ataxia and dysarthria. Regarding his family, one sister and two brothers died from end stage hepatic failure and neurological deterioration between a 15- and an 18-year-old. Abdominal examination revealed ascites, the spleen and liver were not palpable. On neurologic examination, the patient had speech difficulties and extrapyramidal features consisting of rigidity, tremors, slurred speech, emotional lability, exaggerated deep tendon reflexes and ataxia. Slit-lamp examination of his eyes not appears a KF ring. We have treated this patient with a combination of trientine and zinc.

Methods

Written informed consent was obtained from all of the patients' parents before DNA collection in order to perform the genetic study. Genomic DNA (gDNA) was extracted from peripheral blood using Invitrogen Kit (PureLink™ Quick Gel Extraction and PCR Purification Combo Kit/K220001) according to the manufacturer's

instructions. gDNA quantity was measured for each sample with Qubit®3 Fluorometer (Invitrogen™) using Qubit dsDNA HS (High Sensitivity) Assay Kit. For our molecular diagnosis strategy, a targeted gene panel sequencing was performed in the first line using the Ion AmpliSeq On-Demand panel.

The final customized panel of 13 genes associated with different diseases from our consultation includes the *ATP7B* gene (NM_000053.3) responsible for Wilson's disease. This panel was composed of average 532 amplicons divided into two primer pools and in silico covered 100% of regions of interest (ROI). Libraries were prepared from 10 ng of each gDNA sample using Ion AmpliSeq Library Kit v2.0 (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. One of 16 barcodes of the Ion Xpress Barcode Adapters1-16 Kit (Thermo Fisher Scientific Life Sciences Solutions, Carlsbad, CA, USA) was added to each sample. Libraries were quantified with QubitdsDNA HS Assay Kit on Qubit 2.0 Fluorometer (Molecular Probes, Eugene, OR, USA) to get equimolar amounts of each DNA library ready to prepare clonal amplification by Emulsion PCR that performed on OneTouch2 Systems (Life Technologies, Carlsbad, CA, USA) using Ion PGM HI-Q OT2 Kit (Life Technologies, Carlsbad, CA, USA). Templates were enriched using Ion OneTouch ES (Life Technologies, Carlsbad, CA, USA) and prepared for 316v2 chip loading (Life Technologies, Carlsbad, CA, USA). Sequencing runs were performed on Ion Torrent Personal Genome Machine (PGM, Life Technologies) using Ion PGM HI-Q Sequencing Kit according to the manufacturer's instructions.

NGS Raw data in FASTQ format were aligned to the hg19 human reference genome using the Torrent Mapping Alignment Program aligner implemented in v5.10.0 of the Torrent Suite software (Thermo Fisher Scientific). For SNV calling, we used plug-in Torrent Variant Caller v5.2.0.34 (Thermo Fisher Scientific) to generate a variant call format (VCF file). For Torrent Variant Caller analysis, the germline low-stringency parameters including minimal variant frequency of 0.1, minimum variant quality of 10, minimum coverage of 5X, maximum strand bias of 0.98, and minimum variant score of 10 were used with default settings. The variants annotated and analyzed by Ion reporter software were selected if variant frequency was more than 20% and variant coverage was more than 20X.

The candidate variants were assessed in the Human Gene Mutation Database (HGMD, http://www.hgmd.cf.ac.uk/ac/index.php), Clinvar database (http://www.ncbi.nlm.nih.gov/clinvar/), gnomAD (https://gnomad.broadinstitute.org/), 1000 Genomes (https://www.internationalgenome.org/) and in the literature. The predictions of variants pathogenicity were performed using the SIFT algorithm, mutationTaster and PolyPhen2 software tools.

All potential causative variants were confirmed in probands and in the parents DNA sample of patient 1 by target direct Sanger sequencing using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Life technologies). Purification of sequencing reactions was done by Sephadex® (Sigma-Aldrich Co. LLC.) method. Samples were sequenced on an ABI 3130 Genetic Analyzer (Applied Biosystems, Life technologies). The sequences were analyzed by Mutation Surveyor® software.

In this study, NGS analysis identified four pathogenic known *ATP7B* variants in the four WD patients: For the WD-P1, we reported a compound heterozygous variant; the first was a splicing site variant c.3244-2A>G detected in exon 15 coupled to a frameshift c.3664delG (p.Asp1222fs) variant detected in exon 17(Fig. 2a). The segregation analysis in her parents showed that her father and her mother were heterozygous for c.3664delG and c.3244-2A>G, respectively.

For the WD-P2, we identified a homozygous missense variant c.2507G>A (p.Gly836Glu) in exon 10 (Fig. 2b). For the WD-P3, we identified a homozygous nonsense variant c.865C>T (p.Gln289Ter) in exon 2 (Fig. 2c), and for WD-P4, we detected a homozygous missense variant c.3059A>G(p.Lys1020Arg)in exon 13 (Fig. 2d).

Discussion

Wilson's Disease (WD; OMIM #277900) is a rare autosomal recessive disorder of copper metabolism, affecting approximately 1 individual from 30,000 in the general population [9]. Clinical manifestations are highly heterogeneous, the age of onset varies from 2 to 70 years [10, 11]. The main involvements are most commonly hepatic, neurological, and psychiatric, which can range from asymptomatic to fatal form if left untreated [12, 13]. Hepatic involvements occur in 43% of WD patients and vary from clinically asymptomatic patients with mild elevations in levels of the liver enzymes alanine transaminase and aspartate transaminase to hepatic failure and cirrhosis [2]. Neurologic symptoms occur in 35% of WD patients and may range from mild disturbances, dysarthria and trouble articulating to a severe and rapid neurologic and cognitive dysfunction [1]. Because of the progressive copper deposition in the cornea, ophthalmic symptoms can also associated with this disease and lead to formation of a dark rings that appear to encircle the cornea. The KF rings are easily detected by slit lamp examination, but it may present in only 50% of the patients with isolated hepatic involvement but often present in neurological forms in 95% [14]. Thus, WD

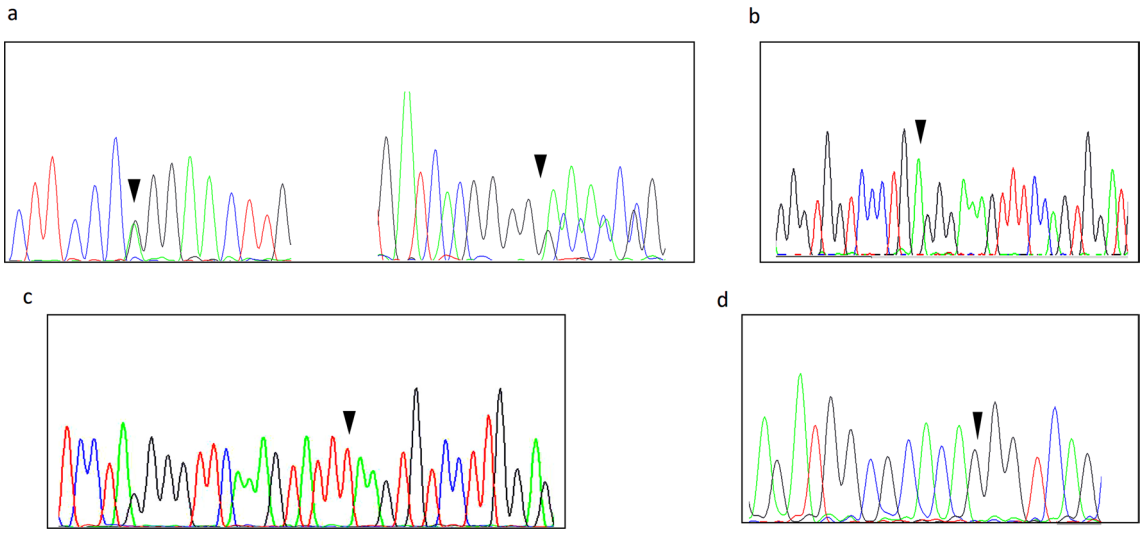

Fig. 2 The electropherograms of the identified variants in the families of this study, the variant base is indicated by an arrow

patients may develop combinations of hepatic and neurological manifestations at the same time [14, 15].

Initially, diagnosis of WD depends primarily on clinical and biochemical parameters which include low ceruloplasmin and serum copper levels and increased 24-h urine copper and liver copper content; but none of the commonly used parameters are pathognomonic and cannot distinguish it from the common liver diseases.

Mutations are distributed throughout the *ATP7B* gene. More than 800 mutations of *ATP7B* gene are reported in the Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff (HGMD; http://www.hgmd.cf.ac.uk/ac/gene.php?gene=ATP7B). However, some mutations hotspots have been highly mentioned by several authors and are considered population specific. Herein some examples, the p.His1069Gln is the most worldwide mutations that occur in 37–63% of Caucasians populations and is often associated with late and neurologic presentation of WD [1]. The p.Arg778Leu and p.Pro992Leu are the most common mutation in Asiatic population which occurs in 50.43% of patients with WD [16]. The p.Cys271Ter is relatively frequent in China's population and touch approximately 19% of patients with WD [17]. However, the spectrum of *ATP7B* mutations in Moroccan patients with WD has never been studied before. As a department of medical genetics providing genetic services and testing for a variety of diseases in low-income countries, molecular diagnostic strategies through NGS for heterogeneous diseases pose a major challenge. The recent introduction of NGS technology in our laboratory has prompted us to adopt a personalized multigene panel approach for genes involved in the most common genetic diseases from our consultation. This strategy might be the best suited for molecular testing and remains our molecular approach in the public health system, facilitating recruitment of NGS candidates, increasing the availability of multiple diagnostic tests and reduce turnaround time and costs of molecular tests. We choose to include in this panel beside 20 other genes required for others Moroccan patients, the *ATP7B* gene causative of WD to analyze the 4 unrelated patients reported in this study. As result, we identified causal mutations in the 4 cases of WD, three at homozygous state and one at heterozygous state.

In the present study, the diagnosis of WD was based on combination of characteristics symptoms, liver biochemical tests and mutation analysis of the *ATP7B* gene according on the Leipzig score established in 2003 by Ferenci et al. [18] and re-evaluated in 2010 [5].

In WD-P1, Next-Generation Sequencing gene panel allowed to detect two pathogenic variants in the *ATP7B* gene, described in compound heterozygosity: c.3244-2A>G and c.3664delG (p.Asp1222fs) in exon 15 and exon 17, respectively. The two variants were once recorded in Clinvar as likely pathogenic [https://www.ncbi.nlm.nih.gov/clinvar/variation/282915/]. The c.3244-2A>G was firstly reported in one Italian patient with WD [18]. Another pathogenic variant in the same splice site mutation, c.3244-2A>C, was also reported in one Chinese patient with early onset age of severe hepatic manifestations and poor prognosis [19].

The missense homozygous pathogenic variant c.2507G>A; p.Gly836Glu of *ATP7B* gene found in patient WD-P2 was firstly reported in 2012 as deleterious in one WD Moroccan patient [18].

The third non-sense homozygous pathogenic variant c.865C>T; p.Gln289Ter of *ATP7B* gene found in patient WD-P3 was recorded in Clinvar as pathogenic/ likely pathogenic in patients with WD [https://www.ncbi.nlm.nih.gov/clinvar/variation/3864/]. This mutation is highly frequent in a small mountain village next to Heraklion city in the island of Crete [20]. The c.865C>T was firstly reported in 2005 in one girl at the age of 6 years, wish rapidly progressing to acute liver failure that required urgent liver transplantation [20]. The patient WD-P3 is clinically quite distinct from the patient described by Dedoussis GV in 2005. Our patient was clinically asymptomatic at 8-year-old and addressed to our consultation for mild elevations in levels of the liver enzymes alanine transaminase and aspartate transaminase, but the patient described in 2005 had already developed hepatic failure and cirrhosis at 6-year-old. This case suggests that diagnosis of WD is a real challenge, and molecular analysis of *ATP7B* gene should be recommended in patients with minimal alterations to establish the final diagnosis of Wilson disease.

The missense homozygous pathogenic variant c.3059A>G; p.Lys1020Arg of *ATP7B* gene found in patient WD-P4 with severe liver and neurological symptoms, was firstly reported in 2012 as deleterious in one French patient with mixed hepato-neurologic presentation of WD. This mutation p.Lys1020Arg was found to be frequent in French patients with two others mutations, Ile1148Thr and Thr1220Met with a frequency of 19% of the total mutations [21].

In our patients, mutations have been found distributed throughout the *ATP7B* gene. The highest reported mutation H1069Q was not observed in our patients. There are a few potential theories. Firstly, several studies showed that H1069Q is associated with late and neurologic presentation of Wilson Disease, but on the other side only one patient of our cohort has neurological signs. Another hypothesis is that H1069Q mutation was not detected in our cohort, probably due to the small sample sizes studied which requires subsequent genetic studies with a large sample sizes for possible identification of this mutation in Moroccan population. Finally, we could suggest that the epidemiology of Wilson's disease in the Moroccan population may be different from those in other continents.

Conclusion

To conclude, the *ATP7B* mutational spectrum in the Moroccan population is diverse and still unexplored. To the best of our knowledge, the present study is the first report of NGS customized multigene panel in the Moroccan patients with Wilson disease. The identification of the genetic substrate in our patients confirmed the clinical diagnosis and allowed us to provide an appropriate management and genetic counseling to the families.

Abbreviations

WD Wilson disease
NGS Next-generation sequencing
KF Kayser–Fleischer

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Author contributions

MS carried out the clinical diagnosis, literature search, and manuscript preparation. AZ and YE analyzed the patients' data and helped in writing the manuscript. ICJ carried out the clinical diagnosis and contributed in writing the manuscript. TM and AS participated in the design of the study and manuscript review. All authors read and approved the final manuscript.

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Availability of data and materials

Available on request.

Declarations

Ethics approval and consent to participate

All ethical issues of the National Institute of Health in Rabat, where is housed the Department of Medical Genetics are in charge of an Intramural Advisory Committee. It gave its approval to publish clinical and molecular results of our patients in anonymous state.

Consent for publication

Written consent for publication was taken from the parents of the study.

Competing interests

The authors declare that they have no competing interests.

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