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Research Study

Sanfilippo A Disease: Biological And Molecular Study Of Moroccan Patients

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ABSTRACT

Background: The mucopolysaccharidosis III type A (MPS IIIA) also known as Sanfilippo A syndrome is an autosomal recessive metabolic disease, caused by the deficiency in the heparan sulfamidase activity. This deficiency due to mutations in the SGSH gene causes progressive accumulation of heparan sulfate in lysosomes.

Objective: The aim of this work was the clinical, biological and molecular studies of the MPS IIIA characteristics in two Moroccan patients.

Patients and methods: Two unrelated patients suspected suffering from Sanfilippo syndrome were studied. The BST, urinary GAGs investigations including measurement of the GAGs rate and their identification by chromatography and measurements of heparan sulfamidase and alpha-D-N-acetyl glucosaminidase activities were carried out. When the deficiency of the heparan sulfamidase was confirmed the sequencing of the SGSH gene was performed on genomic DNA from patients and their parents to characterize mutations

in this gene.

Results: The BST suggested that these patients have mucopolysaccharidosis. The urinary screening indicated that they have MPS III. The Heparan sulfamidase and alpha-D-N-acetyl glucosaminidase assays confirmed the diagnosis of MPS IIIA. The molecular diagnosis by direct sequencing of the SGSH gene identified the missense mutation p.R377C in the homozygous state in the two patients.

Conclusion: The knowledge of the mutational spectrum of the SGSH gene will provide the phenotype/genotype correlation of the disease and will allow a genetic counselling for families at risk.

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INTRODUCTION

The mucopolysaccharidosis III (MPS III) or Sanfilippo disease constitutes a group of chronic, progressive, hereditary and rare diseases. MPS III is the result of a deficiency of four lysosomal enzymes involved in the degradation of heparan sulphate [1]. These enzymes are: heparan sulfamidase, *N*-acetyl-alpha-D-glucosaminidase, alpha-glucosaminide-*N*-acetyltransferase and *N*-acetylglucosamine-6-sulfate sulfatase which deficiencies lead to the four subtypes A, B, C, and D respectively. The occurrence of heparan sulfate in the nervous system explains the difference of symptoms in contrast with other MPS.

Regarding the MPS IIIA, it is a metabolic lysosomal and autosomal recessive disease [2] caused by mutations in the heparan sulfamidase gene or *N*- sulfoglucosamine sulfohydrolase (SGSH) [3].

The purification of the SGSH gene and its characterization open the possibility to investigate various pathogenic mutations in MPS IIIA patients. This gene has an approximate length of 11 kb encoding, located on chromosome 17q25.3 and contains eight exons. Characterized in 1996 it codes for 502 amino acid protein [4]. Up to this date, 147 mutations in the SGSH gene have been identified [5], including 112 missenses mutations, 18 micro-deletions, 9 micro-insertions, 3 splicing site mutations, 3 large deletions, a large insertion and a micro-indels. These mutations may result in a severe, intermediate or moderate phenotype.

In the present work, in addition to the phenotypic diagnosis, we performed the molecular analysis of the SGSH gene in two MPS IIIA Moroccan patients using genomic DNA samples. The aim of this screening is to determine the mutation responsible for this disease. This study is the first genetic research carried out on MPS IIIA to be reported in Morocco.

PATIENTS

This study was conducted on two patients aged 2 and 5 respectively. These patients were born from a consanguineous marriage, and there were no known relationship between the two patient's families. The first case is from the north of the country while the other is from the south. Both patients are recruited from a private pediatric practice in Agadir. Informed consent was obtained from the patient's parents investigated.

The clinical description of both patients (table 1) had shown macrogloria, coarse features, short neck, thick hair, facial dysmorphic syndrome, joints stiffness, abnormal behavior and sleep disorders. As for the 5 years old patient, she also presents hepatosplenomegaly, trapped hands and speech disorder since words she pronounces are incomplete. Her younger sister is healthy but her maternal aunt has died from the same disease. For both patients parents, the abnormal behaviour was the main motive for pediatric consultation.

Table 1: Characteristics of the two studied patients.

	Patient 1	Patient 2
Age (years)	2	5
Gender	Female	Female
Weight (kg)	13	22
Age of 1 st diagnosis (years)	2	5
Age of 1 st symptom (months)	6	24

METHODS

Biochemical Diagnosis

The diagnosis of the MPS IIIA disease was based on the following approach after a clinical suspicion. The phenotypic diagnosis was guided by clinical examinations and then screened by performing urinary tests and enzymatic assays.

Urinary tests

Berry Spot Test

The Berry Spot Test (BST) which is a semi-qualitative analysis, is one of the first tests performed during the diagnosis of mucopolysaccharidosis. The Mabe protocol was followed to perform this test [6].

Quantitation and characterization of urinary GAGs

Urinary glycosaminoglycans (GAGs) were quantified using a method described by Humbel [7]. For that, 200 µl of cetylpyridinium chloride (5%) was added to a 10 ml of patient urine samples. The resulting final precipitate is redissolved in 100 µl of sodium chloride (0.6 M). This solution is used for the determination of hexuronic acids through a colorimetric assay with harmine and the results are expressed in mg of glucuronic acid/ mmol of creatinine.

The chromatography on cellulose acetate plate was carried out to identify the type of accumulated GAGs. The bands were visualized by alcian blue reagent[8].

Heparan sulfamidase and alpha-D-N-acetyl glucosaminidase assays

To determine which subtype of Sanfilippo our patients suffer from, we have measured the two enzymes, heparan sulfamidase and alpha-D-N-acetyl glucosaminidase responsible for the subtype A and B, respectively.

The heparan sulfamidase activity was measured in leucocytes by using the fluorogenic substrate

4-Methylumbelliferyl-2-deoxy-2-sulfamino- α -D-glucopyranoside as described by Karpova [9]. Protein was determined by Bradford method [10]. The rate of 4-methylumbelliferone released was measured in Turner fluorometer Model 450 003 at emission and excitation wavelength 360 nm and 450 nm, respectively. The alpha-D-N-acetyl glucosaminidase activity was measured as described by Yon Figura [11].

Molecular Analysis

DNA extraction and PCR amplification

Genomic DNA was extracted by the phenol/chloroform procedure, from the blood cells of the patients, their parents and controls, then precipitated by saturated alcohol and solubilized in distilled water.

Since in our population, mutations in this gene were so far unknown, we have projected to screen the whole SGSH gene by direct sequencing. We have started by screening the two exons 6 and 8 where most common mutations are found among the North African population.

The exons were amplified by PCR, using the following primer couples (Table 2). The PCR was performed on a total volume of 25 µl using 150 ng of genomic DNA. The PCR characteristics were 0.2 mM of dNTP; 1.5 mM of MgCl₂; 5% DMSO; 0.4 µM of each primer and 0.2 µl (5 units) of Taq DNA polymerase (Bioline).

The DNA samples were denaturated for 1 minute at 95°C and then subjected to 35 amplification cycles. Each cycle included a 15 seconds denaturing step at 95°C. The hybridization lasted 20 seconds at temperatures indicated in table2. The elongation step was performed at 72°C for 15 seconds. The terminal extension lasted 3 minutes at 72°C.

Table 2: Pair primers sequences of the SGSH exons 6 and 8

Exons	Sequences	Hybridization temperature
Exon 6	Sense TGTTCTAAGCCTGGCTCCC Antisense CTGCCACACTGGACCCTC	61°C
Exon 8	Sense TTGGATTGGAGAAGGGAGC Antisense CCGGTAGTAGTAATGACGGAGG	65°C

Identification of mutations by direct sequencing

The PCR products obtained in the presence of the primer pairs were purified using the ExoSAP-IT kit, then sequenced using a sequencing kit (BigDye v3.1) and an automatic sequencer (ABI 3130xl Genetic Analyzer, 16 capillary sequencer, Applied Biosystems).

RESULTS

Phenotypic diagnosis

Berry Spot Test

The presence of a blue spot in the centre of the whatman paper disc in the patients sample in contrast of controls ones suggests that these patients have mucopolysaccharidosis.

Urinary GAGs investigations

Quantitative measurement of GAGs expressed in mg of glucuronic acid/mmol of creatinine yielded values of 4.2 and 3.7 for patient 1 and 2, respectively. For healthy peer aged the value measured in parallel to patients was less than 2.6. The GAGs chromatographic patterns

showed the presence of an abnormal single band corresponding to heparan sulfate, compared to the control case. These results indicate that both patients have type MPS III.

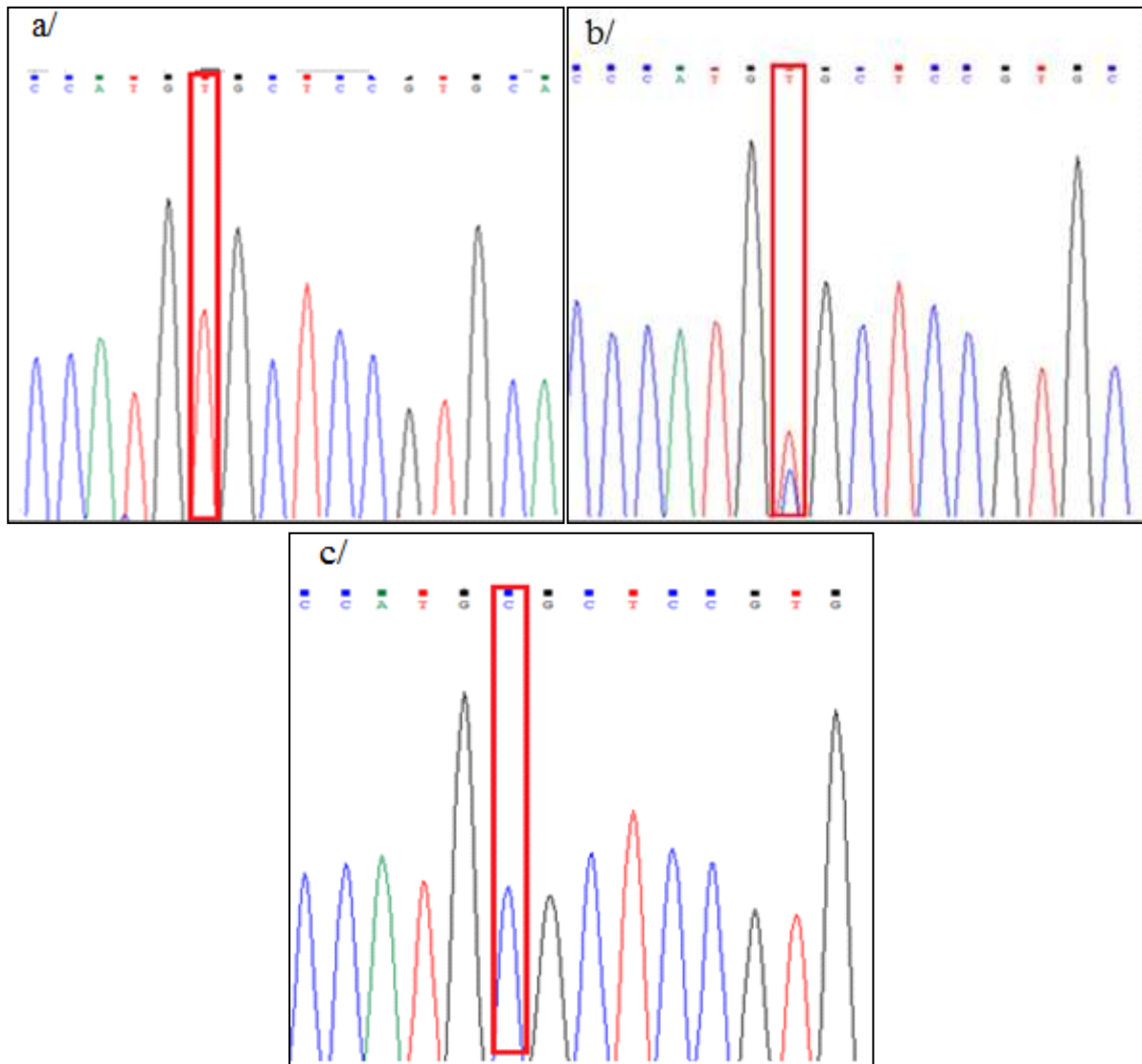
Enzymes assays

The alpha-D-N-acetyl glucosaminidase was normal which excludes subtype B while no activity of heparan sulfamidase was detected. The lack of heparan sulfamidase activity confirmed the subtype MPS IIIA in both our patients.

Molecular Diagnosis

The amplification followed by sequencing of the exon 6 from the two studied cases doesn't show any alteration. The screening of the exon 8 identified, for both of them a missense mutation: p.R377C, due to a C-T transition of the first codon nucleotide 377, at nucleotide position 1129 in the cDNA causing the switch of amino acid from arginine to cysteine. This mutation appears homozygous in both patients and heterozygous in their parents. There are no changes in the control alleles (Figure 1).

Figure 1: SGSH exon 8 sequences profiles showing the p.R377C mutation. a: MPS IIIA patients sequence; b: parents sequence; c: Normal sequence.



DISCUSSION

Sanfilippo A syndrome is an inherited metabolic disorder that causes progressive neurocognitive degeneration. In this study, both patients have a severe clinical phenotype of Sanfilippo A disease with facial dysplasia that was revealed in their first year, neurological complications, behavioral disorders, hyperactivity, sleep disturbances and skeletal abnormalities. The 5 years old patient also has strong hepatosplenomegaly and speech disorder. These clinical manifestations are reported in previous studies that have provided data on disease progression and the development of clinical features for the MPS IIIA such as: language delay (93%), coarse features (92%),

abnormal behaviour (75%), hepatomegaly (51%), autism spectrum disorder (29%), and epilepsy (17%) [12] and similar symptoms; like hyperactivity [13].

In this work we have followed a diagnosis strategy; starting by the BST, which have revealed that the studied patients have the mucopolysaccharidosis. Then, urinary GAGs investigations indicated that they have the MPS III type. After that, enzymatic assays have specified that they have the MPS IIIA subtype. Currently, heparan sulfate concentrations from Sanfilippo patients are measured not only in urine but also in plasma [14]. The concentrations corresponding to the severe phenotype are higher

than those corresponding to the attenuated phenotype. This suggests that measuring these concentrations in blood and urine is a biomarker of the clinical severity of MPS III [15].

All studies, reported elsewhere, are based on clinical phenotypes, biochemical profiling and genetic analysis, but in Morocco all reported cases do not have genetic information [16]. This is the first genetic study reported in Morocco to this date.

Molecular analysis in our MPS IIIA patients shows the presence of the p.R377C mutation in the homozygous state. This mutation was reported to be a pathogenic variant [17]. Each alteration in the R377 residue by the mutation such as R377C, R377H, R377T or R377L resulted in similar changes in the structure of the SGSH protein, resulting in the loss of protein function and the onset of the disease [18].

For the patients 1 and 2, the disease onset has occurred at 6 and 24 months respectively. Furthermore, the progressive character of the disease is well observed, since the older patient has more morbid traits. The p.R377C missense mutation would be then associated with the early age of onset and rapid progression of the disease. Indeed, this mutation was associated with a severe phenotype [17]. The frequency determination of this mutation in our population will be carried out after molecular analysis of other cases of MPS IIIA by enzyme digestion or Single Strand Conformation Polymorphism in addition to sequencing.

The p.R377C mutation was previously reported in Italian patients with other 12 missenses mutations [17]. It was found in a Tunisian patient in the heterozygous state with a large deletion: g.75802301_75809393del7093 bps [19, 20]. This was one of the four mutations identified in two Chinese patients [21].

On the other hand, Sanfilippo A disease is characterized by a high heterogeneity and it was reported that a high number of mutations are geographically specific. We could thought that the p.R377C mutation would be specific to Moroccan patients since one of them is from the north of the country while the other is from the south. In this study, we did not identify the Q365X nor the D477E mutations that appear to be common in the Tunisian population [20] and that

result in a severe phenotype; neither the R245H mutation which is found in 31% of mutant alleles, 35% and 58% in Australia, Germany and Netherland, respectively; nor the S66W mutation which is identified in 29% in a cohort of Italian patients; neither the R74C mutation, which has a frequency of 56% in the Polish population [22,23]; nor the common G205R mutation in Indian patients [24]. As in our patients, molecular defect resulting in a Japanese compound heterozygous patient are on exon 8, these are S347F and D444G mutations [25]. This heterogeneity may explain the important phenotypes variability observed in MPS IIIA patients.

It should be mentioned that both patients are born from first-cousins parents. In fact, inbreeding unions with various degrees between Moroccans are very high, especially in rural areas of the country. The first-degree inbreeding is still the most common one [26]. This type of social and cultural tradition probably promotes the exposure of rare mutations causing some serious diseases such as Sanfilippo A syndrome.

Regarding the prevalence of the Sanfilippo syndrome, it is difficult to establish it with certainty because it is under-diagnosed; more over, it is even less for the subtype A. Nevertheless, the most recent statistics estimates the number of concerned births between 0.08 and 1.16 per 100000 people, depending on the studied country. Indeed, studies carried out in France [12], Germany [27] and Australia [28] estimated it at 0.48, 1.11 and 0.62 respectively. In Morocco, Sanfilippo syndrome, regardless its subtype, accounts for 12% of a 27 MPS patients series [16]. Since 2014, we have been interested in all types of MPS, however, no Sanfilippo B, C nor D were encountered. Among Sanfilippo subtypes, the only cases encountered are the patients studied here. This rarity would be due to misdiagnosis, or to the frequent replacement of the south Moroccan public hospitals clinicians with whom we have established contacts. In addition to that, some families have refused cooperating with this research, others were ashamed of having a handicapped child.

As for the treatment of Sanfilippo A disease it remains only symptomatic and hard. Bone stem cells transplantation has shown to be ineffective

on neurological problems. The substitutive enzyme replacement which appears to be effective in decreasing the progression of the MPS I, II and IV diseases is not yet available or may be under development [29]. Hence, genetic counselling remains the generally proposed preventive solution for families at risk. The knowledge and study of the different mutations, affecting the SGSH gene, will allow us to better understand the clinical and biological heterogeneity of the disease and to consider therapeutic possibilities.

Furthermore, in Agadir city, the medical school has recently opened, and it has shown its interest to our research field, so in cooperation with physicians, we, scientists, will transfer the diagnosis method to the hospital, hence, patients could be early diagnosed and well monitored as it is done in other countries.

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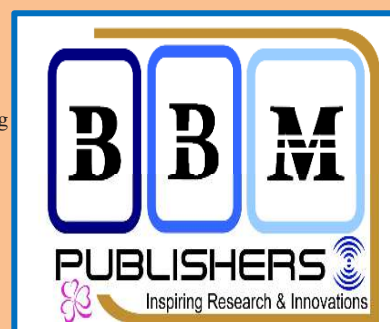
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