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CTNS Molecular Genetics Profile in a Portuguese Cystinosis Population

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Abstract

Background: Cystinosis is a multisystemic autosomal recessive deficiency of the lysosomal membrane transporter protein (cystinosin) caused by mutations in *CTNS* gene. *Objective*: This study summarizes the Portuguese experience in the diagnosis and management of patients with this rare disease over the past few years and reports recurrent mutations in the *CTNS gene*. *Methods*: Unrelated patients from different pediatric and adult hospitals all over Portugal with non-nephrotic proteinuria, hypercalciuria, hypokalemia impaired proximal reabsorption of amino acids, glycosuria and hypophosphatemia, suggestive of a Fanconi syndrome and ocular problems, were studied. Intra-leukocyte cystine levels were determined and molecular analysis was performed, to determine the presence or absence of the 57-kb deletion in *CTNS*, followed by direct sequencing of the coding exons of *CTNS*. *Results*: From 1998 to 2017, twenty-one cystinotic patients were biochemically diagnosed. From the remaining seventeen (four deceased), eleven were studied for *CTNS* gene. Five out of eleven patients were homozygous for the 57-kb deletion (10/22; 45.5%), and other five were compound heterozygous for this variant (15/22; 68.2%). The other mutations found were p.Q128X (c.721 C>T; 2/22), p.S139F (c.755 C>T; 4/22) and c.18-21delGACT (p.T7FfsX7; 1/22). All

of these seventeen cystinotic patients are in treatment. Approximately 84% are adults, 16% are young children, and 54.5% are kidney transplant recipient. *Conclusions*: The authors would like to emphasize the importance of first screening for the 57-kb deletion since it is very common in our population. This genetic study is the first in our country and it could be the basis for future genetic counseling in Portuguese population.

Keywords

Cystinosis, *CTNS* Gene, Mutational Spectrum, Kidney Failure, 57-kb Deletion

1. Introduction

Cystinosis (OMIM #606272 ORPHA213) was first described in literature in 1903 [1] and is a rare autosomal recessive systemic lysosomal storage disease characterized by the abnormal accumulation of lysosomal cystine due to mutations in the cystinosin gene (*CTNS*) [2]. The *CTNS* gene is localized on human chromosome 17p13 contains 12 exons, the first two of which are non-coding [2] [3] and the other ten encode a 367 amino acid protein named cystinosin. The incidence of this disease is estimated to be 1 in 100,000 - 200,000 live births, though exact numbers are difficult to obtain because the disease is often undiagnosed and/or misdiagnosed. Cystinosis is the most frequent cause of inherited Fanconi syndrome and should be considered the initial differential diagnosis. Nevertheless, the severity of Fanconi Syndrome associated with cystinosis requires rigorous treatment that is frequently complex [4]. Although the kidneys are initially affected during the first year of life, in most of the cases, other potentially affected organs include also eyes, thyroid, pancreas, gonads, muscles and CNS over time [3] [5]. The severity of the phenotype is correlated with the genotype. Accurate measurement of intracellular cystine content is obligatory for the diagnosis of cystinosis and still the cornerstone for both diagnosis and therapeutic monitoring of the disease [6].

The most commonly detected cystinosis mutation is a 57-kb deletion that deletes the majority of *CTNS* gene [7] [8] and in general, is related with high doses of cystine in leukocytes. This deletion is present in almost 50% of all the Northern European and North American origin [3] [7]-[13]. However, outside this geographical distribution, the mutation is almost completely absent, especially in the Middle East [14] [15] [16], therefore migration and/or ethnicity could be the explanation for this fact as well as the concept of possible other founder mutation from those regions [3]. Since the cloning of *CTNS* gene, over 200 pathogenic mutations have been reported in different ethnic groups, including mutations in the promoter region, missense, nonsense, deletion, insertion and splice mutations(http://www.hgmd.org/) [13] [17].

Cystinosis has three major clinical presentations: the most serious form, the

infantile nephropathic form or classical nephropathic cystinosis (MIM 219800), a less severe form, the juvenile nephropathic form (MIM 219900) and the ocular non-nephropathic form (MIM 219750) [18]. The infantile or classical cystinosis, comprising about 95% of all cases. The non-nephropathic, or ocular cystinosis manifests exclusively with ocular involvement due to cystine crystals deposition [18] [19] [20]. In some patients, ocular involvement can precede renal manifestation by several years [21]. It has been hypothesized that the underlying differences between the clinical forms and the severity of them is because individuals with infantile cystinosis harbour "severe" mutations (*i.e.* loss of function) on both alleles, whereas individuals with milder forms of cystinosis harbour a specific, non-severe mutation (*i.e.* never observed in cases of infantile cystinosis) either on both alleles or in association with a severe allele (compound heterozygous) that is why is so important to characterized the mutation spectrum of this disease [7] [22] [23] [24]. Molecular analysis of the *CTNS gene* also confirms the diagnosis and offers the advantage of a prenatal diagnosis [25]. Several lines of treatment are available for cystinosis. When started at an early age, cysteamine treatment prevents or postpones the deterioration of renal function and the occurrence of extra-renal complications such as hypothyroidism, diabetes mellitus, retinopathy, encephalopathy and myopathy [26] [27].

The aim of this study was to determine the mutational spectrum of Portuguese population for cystinosis, as well as established a genotype/phenotype correlation to the mutation found with the respective cystine intra-leucocytes value and the clinical presentation. This study is the first one in the Portuguese population.

2. Material and Methods

2.1. Patients

Studies were conducted from 1998 to 2017, where a total of 1,941,372 newborns were registered. In this cohort, 32 unrelated patients from different pediatric and adult hospitals all over Portugal had non-nephrotic proteinuria (<3.5 g protein/24H), hypercalciuria, hypokalemia, impaired proximal reabsorption of amino acids (hyperaminoaciduria), glycosuria and hypophosphatemia, suggestive of a Fanconi syndrome. Later, all developed also ocular complains, that revealed intraocular cystine crystals, a very important find to correlate this Fanconi syndrome with cystinosis. Although most patients had Fanconi syndrome (20/21), one patient had no Fanconi syndrome but an earlier nephrotic chronic disease (with kidney damage and >3.5 g protein/24H) diagnosed only in adolescence (at the age of 15 years). In this particular patient, the diagnosis of the juvenile nephropatic form of cystinosis was done when she got pregnant. In fact, from these 32 patients, 21 patients revealed high values of intra-leukocytes cystine in blood (≥0.50 nmol 1/2 cystine/mg protein). Unfortunately, from these 21 patients (8 males, and 13 females, with ages between 1 - 40 years), four deceased, two we lost the clinical history (*i.e.* emigration) and from the remaining fifteen,

only eleven were characterized at a molecular level. From this study, it seems that generally, not only ocular cystine crystals preceded Fanconi syndrome, but also contributes to the correct final diagnosis. The authors would like to emphasize, that from these 21 positive cystinosis patient, only one patient was diagnosed in adulthood (at the age of 40 years old) due to presence of intraocular cystine crystals, although he have had chronic nephrotic complains (such as proteinuria, calciuria, hypokalemia and hyperaminoaciduria) since pediatric age.

2.2. Ethics Statement

All procedures were followed in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 and approved by the Ethics Committees. Informed consent was obtained from all patients and their families for being included in the study.

2.3. Biochemical Methods

The specific biochemical diagnosis was performed by study of urine and the detection of elevated intra-leukocyte cystine levels (Whole Blood Cells Cystine intra-leukocyte levels; WBC Cis IL) [28]. Prior extraction, cystine quantification was obtained by ion exchange liquid chromatography in a BIOCHROM 30®. The reference values used were:

- Healthy individual: <0.5 nmol 1/2 cystine/mg protein.
- Affected individuals without treatment: ≥0.5 nmol 1/2 cystine/mg protein.
- Individuals treated with good therapeutic control: ≤1.0 nmol 1/2 cystine/mg protein.

2.4. Molecular Diagnosis

Genomic DNA was automatically extracted from whole blood or dried blood spots, using an automated method (EZ1 DNA Blood 350 µl, or EZ1 DNA tissue kit, QIAGEN). The ten protein-coding exons and flanking intronic sequences of *CTNS gene* (NM_004937) were directly sequenced after PCR amplification in an ABI PRISM™ 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) [7]. All patients were first screened for the 57-kb and if negative, whole *CTNS* gene was sequenced. As a control population, we studied 100 healthy (200 alleles) unrelated individuals of Portuguese origin.

3. Results and Discussion

During 19 years, 32 patients with non-nephrotic proteinuria, hypercalciuria, hypokalemia, hyperaminoaciduria, glycosuria and hypophosphatemia, suggestive of a renal Fanconi syndrome and/or ophthalmologic symptoms were biochemically evaluated due to cystinosis clinical suspicion. Usually the diagnosis is made shortly after the onset of the symptoms but some delay may occur due to the low specificity of the symptoms. From those 32 patients suspected, 11 had an

intra-leukocytes cystine value below 0.5 nmol 1/2 cystine/mg protein in two successive samples [4] and they were considered negative for cystinosis and other causes were investigated. In the remaining 21, intra-leukocytes cystine ranging from 0.5 to 8.0 nmol 1/2 cystine/mg protein was confirmed and eleven of these were studied for *CTNS* gene mutations, four pathogenic reported *CTNS* mutations were detected, being the common European 57-kb deletion the most frequent (68.2%; 15/22 alleles). Five out of eleven patients were homozygous for the 57-kb deletion, and five were compound heterozygous for 57-kb deletion (45.5%) and p.Q128X (c.721 C>T; 9.1%), p.S139F (c.755 C>T; 9.1%) and c.18-21delGACT (p.T7FfsX7; 4.5%), mutations (**Table 1**). One patient was homozygous for p.S139F mutation. From the literature, it seems that this mutation in homozygous state is the first time found. We can also conclude that patients with severe or truncating mutations, as the 57-kb deletion and p.T7FfsX7 or p.Q128X had the infantile severe form of the disease, while juvenile cystinosis was associated with at least one mild mutation. We found that patients with the 57-kb deletion had higher cystine intra-leukocyte levels (WBC Cis IL), which is also in concordance with the literature (**Table 1**), because this specific mutation results in the absence of protein [4] [8]. In Portugal, it is estimated that this disease has an incidence of 1/92,000 newborns.

The most common *CTNS* mutation in Northern Europe is the 57-kb deletion, which is in accordance with our results. In fact, the 57-kb deletion accounts for about 50% of cystinosis chromosomes in European populations [29] [30]. It is thought that this deletion has arisen because of a founder effect, originated in Germany around the middle of the first millennium A.D. [3] [7] [9]. The hypothesis of this deletion having arisen in the Northern Europe and having spread throughout Europe toward Northern Africa by migration is consistent with a north to south gradient as already found for the p.F508del cystic fibrosis mutation. In fact, African influence is largely concentrated in the Southern and Western regions of Europe, and Germanic influence is small and limited. Therefore, when comparing our results with other countries from North European and North American origin, the 57-kb deletion in Portugal is also the most prevalent mutation (68.2% of the mutant alleles). This is also true, for other countries, as Spain [12], which has a 57-kb deletion prevalence of 34%, in Netherlands [31] 59% and 65% in Switzerland-Germany population [11], but it decreases to 17% in Italy [14] and is absent in Turkish population. At a first glance, we should expect to have a cystinosis spectrum mutation similar to the Southern Europe population (*i.e.* due to our emigration history), but in fact does not happen. We have a different mutational spectrum for *CTNS* gene comparing with the mutational spectrum of African, Mediterranean and Middle East populations. Indeed, it seems that outside this geographical distribution (North Europe and North America), the 57-kb deletion is almost absent, especially in the Middle East [32] which appears instead to have a common exonic splice site mutation; p.E227E (c.681 G>A) [33]. In fact, this mutation, comprises 39.5% of the Iranian [15], 20% of Turkish [34] and 15.4% of Saudi familial mutant alleles [33]

Table 1. Correlation between the genotype, value of intra-leukocyte cystine at the age of diagnosis and presentation form of cystinosis in the studied patients.

Mutation Status	Patients	Intra-Leukocyte Cystine Value at Diagnosis (nmol1/2cystine/mg protein)	Presentation Form of Cystinosis
57-kb deletion/57-kb deletion	(N = 5)	[2.8 - 8.0]	Infantile Nephropathic form
57-kb deletion/Q128X	(N = 2)	[1.5 and 5.1]	Infantile Nephropathic form
57-kb deletion/p.T7FfsX7	(N = 1)	[7.3]	Infantile Nephropathic form
p.S139F/p.S139F	(N = 1)	[1.7]	Juvenile (late) Nephropathic form
57-kb deletion/S139F	(N = 2)	[2.2 and 3.7]	Juvenile Nephropathic form

and was not detected previously in European or American populations. So, it seems that toward to Mediterranean region, the frequency of the 57-kb deletion decreases. Nevertheless, the 57-kb deletion should be the first mutation for looking for in cystinosis patients from Portugal or as well as from Spain [4]. The other mutations; p.S139F as well as the c.18-21delGACT (p.T7FfsX7), have a Spanish founder gene [12], but an interesting fact is that, the deletion c.18-21delGACT (p.T7FfsX7) has been found in different populations from Europe to Middle East, and with widely disease severities. From the cohort studied, we do not have none ocular non-nephropathic form of cystinosis (**Table 1**), that could be related to the rarity of that form of the disease.

We suggest that this fact is due to the difficulty of established this diagnosis and most ophthalmologists do not suspect of this disease. The prognosis of cystinosis depends on early diagnosis and or the prompt start and good compliance with cysteamine treatment. From our point of view, it is important to reinforce the need to diagnosis cystinosis as earlier as possible, and begin therapy with cystine-depleting agents as soon as the diagnosis is made. In this way, it may be possible to attenuate the renal tubular Fanconi syndrome and significantly slow the progression of glomerular damage. Kidney disease progression, extra-renal complications and shorter life expectancy are more evident in patients who do not adhere to treatment.

4. Conclusions

Four different mutations were identified: one large deletion (57-kb deletion), one small deletion (c.18-21delGACT) that originated a frameshift mutation (p.T7FfsX7), one missense mutation (p.S139F) and one nonsense mutation that originates a premature stop codon (p.Q128X). The 57-kb deletion is the most common mutation found in the Portuguese patients (68.2%).

The authors would like to emphasize the importance of first screening for the

57-kb deletion since it is very common in our population, although the determination of intra-leukocytes cystine (WBC Cis IL) is also an important tool for the cystinosis diagnosis and therapeutic monitorization. Although, it is important to notice that in small infants, WBC Cis IL may result in a false negative. This genetic study is the first in our country and it could be the basis for future genetic counseling and prenatal diagnosis of patients with nephropathic cystinosis in Portuguese population. Prenatal diagnosis allows for early cysteamine treatment. We also like to emphasize that, to those countries with high rate of consanguinity, the incidence of cystinosis is predicted to be higher, we should suggest in a potential universal neonatal screening for cystinosis.

Take Home Message

Mutational spectrum of Portuguese population for cystinosis. This study is the first one in the Portuguese population.

Compliance with Ethical Standards

The submitting author confirms that all procedures were followed in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 and approved by the Ethics Committees.

Informed Consent

Informed consent was obtained from all patients and/or their families for being included in the study.

Author's Contribution

Filipa Ferreira and Laura Vilarinho contributed to all aspects of the study manuscript. Filipa Ferreira involved in the conception, conducting, interpreting of data, drafting the manuscript and revising it critically. Laura Vilarinho involved in planning, revising the manuscript critically for important intellectual content and final approval of the version to be submitted. Célia Carmona; Sónia Ramos; Raquel Neiva, Inês Leal, David Sousa, Teresa Costa, Conceição Mota, Ana Marta Gomes, Daniela Lopes, Maria do Carmo Macário, Isabel Tavares, Helena Pinto, João Paulo Oliveira, Rita Magriço involved in acquisition of data and reviewed manuscript and revising it critically for important intellectual content.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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