Original Article

CTNS Mutations Causing Autosomal Recessive Cystinosis in a Subset of Iranian Population: Report of Two New Variants

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Abstract

Background: Nephropathic cystinosis (NC) is an uncommon autosomal recessive disease with abnormality in lysosomal storage that appearances in patients with mutations in the *CTNS* gene encoding a lysosomal transporter cystinosin. Disrupted function of this transporter is followed by accumulation of cysteine crystals in cells of many various organs. This study aimed to investigate the mutations of the *CTNS* gene in 20 Iranian patients suffering from NC.

Materials and Methods: Twenty Iranian cystinosis patients referring to Imam Hossein Hospital of Isfahan were employed in this case-series study. After extraction of genomic DNA, the promoter and entire coding regions of *CTNS* were analysed using sanger sequencing in all patients. Gap–Polymerase Chain Reaction was used to detect 57 kb deletion in the *CTNS* gene. *In silico* study was performed to analyse variants.

Results: The large deletion was not seen in any NC patients. Molecular analysis which conducted to screen the *CTNS* gene of patients, identified eight different mutations, including two new mutations, c.971_972insC and c.956_956delA, which have not been reported before, and c.681G>A mutation, which was identified as a frequently founded mutation in the Middle East and was observed in 35% of patients. In this study, five other mutations including c.1015G>A, c.922G>A, c.323_323delA, c.433C>T, and c.18_21delGACT were also observed, which have been reported in previous studies.

Conclusion: The mutational spectrum in the Iranian patients is the same as previously reported mutations except that two new mutations were found. The present findings will present suggestions for regular molecular diagnosis of cystinosis in Iran.

Keywords: Genetic variations, lysosomal storage disease, molecular diagnostic testing, nephropathic cystinosis

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Introduction

Cystinosis is an infrequent illness, representing in individuals carrying mutations in both alleles of the *CTNS* gene which is embedded on the 17p13. The *CTNS* gene has 12 exons and exons 3-12 encodes cystinosin, a protein that transports the lysosomal-free cystin.[1] Imperfective cystinosin function make the accumulation of intralysosomal cystine crystals in the cells of various organs, especially the kidney, liver, cornea, lymph nodes, thyroid, bone marrow, and spleen.[2]

Considering the severity of disease and the age of imitation of expression of disease, three various clinical types of cystinosis have been reported. Nephropathic cystinosis (NC) is known as the most common and deleterious form of cystinosis that is represented in infancy and contributes in affecting 95% of

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all patients. Patients represent symptoms including failure to thrive, and symptom related to proximal tubular Fanconi syndrome (glucosuria, proteinuria, acidosis, salt craving, polydipsia, polyuria, electrolyte imbalance, and tetany) at 6-12 months after birth, and if they do not undergo temporary treatments and palliative care, they will suffer renal failure at the end first decade of life. The second type begins at the late phase of adolescence and is known as the intermediate form or nephropathic juvenile cystinosis. The second form typically is seen in adolescence rather than in infancy with more moderate renal symptoms. The third type is an ocular non-nephropathic form appearing with only corneal illness. All three kinds of the illness are generated by alterations in the *CTNS* gene.[2-4]

Regular diagnostic methods for cystinosis are assessment of leukocyte cystine, using the slit lamp examination to look for corneal cystine crystals, and molecular screening of the *CTNS* gene.[5] From 1998 that the gene was uncovered until now, more than 148 various *CTNS* pathogenic and destructive alterations, instantiate short deletions or insertions, substitutions, aberrant stop codons, and splice site mutations, in the coding or noncoding region and also in the promoter have been reported.[2] A 57 kb deletion has been recorded as the most frequent alteration in northern parts of Europe and c.681 >4A; p.E227E is a frequent alteration that has been recorded in various Middle Eastern areas.[2-4] Although, the information related to the mutation profile of the *CTNS* gene in the Middle East, especially in Iran, is restricted.[6] The present investigation aimed to identify the mutations of the *CTNS* gene in some Iranian patients suffering NC.

Materials and Methods

Patients

Twenty Iranian patients, from 18 unrelated families, nine males and 11 females, suffering from infantile NC, were analysed in the present study. All patients had been diagnosed according to clinical signs and symptoms considering lack of adequate growth, presentation of renal Fanconi syndrome-related signs detected by the pediatric nephrology group in the Imam Hossein hospital pediatric center (Isfahan, Iran), and the existence of corneal crystals founded by via slit lamp examination conducted by a practiced ophthalmologist. Genetic counselling was conducted. Afterwards, all the patients signed an informed consent before taking a blood sample. Approvement of assembling of samples and experimental protocols was made by the Isfahan University of Medical Science Ethical Committee (IR.MUI.MED.REC. 1398.669).

Molecular genetic analysis

At first, 1 mL blood sample was acquired from each patient and was kept in Ethylenediaminetetraacetic acid (EDTA) tubes. Extraction of DNA from whole blood carried out by Addprep Genomic DNA extraction kit (Addbio, Korea). Exon-specific primers were provided using Primer3 Plus software (http://www.primer3plus.com) and the promoter region of the *CTNS* gene and 57-kb deletion were amplified with previously reported primer pairs.[7,8] The sequences of primers are represented in Table 1.

Polymerase Chain Reaction (PCR) was performed in a final volume of 25 µL containing 12.5 µL Addstar Taq master mix (2x) (Addbio, Korea), 500 pmol of each primer, and 100 ng of genomic DNA. The PCR program consisted of an initial denaturation at 95°C for 5 min; 30 cycles at 95°C for 20 s, annealing (60-67°C) for 30 s, and 72°C for 45 s; and a final elongation step at 72°C for 5 min. Sanger sequencing of PCR products was performed by ABI 3130XL capillary sequencer (Applied Biosystems, USA).

Validation of mutations

The founded mutations were checked in the 1,000 genomes database, NCBI dbSNP database, and the HapMap database to determine their frequencies and pathogenesis status. Also, the Human Gene Mutation Database (http://www.hgmd.org/) and the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/) were searched to check novelty of detected mutations. Furthermore, the functions of protein carrying the novel alterations were predicted using Mutation Taster, PolyPhen-2, SIFT, and Mutation Assessor.

Results

Twenty patients (nine males and 11 females) from 18 families with infantile NC were evaluated. Assessed patients were aged 3 to 19 years. Indeed, 12 families had consanguineous marriage, and in two families, there were two affected cases. The age of onset of symptoms was 5 months to 2 years. The earliest appeared symptoms in all patients were severe failure to thrive, polyuria, and polydipsia [Table 2]. All patients

Table 1: The employed primers sequence

Primer name	Sequence (5' → 3')	Amplicon size (bp)
del57–kb	F: CCTGGCTGCTCTCCTCTTCTTGCCGTTC R: TCCCTGTGGCTTTCCGTCCTGTTTCCTC	387
Promotor	F: CATGGAGGCTTCTCGTCTTC R: CGGCTAGACTTTGGGAGAGG	405
Cys-Exon3	F: TTGTCTACAGGGAGCTGAGC R: TCCTCTAGCCACCATTTCCC	202
Cys-Exon4	F: TTTGCAGTCCCATCACAGAA R: GCGCATCTGAAATCTGAACA	316
Cys-Exon5	F: TGGAGCACCTAGCATTTCCT R: CATCCCCAAAAGACACCAAC	362
Cys-Exon6-7	F: CAGCGTCTCTCCTTTTGCTT R: TTCTGGAAAGGGTAGGCAGA	633
Cys-Exon8-9	F: CCCTGCCCTGTCTTGTCC R: GCTCTGCCGTGTCTTCTGTC	426
Cys-Exon10	F: AGGTGTGATGCTTTCTGTGG R: TTTCCGTCCTGTTTCCTCAC	478
Cys-Exon11	F: CATGCAGATGAGACGCCTAC R: CCCAGAAAACCACAAAGCCT	500
Cys-Exon12	F: TTCGTAGCTGGAGGCTTTGT R: AAAGAGATGGCGGTGTCAAG	490

Table 2: Clinical manifestations founded in patients suffering cystinosis

Patient/sex	Age in years	Consanguinity	Age of onset of symptoms	Fanconi syndrome	Corneal crystals	Renal transplantation
P1/M	15	First cousin	8 months	+	+	+
P2/M	6	Unrelated	6 months	+	+	−
P3/F	19	Unrelated	9 months	−	+	+
P4/M	14	First cousin	4 months	+	+	+
P5/M	9	Unrelated	8 months	+	+	−
P6/M	10	Second cousin	6 months	+	+	−
P7/F	4	Second cousin	8 months	+	+	−
P8/M	3	First cousin	8 months	+	−	−
P9/M	12	First cousin	4 years	−	−	−
P10/F	2	First cousin	6 months	+	+	−
P11/F	5	First cousin	6 months	+	+	−
P12/F	16	First cousin	2 months	+	+	+
P13/M	12	First cousin	7 months	+	+	−
P14/M	9	First cousin	2 years	+	+	−
P15/F	13	First cousin	5 months	+	+	+
P16/F	7	First cousin	16 months	+	+	−
P17/F	8	First cousin	6 months	+	+	−
P18/F	5	First cousin	12 months	+	+	−
P19/M	13	First cousin	6 months	+	+	+
P20/F	8	First cousin	6 months	+	+	−

were screened for the 57-kb deletion but 57 Kb deletion was observed in no cases in either homozygote or heterozygote state. Sequencing of the promoter region and 10 coding exons of *CTNS* discovered the existence of different nucleotide variations. Eight different variants were identified in the 16 patients [Figure 1a]. Six mutations have been reported in previous studies (c.1015G>A, c.922G>A, c.681G>A, c.323_323delA, c.433C>T, and c.18_21delGACT). Two new mutations were identified in three unrelated families, including one homozygous deletion in exon 11 (c.956_956delA) in two families, leading to a truncation of protein (Q319Rfs*10), and one insertion in exon 12 (c.971_972insC) in one family, leading to truncated protein (Q325Pfs*40) [Figures 2 and 1b]. No mutation was identified in the other cases.

Discussion

An incidence of 1 in 100,000 and 1 in 200,000 is estimated for CTNS in the general population, nevertheless, it is expected that the diseases frequency be higher in the areas which have a higher rate of consanguinity, instantiate the Middle East, Turkey,[9] and Iran.[1] The *CTNS* gene is embedded on chromosome 17p13.3 and contains 12 exons, and the first two exons are noncoding. Cystinosin is an integral lysosomal cystine transporter having seven membrane domains [Figure 1b] and dysfunction of it makes cystine accumulate in all organs and results in cystinosis disease. However, cystinosin has other roles, such as modulation and regulation of mTOR signaling pathway, autophagy, mTOR signaling pathway, lysosomal formation, and vesicle transferring in proximal tubular epithelial cells.[10] Considering the issue that cystinosis is the main cause of renal Fanconi syndrome, its diagnosis is basically using the known criteria are employed to recognize renal Fanconi syndrome, inclusive of growth retardation, dehydration, polydipsia, polyuria, rickets, glucosuria, and electrolyte imbalance.[11] Measuring increased cystine content in the white blood cell is the gold standard for the diagnosis. The validation of the diagnosis made by the presence of corneal cystine crystals and especially molecular analysis of the cystinosin gene. Importantly, diagnosis must be performed as soon as possible not to loss time for initiation of treatment. Until now, the only treatment is the use of oral cysteamine.[5]

This study investigated 20 patients from 18 unrelated families in Iran. At first, we searched for the 57 kb deletion, the most frequently detected pathogenic alteration in North America and Northern Europe and which affects the promoter region and the initiator 10 exons of the *CTNS* gene, in addition to two upstream genes (*TRPV1* and *CARKL*),[12] and have been reported in 65% of patients of northern European descent.[13,14] According to previous reports, this mutation has not been detected in the Middle East, Asia, and Africa,[15] while this mutation was identified in a patient in a recent study in Iran.[2] Hence, initially, we examined patients for this large deletion. None of the investigated patients had the mentioned deletion. Then, screening the coding regions and also exon–intron boundaries of the *CTNS* gene and its promoter revealed eight different mutations, two of which had not been previously identified. The first undiscovered previously mutation founded in this study is the deletion (c.956_956delA; Q319Rfs*10) in exon 11 that was detected in two unrelated families (P2 and P6) [Figure 1a]. The second novel mutation is the insertion (c.971_972insC; Q325Pfs*40) that was detected in patient 15. Both of them make a truncated protein in homozygous states.

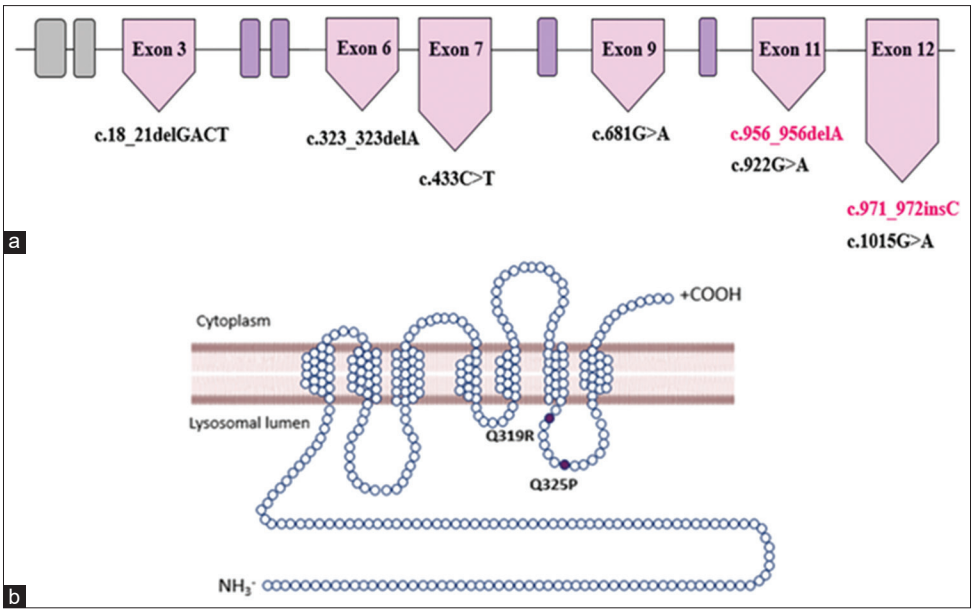

Figure 1: Localization of identified mutations in the study. (a) Four detected mutations are related to exons 3, 6, 7, and 9; while two mutations are occurred in exon 11, and two of them are in exon 12 (b) *CTNS* is a seven-passed transmembrane receptor and we have detected two new mutations affecting residues 319 and 325 in the protein

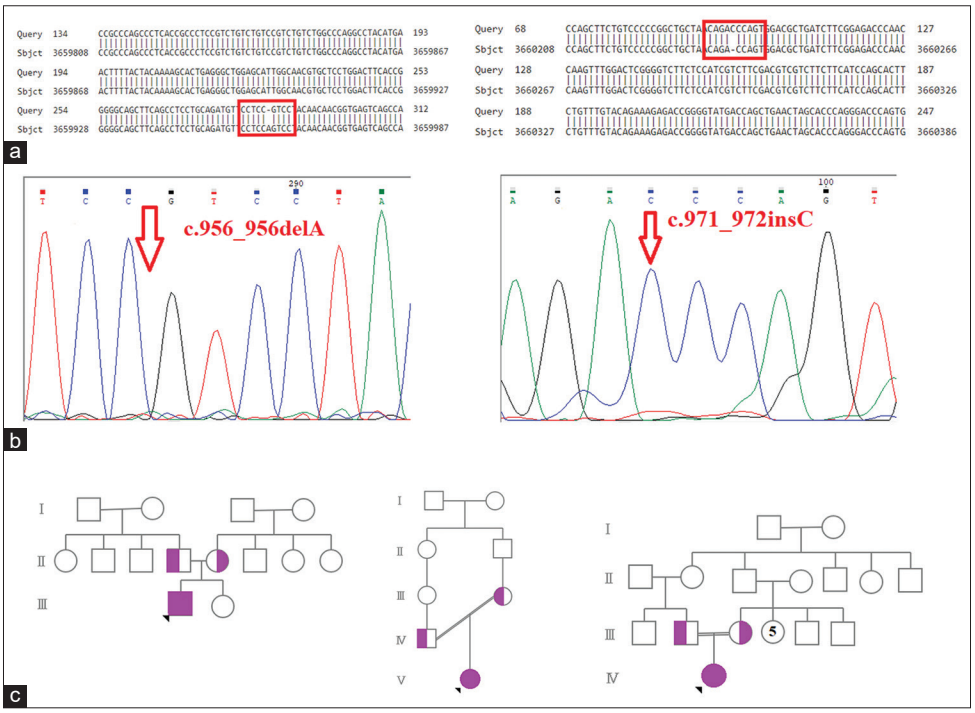

Figure 2: Data of two new mutations. (a) NCBI blast (b) Chromatogram (Chromas software version 2.6.6) for the region containing two novel mutations. The substituted nucleotides are shown by red arrow shows. (a) illustrates a single base pair deletion in nucleic acid position 956 (c.956_956delA) in exon 11, resulting in formation of truncated protein (Q319Rfs*10) which was detected in two patients in homozygous condition. (b) shows an insertion in exon 12 (c.971_972insC) in one family, leading to truncated protein (Q325Pfs*40). (c) Family pedigree of patients

However, the previously discovered splice site mutation, c.681G>A, that has been identified in 35% of all patients in our investigation, is a frequent alteration in the Middle East and also the most founded mutation in Iran based on this study and other studies of cystinosis in Iran.[1,6,16] All the patients carried the same homozygous mutation. This mutation is frequently reported in other Middle Eastern countries, such as Saudi Arabia,[16] Iran,[6] Egypt,[12] and Turkey.[9] It can be considered a "founder mutation" in the Middle East, especially since it has a higher frequency in Iran. Therefore, checking exon 9 should be given priority when trying to identify the responsible mutation in affected patients.

Table 3: The identified mutations in Cystinosis patients

Patient ID	Mutation	Position	AA change	Protein effect	Reference
P1	c.922G>A	Exon 11	G308R	Protein features	[18]
P2	c.956_956delA	Exon 11	Q319Rfs*10	Truncated protein	This study
P3	c.681G>A	Exon 9	E227E	Alternative splicing	[16]
P4	c.681G>A	Exon 9	E227E	Alternative splicing	[16]
P5	c.18_21delGACT	Exon 3	T7Ffs*7	Frameshift at 7→Stop 6 amino acids downstream	[20]
P6	c.956_956delA	Exon 11	Q319Rfs*10	Truncated protein	This study
P7	c.681G>A	Exon 9	E227E	Alternative splicing	[16]
P8	ND	_____	_____	_____	_____
P9	ND	_____	_____	_____	_____
P10	c.1015G>A	Exon 12	G339R	AA change at TM7	[18]
P11	ND	_____	_____	_____	_____
P12	c.681G>A	Exon 9	E227E	Alternative splicing	[16]
P13	c.681G>A	Exon 9	E227E	Alternative splicing	[16]
P14	c.323_323delA	Exon 6	Q108Rfs*10	Premature truncation of the protein	[21]
P15	c.971_972insC	Exon 12	Q325Pfs*40	Truncated protein	This study
P16	c.323_323delA	Exon 6	Q108Rfs*10	Premature truncation of the protein	[21]
P17	ND	_____	_____	_____	_____
P18	c.681G>A	Exon 9	E227E	Alternative splicing	[16]
P19	c.433C>T	Exon 7	Q145*	Truncated protein	[19]
P20	c.681G>A	Exon 9	E227E	Alternative splicing	[16]

Other previously identified mutations were also found in this study, including the c.1015 G4A; G339R[17] in TM7 (exon 12) in patient 10 in both the alleles. The nonsense mutation, c.433C4T, Q145X in exon 7 resulting in generation of an incomplete protein was identified in Russia.[18] This mutation was identified in a homozygous condition in patient 19. One patient represented heterozygous mutation of c.18-21delGACT[19] due to the heredity of this disease; this patient requires further investigation. This mutation results in a T7F substitution and truncation at amino acid 13 which most likely is found in the European population.[20] Two patients had single base pair deletion (c.323delA) in exon 6, in both alleles, followed by generation of short protein (p.Q108RfsX10).[21] Shotelersuk *et al.* have found a missense mutation (922G>A) in a number of patients, which replaces an arginine for the severely conserved glycine residue and leads to disruption of TM6.[17] A summary of all identified mutations during this or previous studies is depicted in Table 3.

Conclusion

However, our new findings make progress to know more about the mutations of the *CTNS* gene in patients suffering NC and could be beneficial for the prenatal or preimplantation genetic diagnosis. In Iran, there is a relatively higher frequency of consanguineous marriages, which can lead to an increased risk of rare autosomal recessive disorders such as cystinosis in offspring. Therefore, to prevent or reduce the rate of these genetic conditions, genetic counselling before marriage and prenatal diagnosis in carriers and high-risk families are highly recommended. To rapidly identify heterozygous carriers, and other patients with similar disease symptoms and genetic diagnosis, primary searching for c.681G>A in high-risk Iranian persons may be useful. However, patients investigated in the present study were collected from patients referring the Imam Hossein hospital pediatric center of Isfahan; hence, further studies needed to screen *CTNS* gene in a larger population of patients from all over the Iran. Additionally, performing functional experiment of novel mutations is our next step study.

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Conflicts of interest

There are no conflicts of interest.

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