Molecular Genetics and Metabolism Reports 5 (2015) 63–66

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism Reports

journal homepage: http://www.journals.elsevier.com/molecular-genetics-and-metabolism-reports/

CTNS mutations in publicly-available human cystinosis cell lines

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ARTICLE INFO

Article history:
Received 11 September 2015
Received in revised form 13 October 2015
Accepted 13 October 2015
Available online 27 October 2015

Keywords:
CTNS
Sequencing
Cystinosis
Mutation
Splicing
Coriell Cell Repository

ABSTRACT

Patient samples play an important role in the study of inherited metabolic disorders. Open-access biorepositories distribute such samples. Unfortunately, not all clinically-characterized samples come with reliable genotype information. During studies directed toward population frequency assessments of cystinosis, a rare heritable disorder, we sequenced the *CTNS* gene from 14 cystinosis-related samples obtained from the Coriell Cell Repository. As a result, the disease genotypes of 7 samples were determined for the first time. The reported disease genotypes of 2 additional samples were found to be incorrect. Furthermore, we identified and experimentally confirmed a novel mutation, c.225 + 5G>A, which causes skipping of the 5th exon and is associated with infantile nephropathic cystinosis.

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1. Introduction

Genetic medicine is based on the identification of genetic lesions leading to human disease. The Coriell Cell Repository is one of the world's largest open-access sources of biological samples from patients with clinically-characterized heritable diseases. Diagnoses of genetic disorders are facilitated by databases containing previously-identified disease-associated mutations. Unfortunately, some Biorepository samples lack genetic characterization.

Cystinosis is a rare, recessive, genetic disorder, which occurs due to mutations in the *CTNS* gene [1,2]. This gene encodes the transmembrane protein cystinosin (also *PQLC4*), which is responsible for transport of lysosomal cystine into the cytosol [3]. Malfunctioning cystinosin leads to accumulation of cystine in lysosomes throughout the body. All affected cells accumulate cystine, however the kidney is the organ that is most functionally affected. Nephropathic cystinosis is commonly associated with end-stage renal disease, where patients often require kidney transplant [4].

Human sample acquisition for research purposes is often complicated. Cystinosis, for example, has been estimated to be present in only 2000 people worldwide [5]. The Coriell Cell Repository is the only publicly-accessible source of cystinosis-related samples. About half of the Coriell cystinosis samples lack genetic characterization. Based on phenotypic severity, the disorder can be generally divided into three groups: infantile nephropathic, intermediate or late-onset nephropathic, and ocular non-nephropathic. The severity of the disease is associated with particular *CTNS* mutations. Large deletions or trans-membrane missense substitutions often lead to infantile nephropathic cystinosis, while other mutations cause the milder, late-onset version, of the disorder [6,7]. Because mutations impact disease course differently, it is important to know a patient's genotype. Here we determined genotypes of 14 cystinosis-related tissue samples.

2. Material and methods

2.1. Sample acquisition

All 14 tissue samples were purchased from the Coriell Cell Repository, including 13 fibroblast cell lines and 1 genomic DNA sample. Ten samples (Table 1) were from patients diagnosed with cystinosis, while the other 4 samples were from unaffected parents. Additionally, for the gross deletion screen, a cystinosis fibroblast cell line (GM002894, Coriell Cell Repository, Camden, NJ, USA) was used as a positive control, and normal fibroblast line (CRL-2529, ATCC, Manassas, VA, USA) as a negative control.

2.2. Targeted capture and sequencing

Genomic DNA from cell lines was extracted using a Qiagen DNeasy Blood and Tissue kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. Fluidigm's Access Array IFC (Fluidigm Corporation, South San Francisco, CA, USA) was utilized for the target enrichment of *CTNS* gene (see Supplementary Table 1 for Fluidigm targeting primers). Enriched DNA was sequenced on a Hiseq 2500 (Illumina Inc., San Diego, CA, USA) with 2 × 100 bp sequence length.

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http://dx.doi.org/10.1016/j.ymgmr.2015.10.007
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Table 1
Identified mutations in the 14 samples. Samples with mutations in bold were genotyped for the first time.

Coriell ID	Clinical phenotype	Allele 1	Allele 2
NA17885	Nephropathic cystinosis	p.G308R/c.922G>A	57 kb deletion
GM17886	Nephropathic cystinosis	p.T7Ffs*7/c.18_21delGACT	p.T7Ffs*7/c.18_21delGACT
GM00760	Nephropathic cystinosis	p.W138*/c.414G>A	**57 kb deletion**
GM00046	Nephropathic cystinosis	p.I69Rfs*5/c.206_210delTCCTT	**57 kb deletion**
GM02066	Nephropathic cystinosis	**p.P48Lfs*7/c.225 + 5G>A**	**p.G339R/c.1015G>A**
GM00908	Nephropathic cystinosis	**p.L158P/c.473T>C**	**57 kb deletion**
GM00909	Unaffected mother of GM00908		**57 kb deletion**
GM00910	Unaffected father of GM00908	**p.L158P/c.473T>C**	
GM00378	Late-onset nephropathic cystinosis	c.970 + 2T>C	p.W138*/c.414G>A
GM00379	Late-onset nephropathic cystinosis	c.970 + 2T>C	p.W138*/c.414G>A
GM00907	Unaffected mother of GM00378 and GM00379	**c.970 + 2T>C**	
GM00906	Unaffected father of GM00378 and GM00379		**p.W138*/c.414G>A**
GM17888	Ocular non-nephropathic cystinosis	p.I69Rfs*5/c.206_210delTCCTT	c.853-3C>G
GM08761	Ocular non-nephropathic cystinosis	**p.G197R/c.589G>A**	**57 kb deletion**

Custom Fluidigm CS1 and CS2 sequencing primers were used for the Hiseq run.

2.3. Sequencing data analysis

Amplification primer sequences were trimmed from the raw data using an in-house perl script. The reads were mapped to human genome version hg19 using the BWA aligner (version 0.7.8) with parameters: "mem" and "-M". Aligned reads were sorted with samtools (version 0.1.19). Variant calling was performed using both GATK (version 3.1.1) using parameters: "-T HaplotypeCaller-stand_emit_conf 10 -stand_call_conf 30 -gt_mode DISCOVERY"; and samtools (version 0.1.19) with parameters: "mpileup -d 3000 -L 3000 -u -M 60". Observed variants were merged and then annotated with snpEff (version 3.6b) using human genome version GRCh37.75, Human Gene Mutation Database (version HGMD 2014.2, BIOBASE, Wolfenbuettel, Germany), dbSNP (version 140), dbNSFP (version 2.4). Variants that matched any of the following parameters were filtered out: DP < 16; QD < 2; CAF ≥ 0.1; and QUAL < 30.

2.4. Zygosity determination

To examine samples for the presence of the 57-kb deletion common in European cystinosis patients, we used primers designed by Anikster et al., where forward (CTAACAGTATCACCGGAGTCTAC) and reverse (GGCCATGTAGCTCTCACCTC) primers flank the deletion, and forward (CTAGGGGAGCGTGTTAGCAT) and reverse (TGTAAGACTGAGGCTGGA GC) primers are located within the deletion [8]. A Qiagen PCR core kit was used to amplify 30 ng of gDNA samples according to the manufacturer's instructions. Amplified samples were run on the 2.2% agarose Lonza flashgel (Lonza, Basel, Switzerland).

RNA from cell line GM002066 was extracted with the Qiagen RNeasy minikit according to manufacturer's instructions. About 6 μg of RNA was reverse transcribed using Qiagen's RT Easy first-strand kit. Lastly, cDNA was purified using Qiagen's PCR purification kit. Retention or deletion of *CTNS* exon 5 was determined by amplification with flanking forward (CCTCACTGTTCCTCCTGTCG) and reverse (TGATCAGCGTGAGGACAACC) primers.

3. Results

We used targeted next-generation sequencing to find mutations in the promoter and exons of the *CTNS* gene in 14 cystinosis related tissue samples. Observed mutations for all genetically-uncharacterized samples were confirmed by subsequent Sanger sequencing. The sequencing approach utilized produces 100 base-pair reads, which precludes detection of long deletions when present in only one allele. To address this issue, we screened samples for the presence of the most common deletion in cystinosis patients of European descent. This 57 kb deletion starts prior to the *CTNS* gene and extends to the end of exon 10. We determined that 6 samples had the 57 kb deletion in heterozygous state (Fig. 1). Samples GM00760 and GM00046 were previously characterized as homozygous for p.W138* and p.I69Rfs*5 mutations, while our results suggest that they were hemizygous for these mutations, because they carry the 57 kb deletion on the other allele (Table 1).

Samples GM00909, GM00910, GM00907 and GM00906 were derived from clinically-unaffected parents, and as expected, we found single heterozygous mutations in each of them. In turn, children can be anticipated to have parental mutations in a compound heterozygous state. Two of these, GM00378 and GM00379, had been previously characterized as being compound heterozygous. One affected child, GM00908, inherited the 57 kb deletion from the mother, and was hemizygous for p.L158P, inherited from the father. Similarly, GM08761 was

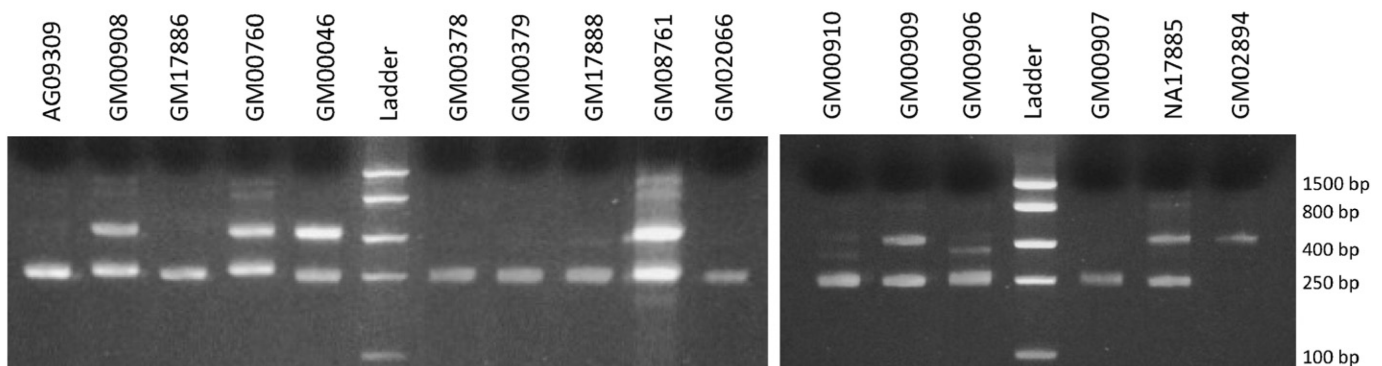

Fig. 1. 57 kb deletion determination. The primer pair that is flanking 57 kb deletion produces a 420 bp amplicon of an allele carrying the deletion. The primer pair that is located within the deletion region produces a 250 bp amplicon of an allele lacking deletion.

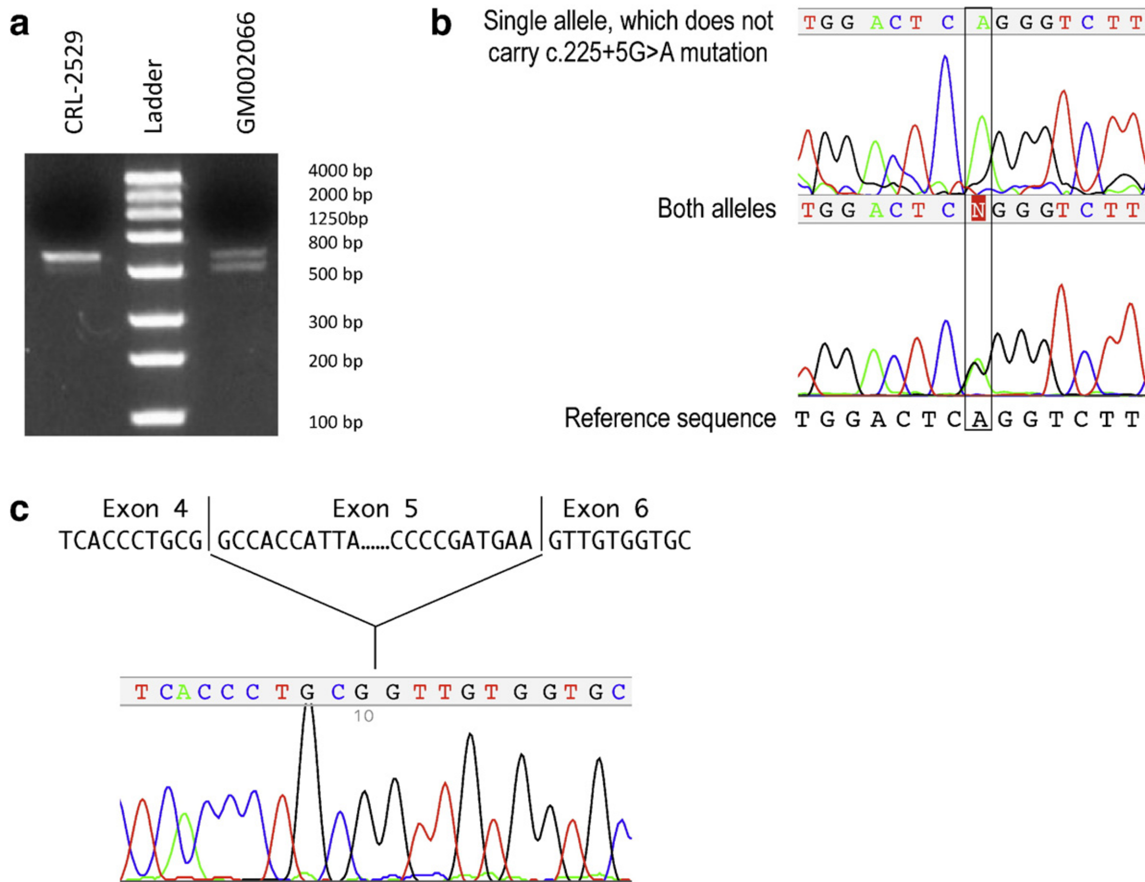

Fig. 2. Genetic characterization of GM02066 sample. (a) PCR of control CRL-2529 and GM02066 samples with primers flanking exon 5. (b) Sanger reads of c.225 + 5G>A mutation. Top panel shows sequence originated from single allele. Bottom panel shows heterozygous state represented by both alleles. (c) Sanger read from single allele with c.225 + 5G>A mutation.

found to be a compound heterozygote of the 57 kb deletion and a p.G197R mutation.

GM02066 was the only cystinosis sample for which we were unable to derive a plausible causative genotype directly from sequencing results. Two heterozygous variants were observed; the previously known mutation p.G339R and a novel variant c.225 + 5G>A. In order to confirm that these mutations are the reason for observed nephropathic cystinosis diagnosis it was necessary to determine if: 1) the novel variant affects normal synthesis of cystinosin protein; 2) both variants are located on different alleles. Novel variant c.225 + 5G>A is overlapping with the previously characterized cystinosis mutation c.225 + 5GT>CC, which leads to skipping of exon 5 [9]. Therefore, we decided to check for the presence of exon 5 in GM02066 by designing primers flanking the exon borders. We found that half of the *CTNS* transcripts of GM02066 did not have exon 5 (Fig. 2a). Exon-skipping causes a shorter, nonfunctional protein to be produced from the allele that carries c.225 + 5G>A mutation.

Next, we checked whether observed heterozygous mutations are located on different alleles. To determine this, we designed primers so that the forward primer was contained within exon 5 and reverse primer was outside of the heterozygous mutation c.1015G>A. Amplicons derived from cDNA using these primers can originate only from one allele, which does not carry c.225 + 5G>A mutation. We found that the amplicon sequences contained adenine at position c.1015 (Fig. 2b), therefore demonstrating that c.1015G>A and c.225 + 5G>A are on different chromosomes. Lastly we have extracted the lower band of the gel represented on Fig. 2a, and sequenced it. As expected, we confirmed that entire exon 5 is missing (Fig. 2c).

4. Discussion

The Coriell Cell Repository is the only biobank distributing cystinosis cell lines. Not all of them have identified mutational genotypes. Here we sequenced the *CTNS* gene of 14 such lines. We were able to establish that two of the samples had been mischaracterized as homozygous because one allele in each case carried the 57 kb deletion. Due to the nature of next-generation sequencing methods, which produce short reads, it is difficult to identify long deletions. This highlights the importance of screening for long deletions, especially those that are frequent, such as the 57 kb deletion in cystinosis patients of European descent.

Characterizing publicly-available samples is not only important for understanding the genotype of research material, but also for identifying novel mutations as in the current study. Knowledge of causative mutations also facilitates disease diagnostics.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ymgmr.2015.10.007.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We would like to thank the following companies for the outstanding service they have provided for this study: Qiagen (Qiagen, Frederick, MD, USA) for genomic DNA extraction; Ambry Genetics (Aliso Viejo,

CA, USA) for next-generation sequencing; and SeqWrite Genomic Services (GE HealthCare, Houston, TX, USA) for Sanger sequencing.

References

[1] V. Shotelersuk, D. Larson, Y. Anikster, G. McDowell, R. Lemons, I. Bernardini, J. Guo, J. Thoene, W.A. Gahl, Am. J. Hum. Genet. 63 (1998) 1352–1362.

[2] L. Forestier, G. Jean, M. Attard, S. Cherqui, C. Lewis, W. van't Hoff, M. Broyer, M. Town, C. Antignac, Am. J. Hum. Genet. 65 (1999) 353–359.

[3] M. Town, G. Jean, S. Cherqui, M. Attard, L. Forestier, S.A. Whitmore, D.F. Callen, O. Gribouval, M. Broyer, G.P. Bates, W. van't Hoff, C. Antignac, Nat. Genet. 18 (1998) 319–324.

[4] G. Nesterova, W.A. Gahl, Pediatr. Nephrol. 28 (2013) 51–59.

[5] V. Kalatzis, S. Cherqui, G. Jean, B. Cordier, P. Cochat, M. Broyer, C. Antignac, J. Am. Soc. Nephrol. 12 (2001) 2170–2174.

[6] M. Attard, G. Jean, L. Forestier, S. Cherqui, W. van't Hoff, M. Broyer, C. Antignac, M. Town, Hum. Mol. Genet. 8 (1999) 2507–2514.

[7] M. Al-Haggar, World J. Nephrol. 2 (2013) 94–102.

[8] Y. Anikster, C. Lucero, J.W. Touchman, M. Huizing, G. McDowell, V. Shotelersuk, E.D. Green, W.A. Gahl, Mol. Genet. Metab. 66 (1999) 111–116.

[9] R. Kleta, Y. Anikster, C. Lucero, V. Shotelersuk, M. Huizing, I. Bernardini, M. Park, J. Thoene, J. Schneider, W.A. Gahl, Mol. Genet. Metab. 74 (2001) 332–337.