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SHORT REPORT

Genetic screening of Congenital Short Bowel Syndrome patients confirms *CLMP* as the major gene involved in the recessive form of this disorder

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Congenital short bowel syndrome (CSBS) is an intestinal pediatric disorder, where patients are born with a dramatic shortened small intestine. Pathogenic variants in *CLMP* were recently identified to cause an autosomal recessive form of the disease. However, due to the rare nature of CSBS, only a small number of patients have been reported to date with variants in this gene. In this report, we describe novel inherited variants in *CLMP* in three CSBS patients derived from two unrelated families, confirming *CLMP* as the major gene involved in the development of the recessive form of CSBS.
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INTRODUCTION

During embryogenesis, the small intestine experiences major growth expanding several times the total length of the whole body. This is a complex process that starts around the fifth week of human development, when the midgut forms a simple linear tube that runs down the midline of the embryo, and is completed by the twentieth week of development.[1] Several pathways have been reported to have a role in intestinal elongation,[2] but to date the molecular mechanism(s) responsible for this process remains unclear.

In congenital short bowel syndrome (CSBS), intestinal elongation is impaired and patients are born with a substantially shortened small intestine (~50 cm in length instead of 250 cm in a normal counterpart).[3] It is a rare disorder for which no cure is available. Total parenteral nutrition is required for long-term survival, but despite considerable efforts to improve treatment, most patients die of starvation or sepsis within the first few days of life.[4] CSBS was first described by Hamilton *et al.*[3] and it has always been considered to have a genetic cause.[3] However, it was only recently that two genes were identified as the cause of CSBS: *CLMP* and *FLNA*.[5,6] *CLMP* encodes for the Coxsackie and adenovirus receptor-like membrane protein, an adhesion molecule that co-localizes with tight junction proteins, but whose function is still unknown.[7] *FLNA* encodes for Filamin-A, an actin-binding protein known to regulate cell shape and to control cell signaling and migration.[8] Pathogenic variants present in *CLMP* and *FLNA* have been linked to the recessive and X-linked forms of CSBS, respectively.

Here, we report the genetic screening of three CSBS patients derived from two unrelated families, and describe the identification of three novel variants in *CLMP*.

MATERIAL AND METHODS

Patient information

In this study, three female patients diagnosed with CSBS were investigated (Table 1). Two of these patients were siblings of consanguineous descent (P1 and P2). The third patient (P3) has been previously described, when a *de novo* translocation was found between chromosome 2 and 11 (46,XX,t(2,11)(q32.2,p12)).[9] However, no gene was identified as the causative factor at that time. A complete description of the patients can be found in Supplementary Data.

Written informed consent was given by the two families reported, and ethical approval was obtained from the Erasmus Medical Center ethical committee (Medisch Ethische Toetsings commissie - METc 2009/364, ABR nr: NL31708.042.10).

Genetic analysis

Genomic DNA was isolated from peripheral blood lymphocytes using standard methods. Exons 1 – 7 of *CLMP* (ENST00000448775) were amplified using 30 ng of genomic DNA as described before.[5] PCR products were purified (ExoSap-IT – GE Healthcare, Eindhoven, The Netherlands), and Sanger sequencing was performed with dye labeled primers (forward and reverse; Big Dye Terminator v3.1 Sequencing Kit, Applied Biosystems, Bleiswijk, The Netherlands) on an ABI 3130XL genetic analyzer (Applied Biosystems). Sanger reads were analyzed using SeqScape software (Fisher Scientific, Landsmeer, The Netherlands) and compared with the reference *CLMP* genomic sequence (ENSG00000166250). Genetic data were submitted to ClinVAr (http://www.ncbi.nlm.nih.gov/clinvar/) and the following

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Table 1 Clinical and molecular features of the CSBS patients included in this study

	P1	*P2*	*P3*
Gender	Female	Female	Female
Age of diagnostic	3 months	38 days	1 week
Ethnicity	Iranian	Iranian	Caucasian
Consanguinity	+	+	No
Malrotation	+	+	+
Length of the small intestine (cm)	76	26	50
Additional features	Intestinal dysmotility UPJO	Intestinal dysmotility UPJO	Very mild mental retardation
CLMP variants	c.508C>T; p.(R170*)	c.508C>T; (p.R170*)	c.410G>A; p.(C137Y) c.29-2 A>G
Zygosity	Homozygous	Homozygous	Heterozygous (two unphased variants)

Abbreviations: CSBS, congenital short bowel syndrome; UPJO, ureteropelvic junction obstruction.

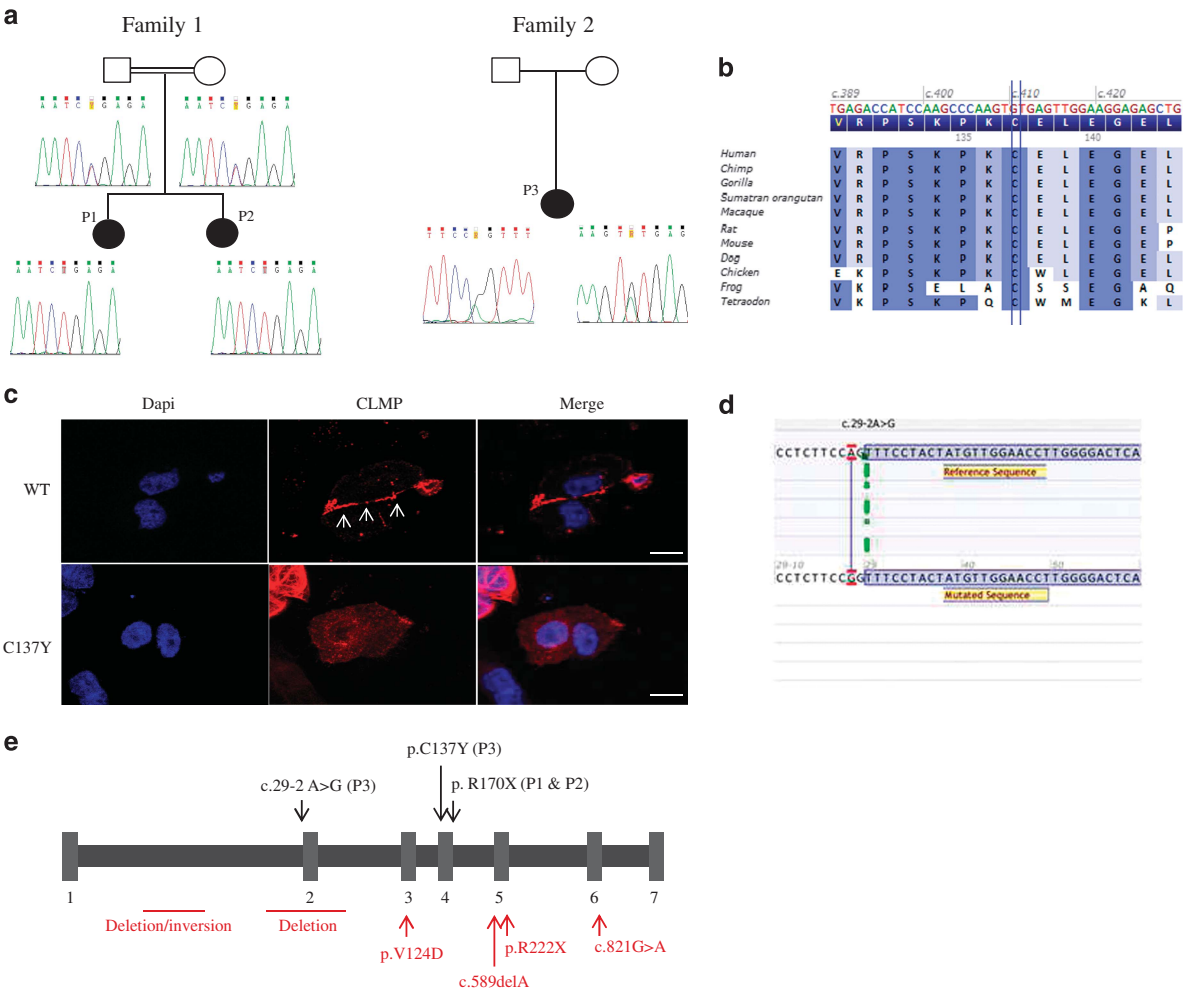

Figure 1 Genetic analysis of three CSBS patients. **(a)** Pedigree of the two families included in this study with Sanger sequencing results showing the presence of variants in *CLMP*. **(b)** Conservation alignments showed that the missense variant p.(C137Y) leads to a change of a cysteine residue highly conserved among vertebrates. **(c)** CHO-K1 cells transfected with constructs expressing wild-type (WT) CLMP or the p.(C137Y) variant show different cellular distribution of CLMP. While the WT protein normally localizes to the tight junctions (see arrows), the mutated protein has a punctuated distribution through the cytoplasm. Scale bars: 30 μm. **(d)** Splicing predictions suggested that the intronic variant identified in patient 3 likely affects normal splicing, as it is predicted to disrupt an acceptor-splicing site (c.29-2A>G). **(e)** Schematic overview of all the variants identified to date in *CLMP*. In black are the variants reported in this report, whereas in red are the variants previously described. **b** and **d** were generated using Alamut prediction software (Interactive Biosoftware, Rouen, France).

accession numbers were obtained: SCV000264793 [p.(R170*)] and SCV000264792 [p.(C137Y) and (c.29-2A>G)].

Expression vectors

Generation of the expression vectors used is described in Supplementary Data.

Immunofluorescence

Chinese hamster cells (CHO-K1) were cultured as described before.[5] Five hundred thousand cells were seeded in a 6-well plate, and transiently transfected with pcDNA-HA-CLMP WT or pcDNA-HA-CLMP C137Y expressing vectors, using GeneJuice (Millipore, Amsterdam, The Netherlands) as transfection reagent. Twenty four hours after transfection, immunofluorescence was performed as previously described.[5] Images were taken in a Leica (AOBS) microscope, and analyzed with the Leica LAS AF Lite software (Leica Microsystems B. V., Eindhoven, The Netherlands).

RESULTS AND DISCUSSION

As the three patients included in this study are females, an X-linked pattern of inheritance involving *FLNA* was considered unlikely, and only *CLMP* was screened. In all patients, we identified previously unreported variants in *CLMP*. None of these variants are listed in any of the available human genome variant databases. Patients P1 and P2 possess a homozygous nonsense variant in exon 4 [c.508C>T; p.(R170*)], leading to the appearance of an early stop codon (Figure 1a). This nonsense variant was inherited from the parents, who were found to be heterozygous. Patient P3 has two unphased heterozygous variants in *CLMP*. Although we suspect that both variants were inherited from the parents, we were unable to investigate segregation in the family due to unavailability of parental DNA. In this patient, a missense variant located in exon 4 was identified leading to an amino acid substitution [c.410G>A; p.(C137Y)], together with a possible splice site variant located two base pairs upstream of exon two (c.29-2A>G) (Figure 1a). Prediction programs used to infer pathogenicity (PolyPhen-2, MutationTaster, SIFT, Human Splicing Finder and Alamut Visual) showed that both variants are likely to disturb protein function. The C137Y variant is located in a highly conserved region present in all mammals (Figure 1b), and *in vitro* assays showed that it affects the cellular localization of CLMP to the tight junctions (Figure 1c). The c.29-2A>G variant is predicted to disrupt normal splicing of exon 2 of *CLMP* (Figure 1d). Considering that both variants likely affect CLMP function, we believe that the *de novo* translocation previously identified in patient P3[9] is an independent event unrelated to CSBS.

Currently, only seven patients have been described carrying pathogenic variants in *CLMP*.[5] All the variants reported were loss of function, leading to the absence of CLMP or to the expression of a mutant protein with a disrupted function (Figure 1e). In this report, we expand the mutational spectrum of *CLMP* variants involved in CSBS, consolidating the involvement of this gene in the development of the recessive form of this disease. We also show that it might be worthwhile to screen previously described patients for whom an X-linked pattern of inheritance was disregarded, for the presence of variants in *CLMP*, as this might shed some light on the genetic basis of the disease in these cases. To date, the role of *CLMP* in intestinal elongation is still largely unknown, making it difficult to determine the molecular mechanisms underlying the development of CSBS. We believe that the results here described reinforce the need to further study the role of *CLMP* in intestinal elongation, as this might contribute for the development of new therapeutic strategies for CSBS patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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