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ORIGINAL INVESTIGATION

De novo *ACTG2* mutations cause congenital distended bladder, microcolon, and intestinal hypoperistalsis

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Abstract Megacystis–microcolon–intestinal hypoperistalsis syndrome (MMIHS) is characterized by prenatal-onset distended urinary bladder with functional intestinal obstruction, requiring extensive surgical intervention for survival. While it is believed to be an autosomal recessive disorder, most cases are sporadic. Through whole-exome sequencing in a child with MMIHS, we identified a de novo mutation, p.R178L, in the gene encoding the smooth muscle gamma-2 actin, *ACTG2*. We subsequently detected another de novo *ACTG2* mutation, p.R178C, in an additional child with MMIHS. *Actg2* transcripts were primarily found in murine urinary bladder and intestinal tissues. Structural analysis and functional experiments suggested that both ACTG2 mutants interfere with proper polymerization of ACTG2 into thin filaments, leading to impaired contractility of the smooth muscle. In conclusion, our study suggests a pathogenic mechanism for MMIHS by identifying causative *ACTG2* mutations.

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Introduction

The etiological diagnosis and the prognosis of a fetus with an abnormally large or distended urinary bladder, also known as megacystis, remain a challenge (Yiee and Wilcox 2008; Freedman et al. 2000). Correct diagnosis is important as some conditions, such as posterior urethral valves, may benefit from in utero treatment. Megacystis–microcolon–intestinal hypoperistalsis syndrome (MMIHS, MIM 249210) was first described by Berdon et al. in 1976 when they reported on five female newborns who had marked dilatation of the bladder without structural obstruction, dilated small intestine and intestinal hypoperistalsis with a diminished caliber of the colon (a.k.a. microcolon) (Puri and Shinkai 2005). Intestinal malrotation was also present in all cases, presumably due to interference of proper rotation by the megacystis. All children died in early infancy because of poor feeding and severe complications. More than 230 cases have subsequently been described in the literature. Most cases are simplex but 15 occurred in siblings, suggesting an autosomal recessive inheritance (McLaughlin and Puri 2013). It is important to make the diagnosis of MMIHS as early as possible, since life expectancy is significantly decreased without extensive surgical operations consisting of multivisceral transplantation which includes small bowel transplantation. At this time, the etiology of the syndrome remains elusive.

Here, we report de novo *ACTG2* mutations as the genetic cause for MMIHS identified in two patients. Previously, an *ACTG2* mutation has been reported as the cause of familial

visceral myopathy with isolated intestinal pseudo-obstruction, a less severe phenotype to that of MMIHS (Lehtonen et al. 2012).

Materials and methods

Families

Two unrelated non-consanguineous families with healthy parents and single affected children diagnosed with MMIHS at the Miller School of Medicine, University of Miami, were included (pedigrees in Fig. 1a).

This study was approved by the University of Miami Institutional Review Board. All participants provided written informed consent prior to enrollment. Written informed consent was obtained from the next of kin on behalf of the children involved in this study.

Exome sequencing

Genomic DNA from the proband of family 1 and her parents were used to sequence the whole-exome as previously described (Diaz-Horta et al. 2012; Gonzalez et al. 2013). The variants were filtered according to different inheritance models, the variant function class, presence and frequency in the dbSNP137 and NHLBI databases (minor allele frequency <0.01), and absence in >2 families in our internal database that includes >1,700 exomes.

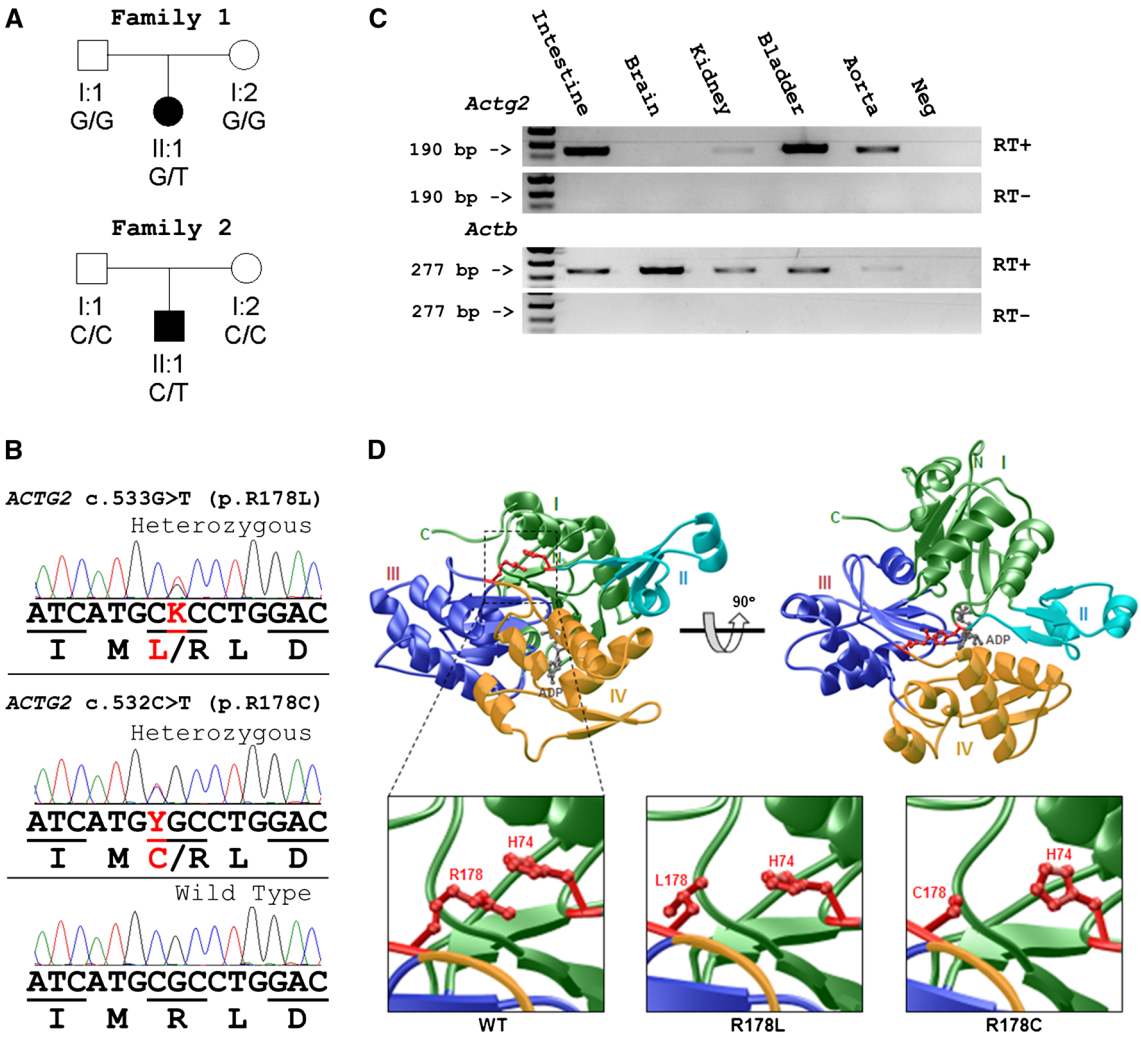

Fig. 1 Detected *ACTG2* mutations in families with MMIHS and *Actg2* transcripts in mouse tissues. **a** Pedigrees of the 2 studied families and results of the *ACTG2* sequencing. **b** Electropherograms showing the identified mutations. **c** *Actg2* transcripts in various murine tissues. **d** Structural models of WT and the two mutants (R178L/R178C) of ACTG2. The four subdomains I (*green*), II (*cyan*), III (*blue*) and IV (*yellow*) of ACTG2 are color-coded for clarity. The *insets* show expanded views of the interactions between side chain moieties (*colored red*) of residues at positions 74 and 178. The ball-and-stick structure of ADP is shown in *gray*

RT-PCR

We performed RT-PCR experiments to evaluate if *ACTG2* is transcribed in the tissues affected in MMIHS using Superscript III (Life Technologies), random hexamers (Promega) and primer pairs shown in Table S1.

Molecular modeling

To further understand the physiological significance of mutations, we built and compared the structural models of wild type (WT) as well as the mutant forms of ACTG2 using MODELLER (Marti-Renom et al. 2000) (Fig. 1d). The crystal structure of the rabbit skeletal muscle alpha actin 1 (ACTA1) bound to ADP (PDB# 1J6Z) was used as a template. The structural models were rendered using RIBBONS (Carson 1991).

Functional analysis of *ACTG2* mutations

The cellular localization of the WT and the two mutated forms of ACTG2 were compared to further investigate if the detected two *ACTG2* mutations affect the incorporation of the mutant ACTG2 into actin filaments. We cloned in-frame *ACTG2* transcript into the pcDNA3.1/CT-GFP-TOPO vector (Invitrogen) to create a C-terminal GFP-tagged variant of the *ACTG2* gene (primers are in Table S1). The two mutated variants were generated using QuikChange® Site-Directed Mutagenesis Kit (Agilent Technologies) and primer pairs given in Table S1. The three plasmids containing the *ACTG2* WT and mutated variants were used to transfect COS7 cells using JetPrime (Polyplus Transfection™). Forty-eight hours post-transfection the cells were fixed, permeabilized, counter-stained for polymerized actin (Phalloidin, Invitrogen) and imaged on an LSM710 laser scanning microscope (Zeiss).

We performed a collagen matrix contraction assay to assess contractibility of ACTG2 WT and mutant overexpressing cells, as previously described (Garrett et al. 2004). The assay is based on the capacity of some cell types to reorganize collagen matrices into a dense, tissue-like structure by pulling together individual collagen fibrils. Tensile strength of the new matrix increases over time, a process previously reported to be dependent on the cellular actin cytoskeleton integrity (Grinnell and Ho 2002; Lehtonen et al. 2012). Briefly, WT or mutant *ACTG2* expressing cells were suspended in type I collagen containing medium and plated in 24-well cell culture plates. The diameter of polymerized collagen matrices was imaged and measured at the indicated times. For these studies the three *ACTG2* variants were cloned into lentiviral vectors to permit the stable transduction of target cells. They were PCR amplified adding *PciI* and *KpnI* restriction sites (Table S1). We used a previously reported system to generate the lentiviruses utilizing vectors pENTR4 no ccdB, pLenti CMV Puro DEST, psPAX2, and pCMV-VSVg (Addgene) (Campeau et al. 2009). U2OS cells were transduced with 1 ml of lentivirus containing supernatants.

Results

Clinical phenotypes

A full-term female (family 1) was born to healthy non-consanguineous parents after the mother's first pregnancy. Postnatally, the baby was found to have megacystis with hydronephrosis as well as malrotation and pseudo-obstruction of the intestine. She became total parenteral nutrition-dependent and developed end-stage liver disease with portal hypertension. She underwent a full thickness biopsy of the large intestine, which showed ganglion cells, excluding Hirschsprung's disease. Biopsy of the jejunum showed a thin outer muscular layer. Evaluation of other systems including an echocardiogram was normal. At 8 months of age, she had an en bloc transplant of stomach, liver, pancreas, and small intestine. She had several post-operative complications, but did well after age 4. She is currently 12 years old and in good health, but does require intermittent urinary catheterization.

A male fetus (family 2) was diagnosed with ultrasound at 19 weeks' gestation to have megacystis, bilateral hydroureters and anhydramnios. A keyhole sign for posterior urethral valve was not distinctly observed. There was no evidence of hydronephrosis, renal cystic dysplasia, or hyperechogenicity of the renal parenchyma. There were no other obvious anomalies. A vesicocentesis showed a sodium concentration of 79 mmol/L, chloride of 71 mmol/L, calcium of 3.9 mg/dL, and an osmolality of 167 mOsm/kg, all of which were below the threshold for renal cystic dysplasia. Fluorescent in situ hybridization on a chorionic villus sample showed no abnormalities for chromosomes 13, 18, and 21 with an XY complement. Fetal cystoscopy at the time of vesicoamniotic shunting at 20 weeks revealed a trabeculated bladder without a dilated urethra, suggesting that bladder distention was not due to a urethral obstruction. Despite the shunt being in place, megacystis and oligohydramnios persisted, for which a repeat vesicoamniotic shunt was placed at 30 weeks' gestation. The mother had ruptured membranes and the child was born at 31 weeks of gestation, weighing 1,460 g with Apgar scores of 7 and 9 at 1 and 5 min, respectively. Postnatal workup included barium enema/upper GI series due to failure to pass meconium, which showed findings consistent with intestinal malrotation, microcolon and a dilated loop of proximal small intestine. Late images did

not show a complete obstruction, but revealed severely delayed emptying of the small intestine. At 5 days of life, the patient was taken to the operating room for vesicoamniotic shunt removal, antegrade urethral stent placement with vesicostomy, exploratory laparotomy, and Ladd's procedure for correction of the intestinal malrotation. Operational findings confirmed the presence of microcolon and megacystis.

Genetic analysis

Sequencing of the whole-exome covered 82, 85, 87 % of the targeted regions at 10× read depth with an average of 64.1×, 67.2× and 72.8× read depth for the proband, the father, and the mother, respectively. The filtering strategy extracted 0 homozygous or compound heterozygous variants for the autosomal recessive inheritance model and only 1 heterozygous variant for the de novo model. The variant was a G>T substitution at chr2:74,140,693 (hg19), corresponding to c.533G>T (p.R178L) in *ACTG2* (NM_001615.3). Sanger sequencing confirmed the presence of this de novo variant (Fig. 1b). The identified variant was absent in the Exome Variant Server (http://evs.gs.washington.edu/EVS/), dbSNP or in our internal database.

Sanger sequencing of the entire *ACTG2* (primers are in Table S1) in the proband and both parents in family 2 showed another de novo variant, c.532C>T (p.R178C) (Fig. 1b). Arginine in ACTG2 is completely conserved in chordates with a −0.929 score of the Conseq server (http://consurf.tau.ac.il). Both variants were predicted to be pathogenic by MutationTaster (http://www.mutationtaster.org/).

RT-PCR in various murine tissues showed higher transcription levels of *Actg2* in tissues containing smooth muscle, particularly intestines and bladder, the major affected organs in both patients (Fig. 1c).

p. R178L and p.R178C mutations in *ACTG2* lead to impaired contractibility

Our structural analysis derived from the molecular modeling revealed that R178, located within the hinge separating domains III and IV, engages in key inter-domain contacts with H74, located within the hinge separating domains I and II (Fig. 1d). Notably, the R178–H74 interaction seems to lock ACTG2 in a conformation best suited for its polymerization into thin filaments (Otterbein et al. 2001). However, the replacement of R178 with leucine (R178L) or cysteine (R178C) would destabilize the R178–H74 interaction (Fig. 1d). This allows domains I–II to fall apart from domains III–IV within ACTG2, rather than remaining in close proximity. Accordingly, unlocking or destabilization of R178–H74 interaction would be expected to result in depolymerization of thin filaments into monomeric actin. Taken together, our structural analysis strongly argues that the R178L and R178C mutations would likely impair polymerization of ACTG2 into thin filaments and thus provide the physical basis of the poor contractility of smooth muscles in patients harboring these mutations.

Confocal images reveal that WT ACTG2 mostly co-localizes with the actin cytoskeleton filament network. However, both ACTG2 mutants show a diffuse localization and poor association with actin filaments (Fig. 2a).

In agreement with the structural/function predictions, cell contractility assessment shows a decreased collagen contraction capacity of cells overexpressing the two ACTG2 mutants (Fig. 2b).

Discussion

Here, we report two simplex cases with MMIHS with de novo mutations in *ACTG2*. MMIHS has previously been believed to be autosomal recessive in its inheritance and a 25 % recurrence risk is given to the couples with an affected child. Despite this, the majority of the reported cases are simplex. Our results show that, at least in some cases, MMIHS is caused by de novo heterozygous *ACTG2* mutations. In this case, a recurrence risk would be approximately 1 % based on the possibility of germline mosaicism. Further studies will determine if there is genetic heterogeneity that may explain previously reported multiple affected siblings with MMIHS.

The *ACTG2* gene is located on chromosome 2 containing two 5-prime untranslated exons and 8 coding exons. The mature protein has 376 amino acids. As other actin proteins, *ACTG2* comprises a nucleotide-binding domain of the sugar kinase/HSP70/actin superfamily between amino acids 9 and 182. Taking into consideration the homology with other proteins of its family, the function may be associated to vital roles in cell integrity, structure, and motility. Balance between monomeric and filamentous gamma actin, a linear polymer of actin subunits may determine the role of this protein (Dominguez and Holmes 2011).

Abnormal expression of smooth muscle actin in patients with MMIHS has been reported in previous studies that suggested a myogenic origin for the syndrome (Piotrowska et al. 2003; Ciftci et al. 1996). Another missense mutation, p.R148S, in *ACTG2* has recently been reported in an autosomal dominant family diagnosed with familial visceral myopathy with intestinal pseudo-obstruction without involvement of other systems (Lehtonen et al. 2012). Similar to MMIHS, familial visceral myopathy is characterized by impaired functions of enteric smooth muscle cells, resulting in abnormal intestinal motility, severe abdominal pain, malnutrition, and often death in the fourth decade of life. Bladder dysfunction is not a typical clinical

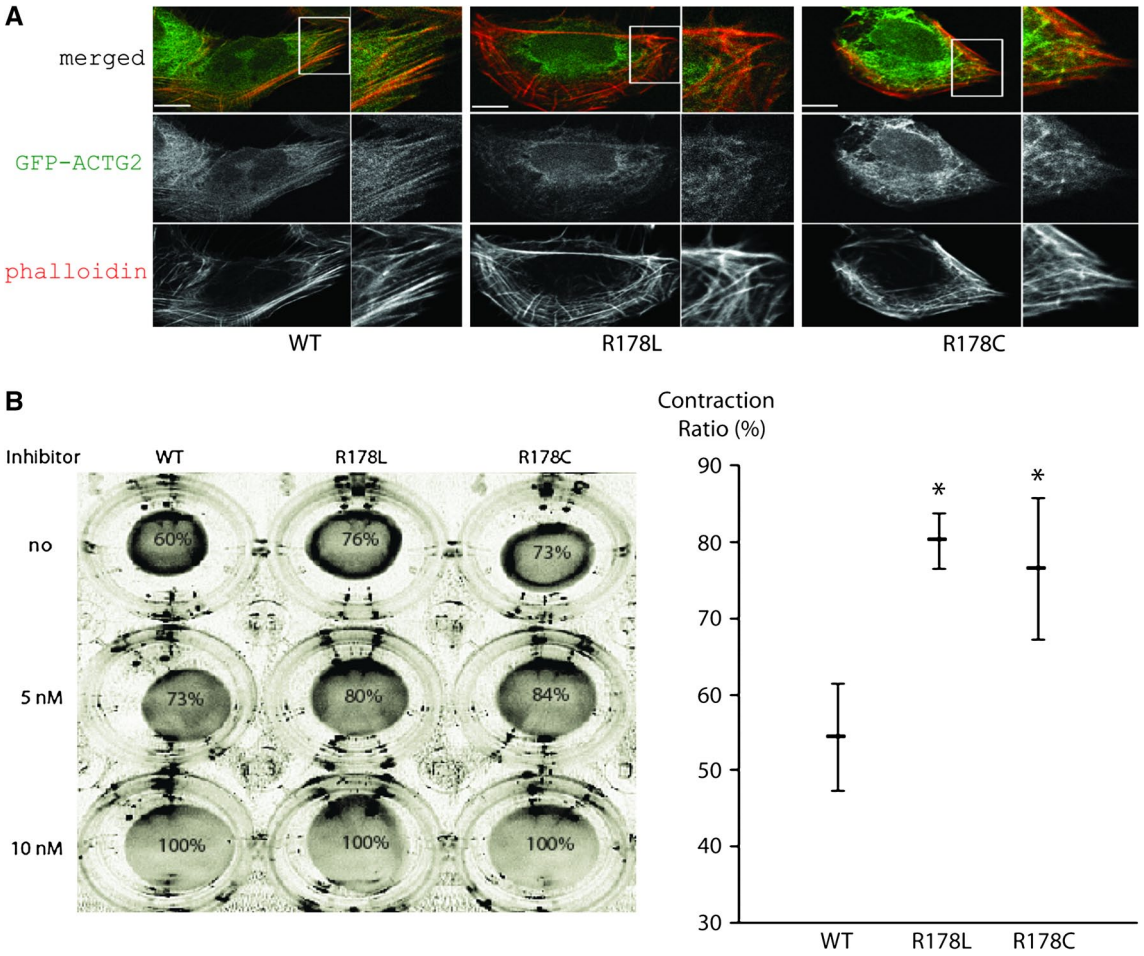

Fig. 2 Cellular localization of WT and mutant ACTG2 and effects on contraction. **a** Confocal scans of COS7 cells showing co-localization of WT ACTG2 but not the mutant proteins with the actin filaments. *Rectangles* indicate the area magnified and presented to the *right* of each image. *Scale bar* 10 μm. **b** Contraction assessment of WT and mutant ACTG2 overexpressing U2OS cells. *Left panel*: areas of collagen matrices containing cells after 48 h in the presence of increasing concentrations of the contraction inhibitor BDM. Each percentage represents the area of contraction relative to the highest inhibitor concentration (10 nM). *Right panel*: *bars* show the mean ±95 % confidence interval of the results of six experiments; *asterisk* indicates Mann–Whitney *U* test *p* value (0.029) between WT and each mutant

presentation. On the other hand, multivisceral transplantation early in life, which includes small bowel transplantation, is often the only treatment option for MMIHS that has only 12–20 % survival rate (Mc Laughlin and Puri 2013). Disruption of the ACTG2 protein is likely to be milder in visceral myopathy compared to those we report causing MMIHS. Thus, MMIHS and the less severe familial visceral myopathy appear to represent a spectrum of one disease caused by different *ACTG2* mutations, the intestinal hypoperistalsis being the common denominator.

A well-known example of genes causing a more severe phenotype associated with de novo mutations is *COLlA1* (MIM 120150) and *COL1A2* (MIM 120160). Approximately 60 % of cases with classic non-deforming osteogenesis imperfecta with blue sclerae, but 100 % of perinatally lethal osteogenesis imperfecta are caused by de novo mutations in these genes (Steiner et al. 1993). This story is also reminiscent of *ACTA2* (MIM 102620) mutations, which can cause autosomal dominant thoracic aortic aneurysms (MIM 611788) without involvement of other systems. However, two de novo mutations involving the arginine residue at position 179 of *ACTA2* can cause a very severe phenotype including persistent ductus arteriosus, cerebrovascular events, mydriasis, intestinal malrotation, and hypotonic bladder (MIM 613834) (Meuwissen et al. 2013; Milewicz et al. 2010) significantly reducing the genetic fitness by shortened survival. These findings suggest that the clinical manifestations caused by *ACTA2* and *ACTG2* mutations present in a spectrum from mild to severe and depend on the degree of protein malfunction and differential expression of these smooth muscle actins.

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