Muscarinic Acetylcholine Receptor M3 Mutation Causes Urinary Bladder Disease and a Prune-Belly-like Syndrome

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Urinary bladder malformations associated with bladder outlet obstruction are a frequent cause of progressive renal failure in children. We here describe a muscarinic acetylcholine receptor M3 (*CHRM3*) (1q41-q44) homozygous frameshift mutation in familial congenital bladder malformation associated with a prune-belly-like syndrome, defining an isolated gene defect underlying this sometimes devastating disease. *CHRM3* encodes the M3 muscarinic acetylcholine receptor, which we show is present in developing renal epithelia and bladder muscle. These observations may imply that M3 has a role beyond its known contribution to detrusor contractions. This Mendelian disease caused by a muscarinic acetylcholine receptor mutation strikingly phenocopies *Chrm3* null mutant mice.

Lower urinary tract and/or kidney malformations account for 40% of childhood end-stage renal failure.[1] Advances have been made in unraveling the genetic pathogenesis of kidney anomalies,[2] but less is known about the genetic origin of malformations of the lower tract. Congenital bladder outflow obstruction (BOO) has several causes, the commonest being posterior urethral valves (PUVs), with a high risk of developing chronic renal insufficiency.[1,3] Recently, mutations of *HPSE2* (MIM 613469; see Web Resources), encoding a heparanase inhibitor expressed in developing urinary tracts, were described in urofacial syndrome (UFS [MIM 236730]), which presents with a dysmorphic, poorly emptying bladder.[4] Also associated with congenital bladder dysfunction is prune belly syndrome (PBS [MIM 100100]). In its rare complete form, it comprises megacystis with disorganized detrusor muscle, cryptorchidism and thin abdominal musculature with overlying lax skin. PUV and PBS generally occur sporadically, although families have been reported with more than one affected member.[3,5,6]

We here describe a homozygous loss-of-function mutation of muscarinic acetylcholine receptor M3 (*CHRM3*) (1q41-q44) in five brothers with a PBS-like syndrome (Figures 1 and 2). *CHRM3* (MIM 118494) encodes the M3 subtype of muscarinic acetylcholine (ACh) receptors, the major receptor mediating urinary bladder contraction upon micturition.[7] We restudied a family reported in 2005[5] when there were four surviving boys with congenital BOO born to consanguineous Turkish parents. A fifth affected male sibling had died soon after birth as a result of renal failure and urosepsis (II-1). He and one affected surviving brother (II-4) displayed marked abdominal wall distension and were assigned as having PBS, whereas their three brothers were considered to have "PUV" because cystopscopy noted urethral valve-like structures. Recently, a sixth brother (II-7) was born, also with a malformed bladder. Evaluation of available cystograms showed massive and irregular-walled bladders with diverticulae (Figure 1). Vesicoureteric reflux was a variable feature. Urethral patency appeared normal in available cystograms. Cystometry performed in four affected boys in the first years of life revealed detrusor hyporeflexia with high residual volumes after micturition. Thus, the collective features in this kindred are most consistent with the PBS spectrum. Affected individuals also had bilaterally impaired pupillary constriction to light (Figure 1) and dry mouths. These examinations were performed after informed consent was obtained from the patients and their parents. Ethical approval for this study was obtained from the ethics review board at the Ruprecht-Karls-University Heidelberg and the collaborating institutions, according to the Declaration of Helsinki.

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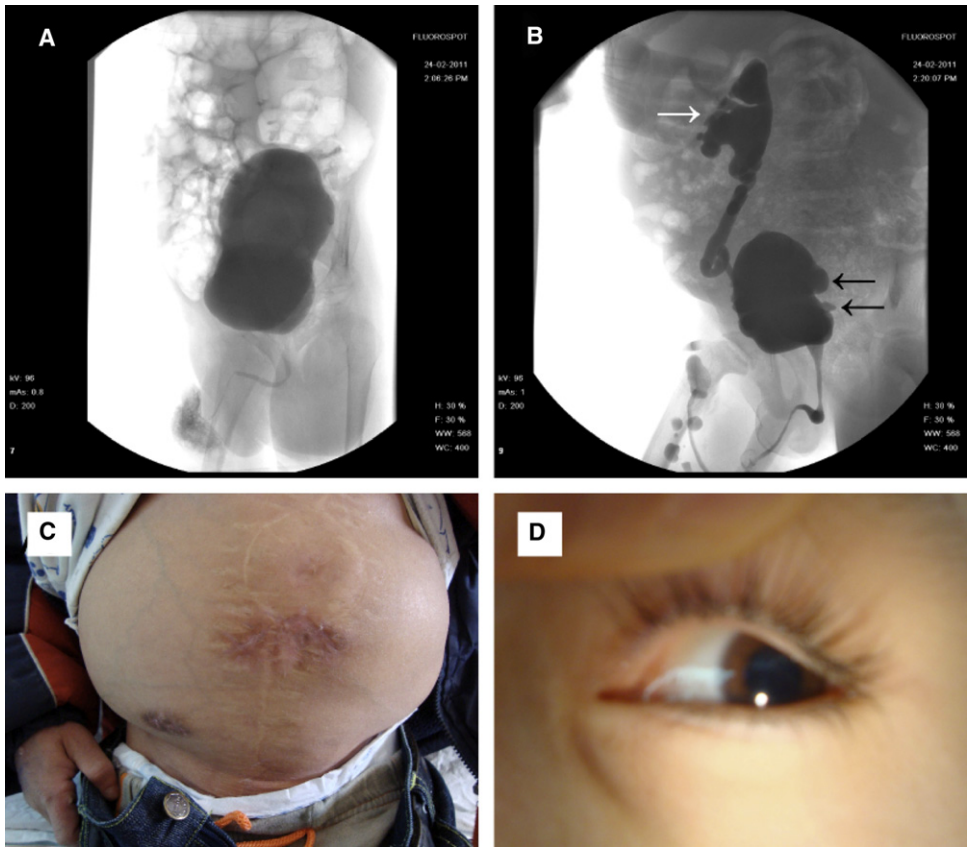

Figure 1. Clinical Features in Family with *CHRM3* Mutation
(A) Cystogram of individual II-7 demonstrating a massively enlarged bladder.
(B) Cystogram of index person II-4 showing an irregular-walled bladder with diverticula (black arrows) and unilateral high-grade vesicoureteral reflux into a distorted renal pelvis (white arrow).
(C) Abdominal wall laxicity of index person II-4.
(D) Impaired pupillary constriction to bright light in index person II-4.

Using a SNP chip-based genome-wide linkage approach, we previously identified two regions of homozygosity on chromosomes 1 (35 cM) and 11 (9 cM) in four affected surviving males of this family (Figure 2).[5] Exon capture and massively parallel sequencing now lead to the identification of the causative genetic defect in *CHRM3*, located in the chromosome 1 critical interval (Figure 2). DNA of index patient II-4 was enriched for protein-coding genes with the Agilent SureSelect Human All Exon 38 Mb kit and ran on two lanes of the Illumina GAIIx Sequencer with the paired-end protocol and a read length of 95 bp at each end, generating ~202 × 10^6 raw reads of which ~193 × 10^6 could be mapped to the human reference sequence. Alignment and variant calling was performed with MAQ for SNP detection (version 0.7.1)[8] and BWA-short (version 0.5.7)[9] in combination with SAMTOOLS (version 0.1.7)[10] for indel detection. On average, 90% of the exome was covered at least 30-fold and 97% ten-fold. Scripts developed in-house were applied to detect protein changes, affected splice sites, and overlaps with known variants (Table S1 available online). In detail, using MAQ and SAMtools, we identified a total of 672,588 variations. After filtering for a minimal phred-like consensus quality score > 15, 517303 variations remained. A stringent filter step discarding known variations with dbSNP, 1,000 genomes data, and the CCG in-house database with ~100 exomes reduced the list to 48811. Further filtering for homozygosity (allele frequency > 75%) together with changes in protein sequence or location ±25 nts inside 3′/5′ splice sites reduced the list to 38 candidate variations. Finally, only two variations were found to overlap with the previously identified regions of significant linkage—a missense change in *HEATR1*:c.6037A > G;p.Lys2013Glu (RefSeq accession number NM_018072.5) and an insertion-deletion frameshift mutation in *CHRM3*: c.1173_1184 delinsT; p.Pro392Ala*fs**43 (RefSeq accession number NM_000740.2). Both variations were not present at the Exome Variant Server, NHLBI Exome Sequencing Project (ESP, Seattle, WA; see Web Resources). *HEATR1* encodes BAP28, a protein involved in rRNA transcription and processing, and knockdown of *Heatr1* in zebrafish causes embryonic death due to CNS degeneration,[11] a phenotype considerably different from the one observed in our index patient. *CHRM3*, however, encodes the human muscarinic acetylcholine receptor M3 (hereafter simply called M3), a G protein-coupled receptor with seven

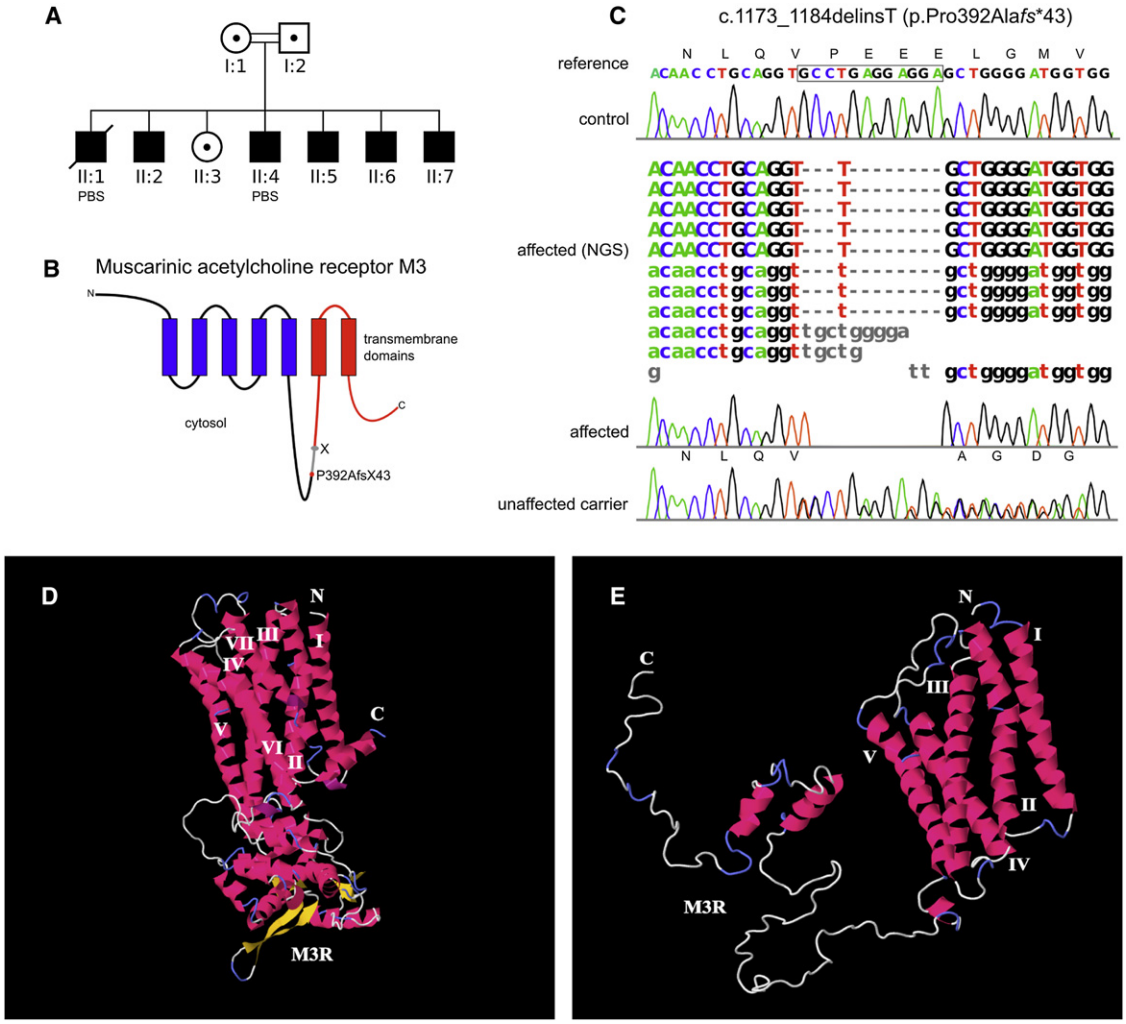

Figure 2. Deletion-Insertion Mutation of *CHRM3*

(A) Family pedigree. The index person II-4 and a deceased boy (II-1) presented with the full picture of PBS. Their male siblings, II-2, II-5, II-6, and II-7, had malformed bladders only. Chronic kidney disease (CKD) grade II was observed in affecteds II-5 and II-6, CKD grade IV in index person II-4.

(B) p.Pro392Ala*fs**43 introduces a premature termination codon in the third intracellular loop (M3R) resulting in a truncated protein that lacks the last two transmembrane domains (shown in red).

(C) A homozygous *CHRM3* frameshift mutation c.1173_1184 delinsT;p.Pro392Ala*fs**43 was identified in index person II-4 by massively parallel sequencing; five forward (upper case) and three reverse reads (lower case) were recognized as an insertion-deletion mutation.

(D and E) Three-dimensional protein structure modeling of wild-type (D) and mutant (E) receptor M3. Transmembrane helices (I-VII) and the amino- (*N*) and carboxy- (*C*) termini are indicated. Structure predictions of mutant M3 suggest complete deterioration of folding of the third intracellular loop (*M3R*) in addition to loss of transmembrane helices VI and VII in mutant M3. Three-dimensional protein structure modeling was obtained as a homology (comparative) protein structure with $(PS)^2$-v2 using Protein Data Bank (PDB) entries 2rh1, 2rh1A and 1r5sA as template.[31] Structures were processed with Jmol. Model reliability is $\geq$75% for wild-type and mutant (excluding the mutant C terminus) and 55%–75% for the mutant C terminus.

transmembrane domains and the major receptor involved in mediating urinary bladder contraction upon micturition.[7] Both the obvious loss-of-function nature of the *CHRM3* mutation and the striking similarity of the human phenotype to *Chrm3* null mutant mice (discussed below) underscored the likelihood of this mutation being the causal genetic lesion in our family. The p.Pro392Ala*fs**43 mutation induces a premature termination codon (PTC) at position 435. If mutant RNA transcripts were stable and translated, a truncated M3 protein would be generated lacking part of the third cytosolic loop, transmembrane domains VI and VII and the C terminus of the receptor. Three-dimensional structure predictions of mutant M3 suggest perturbed folding of the remaining third intracellular loop (Figure 2), known to be essential for signal transduction through G protein activation and scaffold protein interaction.[12] Sanger sequencing was performed for individuals II-2, II-3, II-4, II-5, II-6, II-7, I-1, and I-2 of the family. The c.1173_1184 delinsT; p.Pro392Ala*fs**43 mutation of *CHRM3* cosegregated consistently with the disease phenotype according to a recessive mode of inheritance[5] and was absent from 374 Turkish control chromosomes

(power >95% to distinguish a normal sequence variant given an allele frequency of 0.01[13]). The mutation was also not annotated in the Exome Variant Server SNP database. All affected males were shown to be homozygous for the *CHRM3* mutation whereas both parents and the unaffected sister (II-3) were heterozygous carriers. *CHRM3* mutations and homozygous deletions were not identified by Sanger sequencing in five multiplex PUV families, 38 simplex PUV cases, 19 simplex PBS cases, four simplex megacystis cases, three unrelated individuals affected by UFS lacking *HPSE2* mutations,[4] and a cohort of 86 individuals of the 4C-Kidney Disease Study with bladder and/or urethral anomalies (see Web Resources). Because of the recessive nature of the disease, we did not look for heterozygous deletions in this cohort.

Notably, Pomper et al.[14] recently reported an adult with micturition failure since childhood and with pupils that failed to constrict upon muscarinic stimulation. This phenotype is strikingly similar to that affecting the family in our current report. An absence of M3 protein was found in bladder tissue and a *CHRM3* mutation was postulated. However, sequencing and copy number analyses were normal in the individual presented by Pomper et al., consistent with a yet-to-be-defined genetic or acquired disorder affecting generation of M3 protein and/or its stability.

To date, human mutations of *nicotinic* acetylcholine receptor subunits have been described, e.g., in the fetal akinesia deformation sequence (MIM 208150) where they seem to be involved in morphogenetic signal transduction in addition to skeletal muscle contraction.[15] Our report describes a Mendelian human disease caused by a *muscarinic* acetylcholine receptor mutation, linking a developmental disorder of the renal tract to a component of the parasympathetic nervous system. Key functions of the bladder are to act as a low-pressure reservoir for urine and to expel urine per urethra. The latter is driven by detrusor contractions stimulated by parasympathetic nerves. Although male and female adult human bladders express mRNA of two muscarinic acetylcholine receptor subtypes (M2 and M3),[16,17] only M3 is the critical receptor for micturition,[7] mediating 95% of cholinergic contraction of the detrusor muscle.[18] In mice and rats, M3 is observed in bladders of both young animals and adults.[19,20]

For determining the localization of M3 in embryonic and fetal tissue, immunohistochemistry studies were performed with M3 antibodies in wild-type mice and human embryos (Figure 3).[21] The latter were collected with informed consent under Polkinghorne Committee guidelines and staged by the Carnegie classification or foot length. In embryonic day 13 mice, M3 was present in nascent bladder and urethral epithelia. Later in mouse gestation, when the bladder becomes muscularized, M3 was immunodetected in bladder urothelial and detrusor muscle layers. M3 was also localized in ureteric and metanephric kidney nephron precursors. In normal human embryos, M3 protein was detected in urogenital sinus and urethral epithelia and in the late first trimester bladder in urothelia and muscle cells. M3 protein is therefore not only present in fetal bladder muscle but also in the urogenital sinus and fetal urethral epithelia, concurring with sites of embryonic *Chrm3* mRNA expression according to Mouse Genome Informatics and GUDMAP databases (see Web Resources). GUDMAP also reports *Chrm3* transcripts in the metanephros, and we detected M3 protein in nephron precursors. Collectively, these observations may imply that M3 has a role beyond its known contribution to detrusor contractions. Preliminary GUDMAP data exists that *ChAT* transcripts are expressed in embryonic kidneys as well as in embryonic bladder and urethral mesenchyme. If the encoded protein, choline acetyltransferase (ACh), is also detectable, non-neuronal ACh may be synthesized in the nascent renal tract. In future studies, it will be interesting to address the possibility that ACh is also expressed in non-neuronal tissues.

In homozygous *Chrm3* mutant mice lacking M3, adult detrusor contractility to carbachol ex vivo is impaired and (only) male mutant adult bladders are grossly distended in vivo.[18] Although it is unknown whether megacystis is present in younger *Chrm3* mutant mice, the hypocontractile bladder phenotype is very similar to humans with *CHRM3* mutations, described above. Of note, only male *Chrm3* mice will develop clinical disease. This is, at least in mice most probably, related to differences between male and female urethral anatomy with male bladders being more contractile to force the urine through the longer urethra. Alternatively, female mice (but not males) might have other compensatory physiological mechanisms allowing them to empty their bladders independent of M3 action. Expression and function of M3 in rat and guinea pig urinary bladders do not seem to differ considerably between male and female animals[22] and with regard to overall muscarinic responsiveness this situation appears to be similar in humans.[23] In humans, we can neither rule out nor confirm a male-limited phenotype: the pedigree of the presented index family is not helpful to delineate the phenotype of female homozygous *CHRM3* mutation carriers given that no female individual is affected by such a mutation. Mother and sister of the index person are both heterozygous mutation carriers with apparently normal bladder anatomy and function. With respect to PBS, the full clinical picture, including BOO, cryptorchidism, and abdominal wall distension, is limited to males; however, incomplete forms of PBS have also been described in females.[24,25]

The etiology of abdominal muscle deficiency in individuals with PBS has been a matter of controversy. Either a common underlying defect of mesodermal development is proposed[25] or, as other authors suggest, abdominal muscle deficiency seems to be a nonspecific anatomic defect that is secondary to fetal abdominal wall distention (caused by megacystis, hydronephrosis or constipation).[26] In the present study, RT-PCR experiments were performed on RNA extracted from human mature abdominal wall musculature, but no M3 expression was apparent

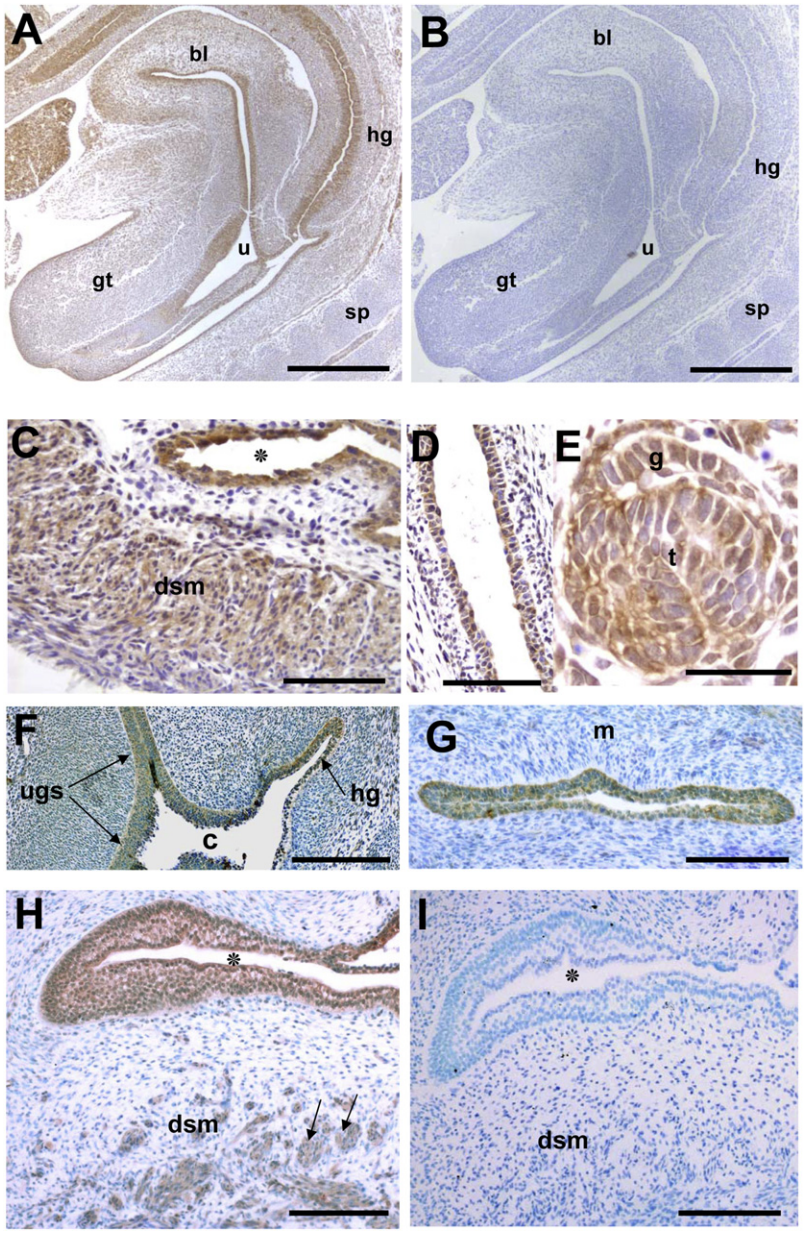

Figure 3. M3 Localization in the Developing Renal Tract

Immunohistochemistry of M3 on sections of normal mouse (A–E) and human (F–I) embryos by respectively applying M3 antibodies Sigma M0194 and Abcam 60981.

(A and B) Embryonic day 13 sagittal sections.

(A) Localization of M3 in epithelia of the urogenital sinus, which has separated from the hindgut (*hg*); the upper (cranial) part of the sinus is forming the bladder (*bl*) and the lower part of the sinus is forming the urethra (*u*) extending into the genital tubercule (*gt*). M3 is also detected in hindgut epithelia. The spine (*sp*) is dorsal to the hindgut.

(B) Adjacent section with primary antibody omitted.

(C) Embryonic day 15 bladder. M3 localization in detrusor smooth muscle (*dsm*) as well as urothelia; the asterisk indicates lumen.

(D) Longitudinal section through embryonic day 14 ureter, when urothelium is surrounded by mesenchyme which has yet to form muscle; M3 is localized in epithelia.

(E) Forming nephron in embryonic day 13 metanephric kidney. The upper part is the precursor of the glomerulus (*g*) while the lower section will form the tubule (*t*); in both M3 is detectable.

(F) Sagittal section at 51 days post conception when the urogenital sinus (*ugs*) and hindgut (*hg*) terminate in a common cloaca (*c*). M3 is localized to both urogenital sinus and hindgut epithelia.

(G) Transverse section through the urethra at the bladder outlet (10 weeks after conception); M3 localization to urothelia while the surrounding mesenchyme (*m*) is negative is shown.

(H) Bladder wall 12 weeks after conception; M3 detection in human detrusor smooth muscle (*dsm*; indicated by arrows) and urothelia; the asterisk indicates lumen.

(I) Adjacent section with primary antibody omitted. Scale bars represent 500 μm (A and B), 100 μm (C), 125 μm (D), 50 μm (E), 200 μm (F), 100 μm (G), and 400 μm (H and I).

(Figure S1). These data are in favor of abdominal wall distension being a secondary event after megacystis and/or urinary stasis due to impaired urinary bladder contraction early in fetal life.

Failure of bladder contractility before birth in humans with homozygous (or compound heterozygous) *CHRM3* mutation might contribute to the observed bladder dysmorphology postpartum. Bladder dysfunction is likely to persist in individuals with *CHRM3* mutation thoughout life, constituting an important risk factor for the progression of renal insufficiency.[3] *Chrm3* mutant mice also display impaired salivation and dilated pupils, consistent with a role for muscarinic ACh actions in serous salivary secretion and pupillary constriction,[18] and a similar phenotype is present when human *CHRM3* is mutated.

Deletions within 17q12 encompassing *hepatocyte nuclear factor 1B* (*HNF1B* [MIM 189907]) have been reported in sporadic PBS,[27] but whether *HNF1B* itself or a nearby disrupted gene is causative remains unclear, and *HNF1B* mutations have not yet been implicated in familial PBS. Furthermore, although *HNF1B* mutations are a not uncommon cause of dysplastic kidneys,[2,28] such patients rarely have overt bladder lesions. Importantly, in our *CHRM3* mutation family, *HNF1B* was normal by sequencing and seeking copy number variants (data not shown). Mice mutant for β2 and β4 or α3 subunits of the nicotinic ACh receptor[29,30] display enlarged, hypocontractile bladders, as a result of compromised neural relaying via autonomic ganglia, and may serve as models for humans with congenital BOO who do not have *CHRM3*, *HPSE2*, or *HNF1B* mutations. We suggest that the next decade

will witness the unraveling of genetic causes of bladder malformations, thus providing insights into the mechanisms of both normal development and explaining how bladder dysmorphology arises in the context of PBS, PUV, UFS and other related syndromes.[3]

Supplemental Data

Supplemental Data include one table and one figure and can be found with this article online at http://cell.com/AJHG/.

Acknowledgments

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Web Resources

The URLs for data presented herein are as follows:

The Cardiovascular Comorbidity in Children with Chronic Kidney Disease Study, http://www.4c-study.org
Exome Variant Server, http://snp.gs.washington.edu/EVS
GUDMAP, http://www.gudmap.org
Mouse Genome Informatics, http://www.informatics.jax.org
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org

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