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Short Report

Expanding the phenotype of CRB2 mutations – A new ciliopathy syndrome?

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Recessive *CRB2* mutations were recently reported to cause both steroid resistant nephrotic syndrome and prenatal onset ventriculomegaly with kidney disease. We report two Ashkenazi Jewish siblings clinically diagnosed with ciliopathy. Both presented with severe congenital hydrocephalus and mild urinary tract anomalies. One affected sibling also has lung hypoplasia and heart defects. Exome sequencing and further *CRB2* analysis revealed that both siblings are compound heterozygotes for *CRB2* mutations p.N800K and p.Gly1036Alafs*43, and heterozygous for a deleterious splice variant in the ciliopathy gene *TTCB21*. *CRB2* is a polarity protein which plays a role in ciliogenesis and ciliary function. Biallelic *CRB2* mutations in animal models result in phenotypes consistent with ciliopathy. This report expands the phenotype of *CRB2* mutations to include lung hypoplasia and uretero-pelvic renal anomalies, and confirms cardiac malformation as a feature. We suggest that *CRB2*-associated disease is a new ciliopathy syndrome with possible digenic/triallelic inheritance, as observed in other ciliopathies. Clinically, *CRB2* should be assessed when ciliopathy is suspected, especially in Ashkenazi Jews, where we found that p.N800K carrier frequency is 1 of 64. Patients harboring *CRB2* mutations should be tested for the complete range of ciliopathy manifestations.

Conflict of interest

All the authors declare they have no conflict of interest relating to this work.

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Recessive *CRB2* mutations were recently described to cause both steroid resistant nephrotic syndrome [focal segmental glomerulosclerosis (FSGS) 9, Online Mendelian Inheritance in Man (OMIM), http://www.omim.org#616220] (1), and prenatal onset ventriculomegaly with cystic kidney disease/congenital nephrotic syndrome (OMIM# 219730) (2). *CRB2* is highly expressed in brain, retina and kidney, and with *CRB1* and *CRB3* forms the Crumbs polarity proteins family. CRB1 and CRB2 have similar protein structure (3), including multiple extracellular EGF-like and laminin-like domains. In *CRB1*, mutations in these domains cause retinal dystrophies (OMIM #600105). CRB3 localizes to cilia and is required for ciliogenesis (4), *CRB3*-related disease has not been reported in humans.

We report two siblings with severe obstructive congenital hydrocephalus, renal anomalies and lung hypoplasia, features clinically consistent with a ciliopathy. Molecular analysis revealed bi-allelic *CRB2* mutations, expanding the phenotypic spectrum of *CRB2* mutations and suggesting that *CRB2*-associated disease may represent ciliary dysfunction.

Patients and methods

The Institutional Review Board and Israeli National Ethics Committee approved this study. Both patients are Ashkenazi Jewish.

Patient 1

This boy is currently 7 years old (Table S1, Supporting Information). Severe ventriculomegaly and left hydronephrosis were noted prenatally. Fetal magnetic resonance imaging (MRI) showed severe bilateral lateral ventricle dilatation and thin corpus callosum (Fig. 1a, Table S1). Maternal serum alpha-fetoprotein (AFP) was not measured during pregnancy, due to maternal preference. Head circumference (HC) at birth was 40 cm [+3.8 standard deviation (SD)]. Mild dysmorphic features at birth included broad forehead, low set ears, retrognathia, widely spaced nipples, bilateral single transverse crease and broad thumbs. At age 3 8/12 years, frontal bossing, high palate and broad first toe were observed. Post-natal MRI showed aqueductal-stenosis and obstructive hydrocephalus (Fig. 1b). A ventriculoperitoneal (VP)-shunt was inserted at age 3 months. Renal ultrasound showed minimal left ureterohydronephrosis at birth, and a mildly enlarged right kidney at age 4 years. He is otherwise healthy, with age-appropriate development. Echocardiogram and ophthalmological examinations were normal, renal function is normal without proteinuria. Chromosomal microarray analysis (CMA) revealed no abnormalities.

Patient 2

The younger sister of Patient 1, is currently 5 1/2 years old (Table S1). Severe ventriculomegaly and mild hydronephrosis were noted prenatally at 35 weeks (pregnancy follow-up was sparse). Maternal AFP was not measured. At birth HC was 47.5 cm (+6 SD). Severe hydrocephalus and respiratory distress necessitated neonatal intensive care admission. Postnatal brain computed tomography (CT) showed severe non-communicating hydrocephalus (Fig. 1c,d), initially treated by external drainage, followed by VP-shunt insertion at age 2 weeks. Respiratory distress was caused by right lung hypoplasia evident on chest X-ray (Fig. 1e) and CT scan. Additional anomalies included bilateral mild ureterohydronephrosis and patent ductus arteriosus (PDA). The PDA, either secondary to or exacerbated by her pulmonary disease, required surgical closure. Several readmissions were required for VP shunt removal/reinsertion, and for treating respiratory exacerbations. A single seizure occurred at 1 3/4 year. At age 2 4/12 years HC was 51 cm (+2.1 SD, 98.5%). Dysmorphic features included: right occipital plagiocephaly, low set left ear, depressed nasal bridge, high narrow palate, mild shield chest with widely spaced nipples and broad first toe. MRI post VP-shunt insertion, showed marked right posterior plagiocephaly, triventricular ventriculomegaly, aqueductal stenosis, very thin and abnormal corpus callosum with absent septum, and relatively normal posterior fossa. She required cranial remodeling surgery. Currently, she has moderate global developmental delay and hypotonia (Table S1). Abdominal ultrasound (2 3/4 years) showed a relatively small right kidney with mild hydronephrosis. Echocardiogram (10 months) showed positional displacement of the heart to the right chest, a small ventriculo-septal defect and a relatively small right pulmonary artery. Ophthalmologic examinations were normal, except for optic disc temporal pallor at 5 years. Visual evoked potentials were normal. Renal function is normal, with minimal proteinuria (50 mg%). Ciliary biopsy exhibited normal motility (light microscopy) and ultrastructure (electron microscopy). Karyotype and CMA showed no chromosomal aberrations.

Molecular analysis

Whole-exome sequencing (WES)

WES was performed in both patients (Appendix S1).

CRB2 *analysis*

CRB2 p.N800K and *CRB2* exons 4 and 10 of were Sanger sequenced (Appendix S1). CRB2 p.N800K allele frequency was extracted from a dataset of 128 control Ashkenazi high-coverage whole-genomes (5).

TTC21B *splice-site variant analysis*

The *TTC21B* c.1088-1G>C variant was confirmed using Sanger sequencing (Appendix S1). Its splicing effects were evaluated using: Human Splice Finder (HSF, http://www.umd.be/HSF/#), Splice Site Prediction by Neural Network (NNSplice, http://www.fruitfly.org/seq_tools/splice.html) and USD SplicePredictor (http://bioservices.usd.edu/splicepredictor/).

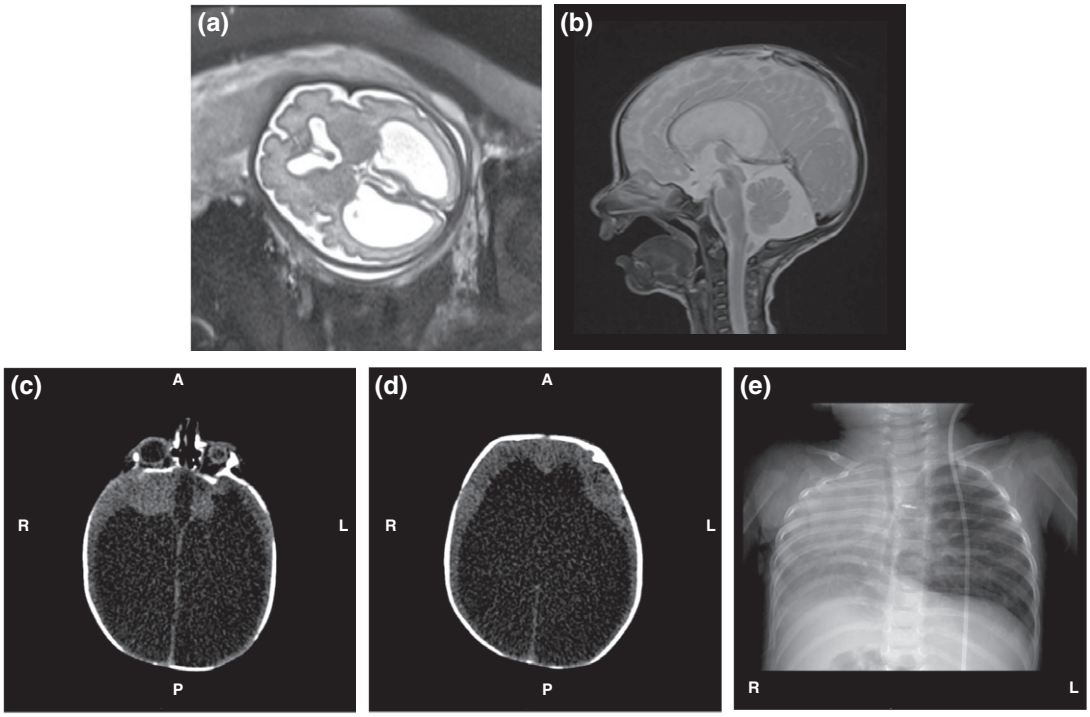

Fig. 1. Aqueductal stenosis, hydrocephalus and lung hypoplasia in patients with bi-allelic CRB2 mutations. Patient 1: (**a**) Fetal magnetic resonance imaging (MRI), showing severe ventriculomegaly with a normal posterior fossa , and (**b**) a post-natal pre-operative MRI (sagittal T2 weighted) showing aqueductal stenosis. Patient 2: (**c**, **d**) Cranial computed tomography (CT) at age 5 days, showing severe obstructive triventricular hydrocephalus – aqueductal stenosis, absence of septum pellucidum with a normal posterior fossa. Differential diagnosis was holoprosencephaly. (**e**) Chest X-ray at age 9 months, showing tracheal deviation because of right lung hypoplasia.

Results

Initial WES of both patients did not identify any shared bi-allelic pathogenic mutations using standard filtering criteria (Table S3). Phenotype-targeted analysis revealed a heterozygous missense mutation in *CRB2*: c.2400C>G, p.N800K (NM_173689 exon 8, genomic position chr9:126,133,821). Sanger sequencing confirmed p.N800K heterozygosity in both affected siblings and their father (Fig. 2a,b). This substitution, in a highly conserved residue (Fig. 2c), is extremely rare in the general population [allele frequency 0.0001439 in the Exome Aggregation Consortium (ExAC) database, http://exac.broadinstitute.org/]. It was previously reported in compound heterozygous state with other *CRB2* mutations in patients with fetal ventriculomegaly, hyperechogenic kidneys and elevated amniotic fluid AFP. All previously reported patients harboring the p.N800K mutation had Ashkenazi Jewish ancestry (2). We therefore examined data from 128 Ashkenazi Jewish control genomes (5). Two CRB2-p.N800K heterozygotes were identified, yielding a 1:64 carrier frequency in Ashkenazi Jews.

Re-examination of WES data in search of a second *CRB2* mutation indicated incomplete *CRB2* coverage, with no reads in exons 4 and 10. Sanger sequencing of these exons revealed a heterozygous 16bp duplication, c.3089_3104dup (genomic position chr9:126,135,899-126,135,914) in both affected siblings and their mother [Fig. 2a,b; confirmed by fluorescently tagged polymerase chain reaction (PCR) (Fig. S1)]. This frame-shifting duplication is predicted to cause premature termination (p.Gly1036Alafs*43), and is not present in large databases (ExAC, Exome Variant Server [EVS: http://evs.gs.ashington.edu/EVS/], 1000 Genomes Project [http://www.1000genomes.org/]), nor in 128 control Ashkenazi genomes (5). It has been reported in a single FSGS patient as a *de novo* mutation in compound heterozygous state with another *CRB2* mutation (1).

WES also revealed that both affected sibs were heterozygous for a *TTC21B* variant: c.1088-1G>C in the exon 10 acceptor splice site (NM_024753, genomic position chr2:166,786,258). This variant is very rare (0.000008333 allele frequency, ExAC), and predicted by all tools to abolish the acceptor site. Sanger sequencing confirmed the variant in both affected siblings, and in their mother (data not shown).

Discussion

Ciliopathies are a large group of inherited diseases resulting from structural or functional ciliary defects. Various ciliated organs are affected, often early in development, especially the respiratory system, central nervous system, retinal photoreceptor cells, reproductive system, kidney, and heart (6). We report two siblings with bi-allelic *CRB2* mutations, presenting with a variable phenotype including severe hydrocephalus and

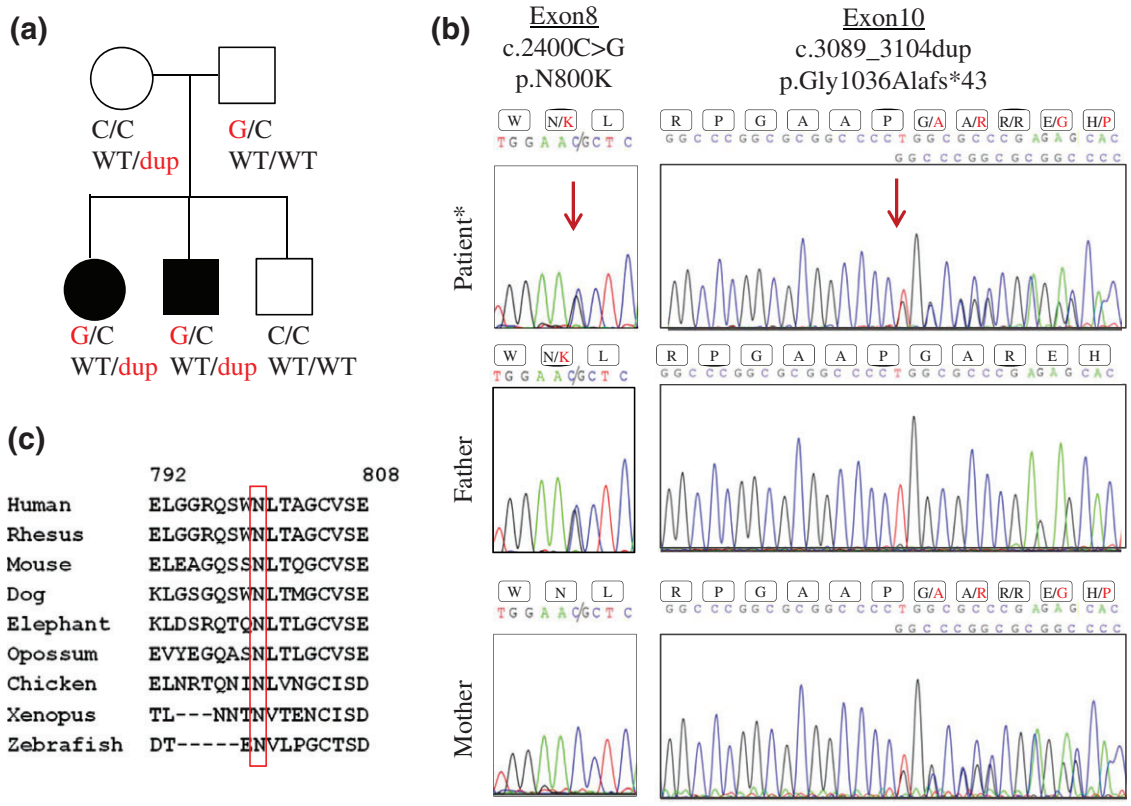

Fig. 2. CRB2 mutations in the patients and their family. (**a**) Family pedigree indicating compound heterozygosity for *CRB2* mutations. Parents are unrelated, of Ashkenazi Jewish origin. Genotype is indicated beneath each individual. Mutant alleles are shown in red, top: c.2400C>G, bottom: c.3089_3104dup. (**b**) Sanger sequencing of *CRB2* exons 8 and 10. Nucleotide and expected amino-acid protein sequence (boxed) are shown above the chromatograms. Mutated positions are indicated with an arrow. *Sequence is the same in both patients. (C) Multiple species alignment of CRB2 amino acid sequence for residues 792–808. Conservation of asparagine at position 800 is indicated by a red rectangle.

other brain anomalies, lung hypoplasia, congenital heart anomalies and mild uretero-pelvic renal defects. Human biallelic *CRB2* mutations have recently been described to cause either postnatal steroid resistant nephrotic syndrome because of podocyte defects (1) or prenatal ventriculomegaly associated with fetal renal anomalies (hyperchogenicity or microcysts) accompanied by elevated AFP (2). Features reported in single patients included brain heterotopia, pericardial effusion and ventricular septal defect (2). Thus, *CRB2*-related manifestations are all consistent with a ciliopathy. Isolated FSGS is also caused by mutations in the *TTCB21* ciliopathy gene (7), affecting podocyte primary cilia which are present in early development. In fact, FSGS may be under-recognized in ciliopathies because nephronophthisis, the archetypic renal phenotype, often develops earlier (8). Hydrocephalus, lung hypoplasia, renal and cardiac structural malformations are all features of ciliopathies, occurring with varying frequency based in part on the underlying genetic defect (9).

CRB2 is a member of the Crumbs family, proteins implicated in planar cell polarity (PCP). PCP proteins (PCPPs) are important for epithelial planar cellular organization, and help align ciliary and cellular components to establish polarized and synchronous ciliary beating (10). In animal models, PCPP mutations result in ciliopathy-like phenotypes: in mice, *Celsr2* mutations cause hydrocephalus (11), and Fat4 knockout causes cystic kidney disease (12). Reciprocally, mice harboring mutations in genes underlying Bardet–Biedl syndrome (BBS) share phenotypic features with PCPP mutants, including open eyelids, neural tube defects and disrupted cochlear stereociliary bundles (13). BBS/PCPP double heterozygote mutant mice show significant embryonic lethality compared with single heterozygotes, implying genetic interactions between BBS and PCPP (e.g. *Vangl2*) genes (13). These data suggest that the functions of PCPPs and cilia are intertwined. PCPPs have also have direct roles in ciliogenesis, as demonstrated for *CRB3*, which also localizes to cilia (4).

Animal models of Crumbs protein deficiencies are reminiscent of ciliopathies or show direct ciliary defects. Conditional *CRB2* knockout in mice retinae resulted in prenatal retinal, postnatal retinal degeneration (14) and features of retinitis pigmentosa (15), characteristic of many ciliopathies. *Crb2*-null zebrafish have pronephric cysts, pericardial edema and smaller eyes, corresponding respectively to renal dysfunction and photoreceptor differentiation defects (1). Direct ciliary effects have been observed with *Crb2b* knockdown in zebrafish. *Crb2b* knockdown resulted in shorter, malpositioned cilia with slower, un-coordinated movement, reflecting both morphological and functional defects (16). Recent studies suggest that CRB2 may act during cilia initiation,

affecting localization of centriolar/peri-centriolar markers (17). Together, the known roles of PCPPs in general and CRB2 in particular, the phenotypic manifestations and ciliary effects in animal models coupled with clinical features in patients with biallelic *CRB2* mutations, suggest that *CRB2*-associated disease can be considered as a new ciliopathy.

Variable expressivity of *CRB2* mutations could be allele-dependent. All known patients with ventriculomegaly/hydrocephalus have extracellular region (residues 643–800) missense mutations: either homozygous (p.R693W), or compound heterozygous (p.R693W; p.N800K), with the second allele harboring either another extracellular region mutation or a termination mutation (2). In contrast, patients with isolated FSGS harbor bi-allelic missense mutations in the EGF-like domain or in the cytoplasmic tail of *CRB2*. A single FSGS patient was compound heterozygous for one EGF-like domain missense mutation, and for the frameshift mutation observed in our patients. This suggests that missense mutations in the *CRB2* extracellular region cause hydrocephalus, whereas missense mutations in EGF-like domains, particularly the 10th domain, cause FSGS. Biallelic nonsense mutations are most probably embryonic lethal, similarly to the *CRB2* knockout mouse (18). Despite the high frequency of p.N800K in Ashkenazi Jews, p.N800K homozygotes are yet to be described. Homozygosity for p.N800K may be lethal, or conversely, this genotype may cause a milder, underdiagnosed phenotype. Phenotypic variability may also be related to digenic/triallelic effects, which have been observed in other human ciliopathies (19). Indeed, in *CRB1* conditional knockout mice, presence of a heterozygous *CRB2* mutation aggravated the retinal phenotype (20). Heterozygous mutations in the ciliopathy gene *TTC21B* are particularly common as a 'third' allele, reported in 5% of ciliopathy cases (21), and we describe such a mutation in both our patients.

To summarize, we suggest that *CRB2* should be considered as a candidate gene for ciliopathy-consistent phenotypes. In patients with Ashkenazi Jewish ancestry, testing should begin with p.N800K. In patients with biallelic *CRB2* mutations, clinical management should include an active search for ciliopathy manifestations, including brain imaging, echocardiogram, and chest X-ray. Because renal and ophthalmological manifestations can evolve within time, urinary tract sonograms, renal function, urinalysis, and routine retinal assessment including ophthalmological examination and electroretinography should be performed at the time of diagnosis and followed periodically.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

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