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Human and mouse studies establish *TBX6* in Mendelian CAKUT and as a potential driver of kidney defects associated with the 16p11.2 microdeletion syndrome

Nan Yang^{1,2,3,19}, Nan Wu^{4,5,6,7,19}, Shuangshuang Dong^{1,2,19}, Ling Zhang^{1,19}, Yanxue Zhao^{4,5}, Weisheng Chen^{4,5}, Renqian Du⁷, Chengcheng Song^{1,2}, Xiaojun Ren^{1,2}, Jiaqi Liu^{4,5,8}, Davut Pehlivan^{7,9}, Zhenlei Liu^{4,10}, Rao Jia^{11,12}, Chunyan Wang^{11,12}, Sen Zhao^{4,5}, Amy M. Breman^{7,13}, Huadan Xue¹⁴, Hao Sun¹⁴, Jianxiong Shen^{4,5,6}, Shuyang Zhang^{5,15}, Jennifer E. Posey⁷, Hong Xu^{11,12}, Li Jin¹, Jianguo Zhang^{4,5,6}, Pengfei Liu^{7,13}, Simone Sanna-Cherchi¹⁶, Guixing Qiu^{4,5,6}, Zhihong Wu^{4,5,17}, James R. Lupski^{7,9,18,20}, Feng Zhang^{1,2,3,20}

¹Obstetrics and Gynecology Hospital, NHC Key Laboratory of Reproduction Regulation (Shanghai Institute of Planned Parenthood Research), State Key Laboratory of Genetic Engineering at School of Life Sciences, Fudan University, Shanghai, China;

²Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, Shanghai, China;

³State Key Laboratory of Reproductive Medicine, Center for Global Health, School of Public Health, Nanjing Medical University, Nanjing, China;

⁴Department of Orthopedic Surgery, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China;

⁵Beijing Key Laboratory for Genetic Research of Skeletal Deformity, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China;

⁶Medical Research Center of Orthopedics, Chinese Academy of Medical Sciences, Beijing, China;

⁷Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas, USA;

Correspondence: Feng Zhang, Obstetrics and Gynecology Hospital, Fudan University, Shanghai 200011, China. zhangfeng@fudan.edu.cn.

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DISCLOSURE

J.R.L. has stock ownership in 23andMe, is a paid consultant for Regeneron Pharmaceuticals and Novartis, and is a coinventor on multiple United States and European patents related to molecular diagnostics for inherited neuropathies, eye diseases and bacterial genomic fingerprinting. Baylor College of Medicine (BCM) and Miraca Holdings Inc. have formed a joint venture with shared ownership and governance of Baylor Genetics (BG, <http://bmgl.com>), which performs clinical exome sequencing and chromosomal microarray genomics assay services. P.L. is an employee of BCM and derives support through a professional services agreement with BG.

⁸Department of Breast Surgical Oncology, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China;

⁹Texas Children's Hospital, Houston, Texas, USA;

¹⁰Department of Neurosurgery, Xuanwu Hospital, Capital Medical University, Beijing, China;

¹¹Department of Nephrology, Children's Hospital of Fudan University, Shanghai, China

¹²Shanghai Kidney Development and Pediatric Kidney Disease Research Center, Shanghai, China

¹³Baylor Genetics, Houston, Texas, USA;

¹⁴Department of Radiology, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China;

¹⁵Department of Cardiology, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China;

¹⁶Division of Nephrology, Department of Medicine, Columbia University, New York, USA;

¹⁷Medical Research Center, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China;

¹⁸Department of Pediatrics, Baylor College of Medicine, Houston, Texas, USA;

¹⁹These authors contributed equally to this work;

²⁰These authors jointly directed this work.

Abstract

Congenital anomalies of the kidney and urinary tract (CAKUT) are the most common cause of chronic kidney disease in children. Human 16p11.2 deletions have been associated with CAKUT, but the responsible molecular mechanism remains to be illuminated. To explore this, we investigated 102 carriers of 16p11.2 deletion from multi-center cohorts, among which we retrospectively ascertained kidney morphologic and functional data from 37 individuals (12 Chinese and 25 Caucasian/Hispanic). Significantly higher CAKUT rates were observed in 16p11.2 deletion carriers (about 25% in Chinese and 16% in Caucasian/Hispanic) than those found in the non-clinically ascertained general populations (about 1/1000 found at autopsy). Furthermore, we identified seven additional individuals with heterozygous loss-of-function variants in *TBX6*, a gene that maps to the 16p11.2 region. Four of these seven cases showed obvious CAKUT. To further investigate the role of *TBX6* in kidney development, we engineered mice with mutated *Tbx6* alleles. The *Tbx6* heterozygous null (i.e., loss-of-function) mutant (*Tbx6*^{+/−}) resulted in 13% solitary kidneys. Remarkably, this incidence increased to 29% in a compound heterozygous model (*Tbx6*^{mh/−}) that reduced *Tbx6* gene dosage to below haploinsufficiency, by combining the null allele with a novel mild hypomorphic allele (*mh*). Renal hypoplasia was also frequently observed in these *Tbx6*-mutated mouse models. Thus, our findings in patients and mice establish *TBX6* as a novel gene involved in CAKUT and its gene dosage insufficiency as a potential driver for kidney defects observed in the 16p11.2 microdeletion syndrome.

Graphical ABSTRACT

Keywords

16p11.2 deletion; allelic series; CNV; compound inheritance; gene dosage and expression; kidney development

INTRODUCTION

Congenital anomalies of the kidney and urinary tract (CAKUT) have an estimated prevalence of approximately 3 – 6 in 1000 live births.^{6,7} CAKUT clinical manifestations represent a heterogeneous group of developmental anomalies that include renal hypoplasia, renal agenesis, renal hypodysplasia (RHD), and vesicoureteral reflux (VUR).^{8,9} Clinically, renal hypoplasia is characterized by abnormally small kidneys with normal morphology and decreased number of nephrons.¹⁰ Renal agenesis is characterized by unilateral or bilateral fetal kidney developmental failure. RHD is described by a small kidney size, reduced nephron number, and/or disorganized renal tissue.¹¹ VUR is caused by abnormal insertion of the ureter within the bladder wall, which can result in susceptibility to acute kidney infection and chronic kidney disease.¹² Importantly, CAKUT are the main cause of end-stage renal disease in childhood.^{13,14}

Evidence for the genetic contribution to CAKUT is substantive.¹⁵ For example, variants in *EYA1*, *GREB1L*, *HNFB1B*, *PAX2* and *TBX18* can cause CAKUT in humans.^{16–19} Nicolaou *et al.* summarized 208 candidate genes involved in CAKUT.²⁰ Besides gene variants due to single nucleotide variants and indels, genomic copy number variants (CNVs) can also cause CAKUT.^{21–25} Of note, the 17q12 deletion was found in 2.2% of RHD patients.^{21,26} The 22q11.2 deletion was found in 1% of CAKUT patients.²⁶ Previously, nine CAKUT cases and five population controls with overlapping 16p11.2 deletions were reported in a genome-wide CNV study based on 2,824 CAKUT cases and 21,498 controls (0.32% versus 0.02%; OR 13.7, 95% CI 4.1–52.2; $P = 4.39 \times 10^{-6}$).²⁷ The human *TBX6* gene maps within the 16p11.2 deletion interval. Our previous experiments have suggested that *TBX6* dosage insufficiency might be involved in kidney defects associated with the 16p11.2 microdeletion

syndrome.²⁷ However, to establish a single-gene causal effect it remains to be demonstrated whether a) *TBX6* coding variants are observed in individuals with CAKUT; b) haploinsufficiency is sufficient to cause human kidney defects; and c) *TBX6* gene dosage has an effect on the penetrance of renal and extrarenal phenotypes in human CAKUT.

Here we recruited human subjects molecularly diagnosed to be due to 16p11.2 deletion²⁸ from worldwide multi-center cohorts and retrospectively analyzed available clinical data for renal system abnormalities. Our data further suggested 16p11.2 deletion as a genetic factor contributing to CAKUT across different populations. We also identified heterozygous null *TBX6* carriers who manifest CAKUT supporting the contention that *TBX6* in 16p11.2 is the gene potentially driving the perturbations in kidney and urinary tract system development. Finally, the genetics of *TBX6*-associated CAKUT were further explored using mouse models of novel *Tbx6* allelic series that closely recapitulate the down-regulation of *TBX6* expression in humans. Our mouse model experiments provide evidence for *Tbx6* gene-dosage alterations and a dosage-dependent risk of CAKUT. In aggregate, these new human and mouse studies establish *TBX6* variants as a novel Mendelian cause of CAKUT, and implicate *TBX6* as a potential driver of the 16p11.2-associated CAKUT. Moreover, our allelic series support the contention of a role for a compound inheritance model as the modifier of disease penetrance of CAKUT, a genetic model similar to what we previously described in congenital scoliosis (CS) and in lung developmental defects.^{1–3,29}

RESULTS

Recurrence of CAKUT with 16p11.2 deletion in different ethnicities

Our previous study showed an association between 16p11.2 deletion and CAKUT.²⁷ However, we have also observed that the coexistence of CNV and single nucleotide polymorphisms (SNPs) at a locus can result in distorted calculations of the significance in associating SNPs with disease.³⁰ To further explore this association of 16p11.2 with CAKUT, we conducted reverse phenotyping for renal morphology and function of 24 patients with CS, caused by 16p11.2 deletion.^{2,31} These 16p11.2-deleted subjects were collected from the Peking Union Medical College Hospital (PUMCH, China). Detailed clinical information was available from 12 deletion carriers (Table 1). Three (25%) subjects (XH265, XH300, and XR636) had renal hypoplasia; reductions in kidney volumes of > 2 standard deviations below the mean in controls were considered as renal hypoplasia (Tables 1 and Supplementary Table S1 and Figure S1).^{32,33} Thus, the renal phenotypes of CAKUT were observed in at least 3/12 (25%; subjects XH265, XH300 and XR636 underlined in Table 1) Chinese 16p11.2 deletion carriers available for objective evaluation of kidney morphology and function.

Furthermore, in terms of kidney symmetry, two additional subjects XR330 and XR402 had symmetry values (i.e., volume ratio of the small kidney to the contralateral large kidney in each patient) < 0.8, and the value of the aforementioned subject XH265 was < 0.6, also suggesting unilateral renal hypoplasia (Table 1). Thus, the rate of kidney defects in 16p11.2 deletion carriers would be further increased to 5/12 (42%; subjects XH265, XH300, XR330, XR402 and XR636 in Table 1), when considering kidney asymmetry.

We next investigated 78 anonymized subjects of Caucasian/Hispanic ancestry with 16p11.2 deletion from Baylor College of Medicine (BCM) in Houston, USA. Imaging and clinical data assessing the renal system were available for 25 subjects. Interestingly, five (20%) subjects (BCM-1 to BCM-5) with 16p11.2 deletions were identified with various renal or urinary tract anomalies (Figure 1a, Table 2), including solitary kidney (subject BCM-5), VUR (subjects BCM-1 and BCM-2), hydroureteronephrosis (subject BCM-5), complex cystic structure in the left kidney (subject BCM-2), trace bilateral pelvicalyceal dilation (subject BCM-4), and bilateral small kidneys indicating renal hypoplasia (subject BCM-3). Since the exact data of kidney volumes of subject BCM-3 are not available, this case is not categorized into the CAKUT group in this study (shown as “suggestive” in Table 2). Thus, renal phenotypes consistent with CAKUT were observed in at least 4/25 (16%) carriers (BCM-1, BCM-2, BCM-4 and BCM-5) of 16p11.2 deletion available for objective evaluation (Figure 1a, Table 2). The 16p11.2 deletion associated renal phenotypes in Caucasian/Hispanic subjects are consistent with our observations in the Chinese population.

In our recent study on 42 subjects with 16p11.2 deletion from the Children’s Hospital of Philadelphia (CHOP), clinical data assessing the kidney and urinary tract were available for 15 cases, and 6 (40%) of them showed CAKUT phenotypes.²⁷

Collectively, our observations on the Chinese, BCM and CHOP cohorts revealed CAKUT-related phenotypes in 13/52 (25%) cases, strongly implicating a potential gene dosage effect at this 16p11.2 locus as an important susceptibility factor for CAKUT.

Heterozygous loss-of-function variants of *TBX6* constitute a novel form of human monogenic CAKUT

Among genes mapping within 16p11.2 (Supplementary Figure S2), *TBX6* is a well-known transcription factor, in which genetic and genomic variants at the locus have been associated with multiple developmental disorders.³⁴ A recent study showed *Tbx6*-expressing cells in the mesonephric duct and in the cells that would later give rise to the metanephric mesenchyme, the latter being the forerunner of renal tubules, of mouse embryos.³⁵ We previously reported that *TBX6* in 16p11.2 is a candidate gene responsible for kidney developmental anomalies.²⁷ However, no loss-of-function variants of *TBX6* in CAKUT patients have been reported to date.

Here we reviewed the renal morphology and function of human subjects with heterozygous loss-of-function variants in *TBX6*. Seven Chinese cases were previously collected from PUMCH and 3 Caucasian/Hispanic cases were newly collected from BCM.^{2,31} Imaging and clinical data assessing the renal system of 4 subjects from PUMCH and 3 subjects from BCM were available for review of the data.

In total, by reverse phenotyping of these 7 *TBX6* variant allele carriers (XH122, XH170, XR341, XR625, BCM-6, BCM-7 and BCM-8), we identified clear CAKUT phenotypes in 4 (57%) subjects (XH122, XH170, XR341 and BCM-6 in Tables 1 and 2). Two additional variant carriers (XR625 and BCM-7) had milder abnormalities suggestive of CAKUT (Tables 1 and 2).

Specifically, among Chinese patients with heterozygous *TBX6* loss-of-function variants (Table 1, Supplementary Tables S1 and S2), subject XH122 (c.266dupC, p.V91Gfs*80) had unilateral renal hypoplasia and bilateral delayed renal tissue tracer transit in ^{99m}Tc -diethylenetriamine pentaacetic acid (^{99m}Tc -DTPA) renal dynamic imaging suggesting a urinary obstruction consistent with ureteropelvic junction obstruction, another form of CAKUT. Subjects XH170 (c.704dupG, p.M236Hfs*44), and XR341 (c.1179_1180delAG, p.G395Lfs*91) had evidence for renal hypoplasia by volume measurements and in comparison to age-matched control values.^{31–33} Subject XR625 (c.933C>A, p.C311*) had asymmetric kidney volume but the smaller left kidney measured to the lower limit or normal size.

Among Caucasian/Hispanic cases with heterozygous *TBX6* loss-of-function variants (Figure 1b, Table 2), subject BCM-6 (c.1018_1019delCT, p.L340Vfs*7) had CAKUT characterized by right renal agenesis with contralateral compensatory hypertrophy. Subject BCM-7 (c.138_141dupGGAT, p.C48Gfs*30) had recurrent urinary tract infections suggestive of CAKUT (in particular VUR) but no definitive imaging studies were available for confirmation.

Thus, in aggregate, our data strongly implicate *TBX6* haploinsufficiency as a cause of CAKUT phenotypes across human populations of different race and ethnicity.

Reduced gene dosage of *Tbx6* induced CAKUT phenotypes in mice

No homozygous null alleles have been reported in humans while homozygosity for a null allele of *Tbx6* is embryonic lethal in mice.^{2,36} We recently demonstrated that severe reduction of *Tbx6* gene dosage in the ‘*rib-vertebrae*’ (*rv*) mutant mice resulted in high penetrance of CAKUT.²⁷ In order to study a model that more closely recapitulates the effect of human heterozygous 16p11.2 deletions or *TBX6* loss-of-function variants, we sought to study mouse models of milder reduction of *Tbx6* gene dosage. We previously generated a *Tbx6* null allele (*Tbx6*[−]) and a mild hypomorphic allele of *Tbx6* (*Tbx6*^{mh}) in mice using the CRISPR-Cas9 technology.² The *Tbx6*^{mh} allele resulted in the reduction of *Tbx6*-expression levels to ~ 65% of the wild-type (WT) allele, as determined using a gene-expression reporter system.² The *Tbx6*^{mh} allele mimics the human *TBX6* hypomorphic allele in function (Supplementary Figure S3).²

To explore the phenotypic consequences of combinations of *Tbx6* alleles for renal system development, mice bearing different combinations of alleles were studied. In a pilot experiment we dissected 10 compound heterozygous *Tbx6*^{mh/−} adult mice, and identified solitary kidneys in 20% (2/10) as compared to 0/10 WT mice. This incidence of solitary kidney was much greater than expected in mice and also significantly higher than that in humans (~ 1/1000 found in humans at autopsy; Figure 2a–c).³⁷ Five different mouse strains with distinct combinations of genotypes at the *Tbx6* locus (*Tbx6*^{+/+}, *Tbx6*^{+/mh}, *Tbx6*^{mh/mh}, *Tbx6*^{+/−}, and *Tbx6*^{mh/−}), aged 35–45 days, were obtained and examined. This allelic series allowed examination of diminishing gene dosages at the *Tbx6* locus. The strain *Tbx6*^{+/mh} had the smallest reduction in gene dosage, and the strain *Tbx6*^{+/−} represented 50% reduction at the locus.

Significantly more solitary kidneys were observed in *Tbx6*^{+/-} mice (7/55, 13%; $P = 0.013$ versus WT, Fisher's exact test) and *Tbx6*^{mh/-} mice (16/56, 29%; $P = 1.0 \times 10^{-5}$ versus WT, Fisher's exact test). But no solitary kidneys occurred in *Tbx6*^{+/+} (n=52), *Tbx6*^{+mh} (n=58), or *Tbx6*^{mh/mh} (n=58) mice (Figure 2d).

In addition, we directly and quantitatively measured mouse 'kidney symmetry' calculated as the weight ratio of the small kidney to the large kidney for each mouse. The theoretical symmetry values range from 0 to 1 (Figure 2e). We also excluded mice with a solitary kidney and investigated kidney symmetry and potential renal hypoplasia only in mice with both kidneys. The symmetry values of the *Tbx6*^{+/-} (0.86 ± 0.12) and *Tbx6*^{mh/-} (0.84 ± 0.10) groups were significantly less than that of the WT group ($P = 2.8 \times 10^{-6}$ and $P = 7.4 \times 10^{-7}$ by Wilcoxon's rank-sum test, respectively; Figure 2e), indicating unilateral renal hypoplasia. No significant difference in kidney symmetry was observed in the *Tbx6*^{+mh} or *Tbx6*^{mh/mh} groups ($P = 0.58$ and $P = 0.19$ by Wilcoxon's rank-sum test, respectively) when compared with the WT group (Figure 2e).

Given that the mice with kidney symmetry values of < 0.7 are suggestive of renal hypoplasia, we studied the frequencies of renal hypoplasia across genotypes with diminished *Tbx6* gene dosage. Renal hypoplasia and solitary kidneys was found in none of 52 WT mice (*Tbx6*^{+/+}), in none of 58 *Tbx6*^{+mh} mice, in only 1/58 (2%) *Tbx6*^{mh/mh} mice, in 12/55 (22%) *Tbx6*^{+/-} mice ($P = 2.6 \times 10^{-4}$ versus WT, Fisher's exact test), and in 19/56 (34%) *Tbx6*^{mh/-} mice ($P = 7.6 \times 10^{-7}$ versus WT, Fisher's exact test) (Supplementary Figure S4). Our renal phenotype observations in the *Tbx6* allelic series of mouse models show a statistically significant correlation of *Tbx6* gene dosage levels with the rates of renal hypoplasia and agenesis.

Severe renal phenotypes may result in mouse death before 35–45 days old. Therefore, we also investigated renal phenotypes in mouse E18.5 embryos. The *Tbx6*^{mh/-} embryos showed unilateral renal hypoplasia and renal agenesis (Fig. 3a–c), which are similar to those in adult mice of 35–45 days old. Hematoxylin-eosin stained renal sections showed no obvious abnormality in renal cortex and kidney medulla (Fig. 3e).

Altered expression of CAKUT-related genes in mouse embryos with *Tbx6* dosage insufficiency

RNA sequencing was conducted to analyze differential expression of the genes potentially involved in renal development between WT and *Tbx6* low-dosage (*Tbx6*^{mh/-}) embryos (Supplementary Table S3).²⁰ Mouse ureteric buds branch into the metanephric mesenchyme at E11.5, and renal vesicles and S-shaped bodies develop from E12.5 to E16.5.^{38,39} Because both stages are crucial for renal hypoplasia etiology, we harvested mouse embryos at E11.5 and E14.5, and collected renal tissues for RNA sequencing. *Hoxd11* was significantly up-regulated at E11.5, and 5 genes involved in kidney development (*Cdh6*, *Dach1*, *Frem1*, *Lhx1* and *Slit2*) were significantly down-regulated in *Tbx6*^{mh/-} embryos, when compared with the expression levels in WT mice ($Q < 0.05$ with false-discovery-rate correction) (Supplementary Table S4). Because *Tbx6* expression was very low in renal tissues from embryos at E11.5 and E14.5, altered expression of the above genes could be an indirect consequence of kidney development in *Tbx6* mutants, rather than a direct regulation by

Tbx6. Ectopic activation of mouse *Hoxd11* altered the morphology and differentiation of mesonephric tubules.⁴⁰ Furthermore, *Lhx1* is a marker of the anterior intermediate mesoderm, essential for mammalian kidney development.⁴¹ Experimental evidence from animal models suggests that *Tbx6* acts upstream of *Lhx1*.^{42,43}

DISCUSSION

The 16p11.2 deletion has a population frequency of ~ 0.03% worldwide.⁴⁴ Our previous studies only revealed CS in the carriers of 16p11.2 deletion.^{1–3,45} In a large genome-wide study for CNV, we recently showed a significant enrichment of this deletion in CAKUT cases as compared to population controls (0.32% versus 0.02%; OR 13.7, $P = 4.39 \times 10^{-6}$), and showed that 40% of North Americans with 16p11.2 deletion had evidence for CAKUT.²⁷ Here, we found that at least 25% of 16p11.2 deletion carriers of Chinese ancestry and 16% of carriers of Caucasian/Hispanic ancestry had clinical features consistent with CAKUT. These experimental observations, combined with our previous work²⁷ indicate an overall prevalence of CAKUT in at least 25% (13/52) of 16p11.2 deletion carriers, further supporting the contention that this deletion is an important genetic risk factor contributing to CAKUT across world populations of different ethnicities.²⁷

Tbx6 is expressed in the mesonephric duct of mouse embryos,^{35,46} suggesting that *Tbx6* is associated with renal development. However, it remained unclear whether *TBX6* variants represent a monogenic form of CAKUT in subjects without 16p11.2 deletion. Our previous work also showed non-coding variants of *TBX6* in CAKUT;⁴⁷ however, the causality of *TBX6* coding variants in CAKUT was not established in that study. Here we provide evidence that heterozygous *TBX6* loss-of-function variants can also cause monogenic CAKUT on their own with incomplete penetrance. In fact, by screening worldwide populations, we found that at least 3 Chinese subjects (XH122, XH170 and XR341) and 1 Caucasian/Hispanic subject (BCM-6) carrying *TBX6* frame-shift variants also had clinical phenotypes consistent with CAKUT. Moreover, an additional analysis of our new mouse models with milder reduction of *Tbx6* gene expression (hence more closely recapitulating the gene dosage effect of 16p11.2 deletion in humans) provide strong evidence that *TBX6* is a genetic driver of the renal system birth defect phenotypes observed in patients with the 16p11.2 deletion syndrome. Also, we cannot exclude the genetic contributions of other 16p11.2 genes to CAKUT. It has been recently reported that *MAZ* in 16p11.2 is involved in CAKUT.⁴⁸

Pathogenic variants can have pleiotropic effects. For example, heterozygous *JAG1* null variants lead to heart, liver, skeletal, and eye malformations in humans.^{49–51} Heterozygous *NOTCH2* null variants cause human renal and vertebral malformations.^{52,53} Homozygous null variants in *HAAO* or *KYNU* cause cardiac, vertebral, and renal defects in humans and mice.⁵⁴ Similarly, *TBX6* variants are also pleiotropic.

The relationship between CAKUT phenotypes and CS in the *TBX6*-mutated subjects as well as the genetic model underlying these traits was unclear until now. *TBX6* gene-dosage reduction can lead to CS through a compound inheritance model.^{2,3,31,55} Here, we found that subject BCM-6 carrying *TBX6* loss-of-function variants only showed CAKUT without

any symptoms of CS. Our previous study showed that among the 15 CAKUT patients with 16p11.2 deletions, only two had CS, suggesting different penetrance for CAKUT and CS, where CAKUT manifestation requires haploinsufficiency at the 16p11.2 locus and, mainly *TBX6*.²⁷ Consistently, our *Tbx6*^{+/-} mice also showed frequent renal hypoplasia with no CS-related phenotypes. Therefore, the consistent renal phenotypes observed in humans and mice harboring *TBX6* variants were not dependent on CS, but were associated with *TBX6* dosage reduction. Our study will facilitate a more comprehensive molecular diagnosis of the disorder and genetic counseling for human subjects carrying 16p11.2 deletion or a *TBX6* null variant.

Tbx6^{v/rv} mice, with a 182-bp insertion at 240 bp upstream of the translational initiation site of *Tbx6*,⁵⁶ manifested high penetrance of kidney and urinary tract malformations in our previous study.²⁷ Nevertheless, the 'rv' mutant is a severe hypomorphic allele that can strongly reduce *Tbx6* expression to much less than 50% of that in WT. In contrast, the common variant hypomorphic allele at human *TBX6* is mild, which reduces *TBX6* expression level to ~ 70% of the WT allele.³¹ Furthermore, the human mild hypomorphic *TBX6* allele is very common in human populations, and its homozygous carriers had no clinical phenotypes.³¹ Therefore, the severe *rv* mutant in mice does not readily mimic the common variant mild hypomorphic *TBX6* allele in humans. Here, we generated a mild hypomorphic mutant allele (*Tbx6*^{mh}) in mice. The expression of *Tbx6*^{mh}, which was ~ 65% that of the WT allele, closely matched the expression level of human hypomorphic *TBX6* allele.² Here we showed that the compound heterozygous alleles *Tbx6*^{mh/-} caused a further reduction in *Tbx6* gene dosage and led to a higher rate of solitary kidney and renal hypoplasia when compared with the *Tbx6*^{+/-} mice (Figure 2). This observation suggests that the common mild hypomorphic *TBX6* allele in human populations may be an important genetic modifier in human CAKUT.

Our previous study suggested that the combination of a rare null allele with a hypomorphic common variant allele of *TBX6/Tbx6* resulted in CS in humans and mouse.¹⁻³ Such a compound inheritance at the same locus can result in gene dosage reduction and modify penetrance of diseases. In addition to *TBX6*, more loci are potentially involved in the compound inheritance model.⁵⁷ While haploinsufficiency in *TBX6* clearly contributes to the penetrance of CAKUT, it may be that common variation at a downstream gene contributes to a compound inheritance model. Such a digenic mutational burden in a developmental pathway has been proposed for penetrance of craniosynostosis.⁵⁸

Our data emphasize the gene-dosage effect and its important contribution to human birth defects. The molecular mechanism whereby *TBX6* causes CAKUT and CS showed different dosage sensitivities. Here, we found that half of normal *TBX6* gene dosage can result in high-to-moderate penetrance of CAKUT. However, this half dosage of *TBX6* was not sufficient to manifest CS. Further reduction in *TBX6* gene dosage to levels less than haploinsufficiency can cause CS.^{2,3,31} Furthermore, we found that *Tbx6* gene dosage was related to the penetrance of CAKUT in mouse models. With a further reduction of *Tbx6* dosage, an increasing proportion and increasing asymmetric severity of CAKUT occurred in mice. It was reported that different VUR percentages in different mouse strains.⁵⁹ Therefore, the *Tbx6* mouse models were not assayed for VUR in this study.

In short, our study revealed three major findings. Firstly, we found that at least 25% of 16p11.2 deletion carriers of Chinese and Caucasian/Hispanic ancestries had clinical features consistent with CAKUT, a different phenotype from well-known 16p11.2-associated CS.^{1–3,45} This study extends what was newly reported that 16p11.2 deletion is an important genetic risk factor of CAKUT.²⁷ Secondly, this current study provided human and mouse experimental evidence to support that *TBX6* is a potential genetic driver of CAKUT in cases with 16p11.2 deletion. Thirdly, using a novel mouse allelic series, we provide evidence for *Tbx6* gene dosage insufficiency as the main mechanism for CAKUT determination and for a *Tbx6* dosage-dependent penetrance of CAKUT.

MATERIALS AND METHODS

CAKUT cohort of patients

Han Chinese cases were collected from PUMCH. The institutional review boards at Fudan University and PUMCH approved this work. Informed consents were obtained from their guardians (for participants < 18 years of age). Quantitative polymerase chain reaction (qPCR) was conducted to screen for 16p11.2/*TBX6* deletions in human subjects, and comparative genomic hybridization (CGH) microarrays (Agilent, USA) were used to verify 16p11.2/*TBX6* deletions. The experimental details of qPCR, Sanger sequencing, and CGH microarrays were provided in our previous works.^{2,31} Three-dimensional kidney reconstructions were conducted using computed tomography and magnetic resonance imaging (MRI). The methodological details were provided in Supplementary Figure S1.

Data from additional anonymous subjects with *TBX6* deletions and loss-of-function variants were obtained from BCM (Houston, USA). The institutional review board at BCM approved this work. These subjects were initially referred for clinical chromosomal microarray analysis or exome sequencing, due to various medical problems.^{60,61} Most subjects in the BCM cohort are of Caucasian/Hispanic ancestry.

Renal hypoplasia analysis

For renal hypoplasia analysis in human subjects, three-dimensional kidney reconstructions were conducted using computed tomography (CT) and MRI (Supplementary Figure S1). $\text{Volume} = 0.5233 \times \text{length} \times \text{width} \times \text{thickness}$.⁶² Normal parameters referred to the reported kidney volume of Chinese children without a history of renal disease or pathological abnormalities.⁶³ The left and right kidneys were investigated separately. Differences in kidney volumes of > 2 standard deviations below the means in age-matched and ethnically matched controls were considered to reflect renal hypoplasia.¹⁰

As for renal phenotypic analysis of adult mice, mice were sacrificed by cervical dislocation at aged 35–45 days before kidney images were taken. Mouse kidneys were removed from individual cadaveric animals, and the weights were determined between each kidney with the ratio used to determine renal hypoplasia. As for mouse renal hypoplasia analysis, mouse ‘kidney symmetry’ was calculated as the weight ratio of the small kidney to the large kidney for each mouse. The mice with kidney symmetry values of < 0.7 were suggestive of unilateral renal hypoplasia in this study.

The kidneys of E18.5 mouse embryos were taken images and fixed overnight in 4% paraformaldehyde and dehydrated through a graded ethanol series (75–85–90–95–100%), then washed with xylene. The embryos were incubated in two changes of paraffin wax at 55°C under vacuum. Then the embryos were embedded in paraffin wax and sectioned at 4 μ m. Sections were dewaxed in xylene, rehydrated through ethanol (100–95–90–80–70%), then rehydrated with deionized water, stained with hematoxylin and eosin and dehydrated before mounting in Cytoseal.

Sanger sequencing and *TBX6* haplotyping in human subjects

The entire *TBX6* gene and its ~ 1 kb upstream region were amplified by long-range PCR, and the PCR products were analyzed by Sanger sequencing. Clone sequencing was performed to verify common *TBX6* variants. Supplementary Tables S5 and S6 show the amplification and sequencing primers. The experimental details were provided in our previous work.³¹

Generation of *Tbx6*-mutated mice

Animal experiments were approved by the institutional review board at Fudan University. The FVB/NJ mouse strain (the Jackson Laboratory, #001800) was used for animal experiments. CRISPR-Cas9 technology was employed to edit *Tbx6* in mice (Supplementary Table S7). Mouse models and strain construction were described in our previous work.² Mouse genotyping was performed by PCR and Sanger sequencing, using DNA from toe clips. The amplification and sequencing PCR primers are shown in Supplementary Table S8. The experimental details were provided in our previous work.²

RNA sequencing

Female *Tbx6*^{+/mh} mice were crossed with male *Tbx6*^{+/-} mice to produce *Tbx6*^{+/+} and *Tbx6*^{mh/-} embryos. Mouse embryos were harvested at E11.5 and E14.5, three of which were randomly selected for the *Tbx6*^{+/+} and *Tbx6*^{mh/-} genotypes. The renal region of E11.5 embryos and the left kidney tissue from E14.5 embryos were collected for total RNA extraction using the RNeasy Mini Kit (Qiagen). Total RNA (2 μ g) was used to construct a cDNA library for each embryo. DNA-sequencing libraries were prepared using the TruSeq Nano DNA HT Library Prep Kit (Illumina). All cDNA libraries were sequenced using the Illumina HiSeq X-TEN platform. At least 10 Gb of raw data were obtained for each library. The expression levels of candidate genes were normalized and presented as fragments per kilobase of transcript per million fragments mapped to provide estimated gene-transcript abundances.⁶⁴

Statistical analysis

We used the Fisher's exact test to investigate the different prevalence of CAKUT phenotypes between WT and *Tbx6*-mutated mice. The Wilcoxon rank-sum test was performed using R software for statistical analysis of kidney symmetry. Gene expression level changes between *Tbx6*^{+/+} and *Tbx6*^{mh/-} embryos were analyzed using the Cuffdiff program in the Cufflinks software.⁶⁵ The *P* values calculated for each gene were adjusted to *Q* values using the false-discovery rate method for multiple testing corrections.⁶⁶

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Wu N, Ming X, Xiao J, et al. TBX6 null variants and a common hypomorphic allele in congenital scoliosis. *N Engl J Med*. 2015;372:341–350. [PubMed: 25564734]
2. Yang N, Wu N, Zhang L, et al. TBX6 compound inheritance leads to congenital vertebral malformations in humans and mice. *Hum Mol Genet*. 2019;28:539–547. [PubMed: 30307510]
3. Liu J, Wu N, Yang N, et al. TBX6-associated congenital scoliosis (TACS) as a clinically distinguishable subtype of congenital scoliosis: further evidence supporting the compound inheritance and TBX6 gene dosage model. *Genet Med*. 2019;21:1548–1558. [PubMed: 30636772]
4. Sparrow DB, McInerney-Leo A, Gucev ZS, et al. Autosomal dominant spondylocostal dysostosis is caused by mutation in TBX6. *Hum Mol Genet*. 2013;22:1625–1631. [PubMed: 23335591]
5. Takeda K, Kou I, Kawakami N, et al. Compound Heterozygosity for Null Mutations and a Common Hypomorphic Risk Haplotype in TBX6 Causes Congenital Scoliosis. *Hum Mutat*. 2017;38:317–323. [PubMed: 28054739]
6. Sanna-Cherchi S, Ravani P, Corbani V, et al. Renal outcome in patients with congenital anomalies of the kidney and urinary tract. *Kidney Int*. 2009;76:528–533. [PubMed: 19536081]
7. Hildebrandt F Genetic kidney diseases. *Lancet*. 2010;375:1287–1295. [PubMed: 20382325]
8. Rasouly HM and Lu W. Lower urinary tract development and disease. *Wiley Interdiscip Rev Syst Biol Med*. 2013;5:307–342. [PubMed: 23408557]
9. Nicolaou N, Renkema KY, Bongers EM, et al. Genetic, environmental, and epigenetic factors involved in CAKUT. *Nat Rev Nephrol*. 2015;11:720–731. [PubMed: 26281895]
10. Cain JE, Di Giovanni V, Smeeton J, et al. Genetics of renal hypoplasia: insights into the mechanisms controlling nephron endowment. *Pediatr Res*. 2010;68:91–98. [PubMed: 20421843]
11. Weber S, Moriniere V, Knuppel T, et al. Prevalence of mutations in renal developmental genes in children with renal hypodysplasia: results of the ESCAPE study. *J Am Soc Nephrol*. 2006;17:2864–2870. [PubMed: 16971658]
12. Murawski IJ and Gupta IR. Gene discovery and vesicoureteric reflux. *Pediatric Nephrology*. 2008;23:1021–1027. [PubMed: 18253765]
13. Ardissino G, Dacco V, Testa S, et al. Epidemiology of chronic renal failure in children: data from the ItalKid project. *Pediatrics*. 2003;111:e382–387. [PubMed: 12671156]
14. Mong Hiep TT, Ismaili K, Collart F, et al. Clinical characteristics and outcomes of children with stage 3–5 chronic kidney disease. *Pediatr Nephrol*. 2010;25:935–940. [PubMed: 20148340]
15. Sanna-Cherchi S, Westland R, Ghiggeri GM, et al. Genetic basis of human congenital anomalies of the kidney and urinary tract. *J Clin Invest*. 2018;128:4–15. [PubMed: 29293093]

16. Thomas R, Sanna-Cherchi S, Warady BA, et al. HNF1B and PAX2 mutations are a common cause of renal hypodysplasia in the CKiD cohort. *Pediatr Nephrol*. 2011;26:897–903. [PubMed: 21380624]
17. Spahiu L, Merovci B, Ismaili Jaha V, et al. Case report of a novel mutation of the EYA1 gene in a patient with branchio-oto-renal syndrome. *Balkan J Med Genet*. 2016;19:91–94.
18. De Tomasi L, David P, Humbert C, et al. Mutations in GREB1L Cause Bilateral Kidney Agenesis in Humans and Mice. *Am J Hum Genet*. 2017;101:803–814. [PubMed: 29100091]
19. Vivante A, Kleppa MJ, Schulz J, et al. Mutations in TBX18 Cause Dominant Urinary Tract Malformations via Transcriptional Dysregulation of Ureter Development. *Am J Hum Genet*. 2015;97:291–301. [PubMed: 26235987]
20. Nicolaou N, Pulit SL, Nijman IJ, et al. Prioritization and burden analysis of rare variants in 208 candidate genes suggest they do not play a major role in CAKUT. *Kidney Int*. 2016;89:476–486. [PubMed: 26489027]
21. Mefford HC, Clauin S, Sharp AJ, et al. Recurrent reciprocal genomic rearrangements of 17q12 are associated with renal disease, diabetes, and epilepsy. *Am J Hum Genet*. 2007;81:1057–1069. [PubMed: 17924346]
22. Hoefele J, Gabert M, Heinrich U, et al. A novel interstitial deletion of 10q24.2q24.32 in a patient with renal coloboma syndrome. *Eur J Med Genet*. 2012;55:211–215. [PubMed: 22361651]
23. Sanna-Cherchi S, Kiryluk K, Burgess KE, et al. Copy-number disorders are a common cause of congenital kidney malformations. *Am J Hum Genet*. 2012;91:987–997. [PubMed: 23159250]
24. Caruana G, Wong MN, Walker A, et al. Copy-number variation associated with congenital anomalies of the kidney and urinary tract. *Pediatr Nephrol*. 2015;30:487–495. [PubMed: 25270717]
25. Westland R, Verbitsky M, Vukojevic K, et al. Copy number variation analysis identifies novel CAKUT candidate genes in children with a solitary functioning kidney. *Kidney Int*. 2015;88:1402–1410. [PubMed: 26352300]
26. Lopez-Rivera E, Liu YP, Verbitsky M, et al. Genetic Drivers of Kidney Defects in the DiGeorge Syndrome. *N Engl J Med*. 2017;376:742–754. [PubMed: 28121514]
27. Verbitsky M, Westland R, Perez A, et al. The copy number variation landscape of congenital anomalies of the kidney and urinary tract. *Nat Genet*. 2019;51:117–127. [PubMed: 30578417]
28. Shinawi M, Liu P, Kang SH, et al. Recurrent reciprocal 16p11.2 rearrangements associated with global developmental delay, behavioural problems, dysmorphism, epilepsy, and abnormal head size. *J Med Genet*. 2010;47:332–341. [PubMed: 19914906]
29. Karolak JA, Vincent M, Deutsch G, et al. Complex Compound Inheritance of Lethal Lung Developmental Disorders Due to Disruption of the TBX-FGF Pathway. *Am J Hum Genet*. 2019;104:213–228. [PubMed: 30639323]
30. Liu J, Zhou Y, Liu S, et al. The coexistence of copy number variations (CNVs) and single nucleotide polymorphisms (SNPs) at a locus can result in distorted calculations of the significance in associating SNPs to disease. *Hum Genet*. 2018;137:553–567. [PubMed: 30019117]
31. Wu N, Ming X, Xiao J, et al. TBX6 null variants and a common hypomorphic allele in congenital scoliosis. *N Engl J Med*. 2015;372:341–350. [PubMed: 25564734]
32. Han BK and Babcock DS. Sonographic measurements and appearance of normal kidneys in children. *Am J Roentgenol*. 1985;145:611–616. [PubMed: 3895872]
33. Shi B, Yang Y, Li SX, et al. Ultrasonographic renal volume in Chinese children: Results of 1683 cases. *J Pediatr Surg*. 2015;50:1914–1918. [PubMed: 26117811]
34. Chapman DL, Agulnik I, Hancock S, et al. Tbx6, a mouse T-Box gene implicated in paraxial mesoderm formation at gastrulation. *Dev Biol*. 1996;180:534–542. [PubMed: 8954725]
35. Concepcion D, Washkowitz AJ, DeSantis A, et al. Cell lineage of timed cohorts of Tbx6-expressing cells in wild-type and Tbx6 mutant embryos. *Biol. Open* 2017;6:1065–1073. [PubMed: 28606934]
36. Chapman DL and Papaioannou VE. Three neural tubes in mouse embryos with mutations in the T-box gene Tbx6. *Nature*. 1998;391:695–697. [PubMed: 9490412]
37. Doroshow LW and Abeshouse BS. Congenital unilateral solitary kidney: report of 37 cases and a review of the literature. *Urol Surv*. 1961;11:219–229. [PubMed: 13887435]

38. Wang P, Pereira FA, Beasley D, et al. Presenilins are required for the formation of comma- and S-shaped bodies during nephrogenesis. *Development*. 2003;130:5019–5029. [PubMed: 12930775]
39. Kobayashi H, Kawakami K, Asashima M, et al. Six1 and Six4 are essential for Gdnf expression in the metanephric mesenchyme and ureteric bud formation, while Six1 deficiency alone causes mesonephric-tubule defects. *Mech Dev*. 2007;124:290–303. [PubMed: 17300925]
40. Mugford JW, Sipila P, Kobayashi A, et al. Hoxd11 specifies a program of metanephric kidney development within the intermediate mesoderm of the mouse embryo. *Dev Biol*. 2008;319:396–405. [PubMed: 18485340]
41. Takasato M, Er PX, Chiu HS, et al. Kidney organoids from human iPS cells contain multiple lineages and model human nephrogenesis. *Nature*. 2015;526:564–568. [PubMed: 26444236]
42. Grieshammer U, Cebrian C, Ilagan R, et al. FGF8 is required for cell survival at distinct stages of nephrogenesis and for regulation of gene expression in nascent nephrons. *Development*. 2005;132:3847–3857. [PubMed: 16049112]
43. Li HY, Bourdelas A, Carron C, et al. FGF8, Wnt8 and Myf5 are target genes of Tbx6 during anteroposterior specification in *Xenopus* embryo. *Dev Biol*. 2006;290:470–481. [PubMed: 16343478]
44. Rosenfeld JA, Coppinger J, Bejjani BA, et al. Speech delays and behavioral problems are the predominant features in individuals with developmental delays and 16p11.2 microdeletions and microduplications. *J Neurodev Disord*. 2010;2:26–38. [PubMed: 21731881]
45. Ren X, Yang N, Wu N, et al. Increased TBX6 gene dosages induce congenital cervical vertebral malformations in humans and mice. *J Med Genet*. 2019, in press.
46. Chapman DL. Impaired intermediate formation in mouse embryos expressing reduced levels of Tbx6. *Genesis*. 2019;57:e23270. [PubMed: 30548789]
47. Dong S, Wang C, Li X, et al. Noncoding rare variants of TBX6 in congenital anomalies of the kidney and urinary tract. *Mol Genet Genomics*. 2019;294:493–500. [PubMed: 30604070]
48. Haller M, Au J, O'Neill M, et al. 16p11.2 transcription factor MAZ is a dosage-sensitive regulator of genitourinary development. *Proc Natl Acad Sci U S A*. 2018;115:E1849–E1858. [PubMed: 29432158]
49. Oda T, Elkahoul AG, Pike BL, et al. Mutations in the human Jagged1 gene are responsible for Alagille syndrome. *Nat Genet*. 1997;16:235–242. [PubMed: 9207787]
50. Boyer J, Crosnier C, Driancourt C, et al. Expression of mutant JAGGED1 alleles in patients with Alagille syndrome. *Hum Genet*. 2005;116:445–453. [PubMed: 15772854]
51. Guegan K, Stals K, Day M, et al. JAG1 mutations are found in approximately one third of patients presenting with only one or two clinical features of Alagille syndrome. *Clin Genet*. 2012;82:33–40. [PubMed: 21752016]
52. McDaniel R, Warthen DM, Sanchez-Lara PA, et al. NOTCH2 mutations cause Alagille syndrome, a heterogeneous disorder of the notch signaling pathway. *Am J Hum Genet*. 2006;79:169–173. [PubMed: 16773578]
53. Kamath BM, Bauer RC, Loomes KM, et al. NOTCH2 mutations in Alagille syndrome. *J Med Genet*. 2012;49:138–144. [PubMed: 22209762]
54. Shi H, Enriquez A, Rapadas M, et al. NAD Deficiency, Congenital Malformations, and Niacin Supplementation. *N Engl J Med*. 2017;377:544–552. [PubMed: 28792876]
55. Takeda K, Kou I, Kawakami N, et al. Compound Heterozygosity for Null Mutations and a Common Hypomorphic Risk Haplotype in TBX6 Causes Congenital Scoliosis. *Hum Mutat*. 2017;38:317–323. [PubMed: 28054739]
56. Watabe-Rudolph M, Schlautmann N, Papaioannou VE, et al. The mouse rib-vertebrae mutation is a hypomorphic Tbx6 allele. *Mech Dev*. 2002;119:251–256. [PubMed: 12464437]
57. Albers CA, Paul DS, Schulze H, et al. Compound inheritance of a low-frequency regulatory SNP and a rare null mutation in exon-junction complex subunit RBM8A causes TAR syndrome. *Nat Genet*. 2012;44:435–439. [PubMed: 22366785]
58. Timberlake AT, Choi J, Zaidi S, et al. Two locus inheritance of non-syndromic midline craniosynostosis via rare SMAD6 and common BMP2 alleles. *Elife*. 2016;5.

59. Murawski IJ, Maina RW, Malo D, et al. The C3H/HeJ inbred mouse is a model of vesico-ureteric reflux with a susceptibility locus on chromosome 12. *Kidney Int.* 2010;78:269–278. [PubMed: 20407478]
60. Boone PM, Bacino CA, Shaw CA, et al. Detection of clinically relevant exonic copy-number changes by array CGH. *Hum Mutat.* 2010;31:1326–1342. [PubMed: 20848651]
61. Yang Y, Muzny DM, Xia F, et al. Molecular findings among patients referred for clinical whole-exome sequencing. *JAMA.* 2014;312:1870–1879. [PubMed: 25326635]
62. Han BK and Babcock DS. Sonographic measurements and appearance of normal kidneys in children. *Am J Roentgenol.* 1985;145:611–616. [PubMed: 3895872]
63. Shi B, Yang Y, Li SX, et al. Ultrasonographic renal volume in Chinese children: Results of 1683 cases. *J Pediatr Surg.* 2015;50:1914–1918. [PubMed: 26117811]
64. Trapnell C, Williams BA, Pertea G, et al. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat Biotechnol.* 2010;28:511–515. [PubMed: 20436464]
65. Trapnell C, Roberts A, Goff L, et al. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat Protoc.* 2012;7:562–578. [PubMed: 22383036]
66. Benjamini Y and Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Statist Soc B.* 1995;57:289–300.

Figure 1]. The 16p11.2/*TBX6* deletions and *TBX6* frame-shift variants in the BCM cohort.

(a) Common proximal 16p11.2 deletions (29.6 – 30.2 Mb, the genome reference consortium human build 37 [GRCh37]) were identified in subjects BCM-1, BCM-2, BCM-3, and BCM-5. Blue shadowing indicates the common deletion region. The deletion in subject BCM-4 is larger (21.7 – 30.2 Mb) than the common deletion. The red circle indicates the location of *TBX6*. The microarrays applied with samples from these individuals were based on different versions of customized oligonucleotide CGH arrays with different numbers of oligonucleotide probes. (b) A heterozygous frame-shift variant of *TBX6* (c.1018_1019delCT; p.L340Vfs*7) in subject BCM-6. The red box indicates the deleted nucleotides. (c) A heterozygous frame-shift variant of *TBX6* (c.138_141dupGGAT; p.C48Gfs*30) in subject BCM-7. Red shadowing indicates the duplicated nucleotides. (d) A heterozygous splice-site variant of *TBX6* (c.118+1G>C) in subject BCM-8. The red arrow indicates the mutated nucleotide. Abbreviation: WT, wide type.

Figure 2]. CAKUT phenotypes were frequently observed in the mice with *Tbx6* gene-dosage insufficiency.
(a) Normal kidneys. **(b)** Solitary kidney (renal agenesis). **(c)** Asymmetric kidneys (renal hypoplasia). **(d)** The frequencies of solitary kidney in the $Tbx6^{+/+}$, $Tbx6^{+mh}$, $Tbx6^{mh/mh}$, $Tbx6^{+/-}$, and $Tbx6^{mh/-}$ mice. **(e)** Kidney symmetry in mice with different genotypes (excluding mice with solitary kidneys). Bar heights indicate mean values. Error bars indicate 95% confidence intervals. * $P < 0.05$; **** $P < 0.0001$; ns, not significant.

Figure 3|. Analysis of CAKUT phenotypes in E18.5 *Tbx6^{mh/-}* embryos.

(a–c) Whole mounts of urogenital tracts. (a) Normal kidneys in a WT mouse embryo. Renal hypoplasia (b) and solitary kidney (c) were observed in *Tbx6^{mh/-}* embryos. (d–f)

Hematoxylin and eosin stained sagittal sections of the right kidneys of samples in a–c. (d) Renal section of the WT embryo. (e) Renal section of the *Tbx6^{mh/-}* hypoplasia kidney. (f) Renal section of the *Tbx6^{mh/-}* solitary kidney. The arrow points to the undeveloped kidney. Abbreviations: B, bladder; K, kidney; m, kidney medulla; rc, renal cortex; U, ureter.

Table 1

Genetic and renal characteristics of Chinese subjects with 16p11.2/*TBX6*-associated CS

Human subject	Sex	Age (yr) ^a	<i>TBX6</i> gene		Second hypomorphic allele ^b	Renal evaluation			Renal parenchyma damage	Phenotype
			First null allele	Kidney volume (cm ³)	Kidney symmetry	Renal hypoplasia ^c	Kidney symmetry	Renal hypoplasia ^c		
				Left	Right					
XH004	F	4	16p11.2 del	55.25	55.96	0.99	Left: no Right: no	NA	Isolated CS	Isolated CS
XH186	M	4	16p11.2 del	37.27	33.21	0.89	Left: suggestive Right: suggestive	No	Isolated CS	Isolated CS
XH265	M	3	16p11.2 del	47.99	27.82	0.58	Left: no Right: yes	No	Syndromic (CAKUT+CS)	Syndromic (CAKUT+CS)
XH292	F	12	16p11.2 del	103.94	115.53	0.9	Left: no Right: no	No	Isolated CS	Isolated CS
XH300	M	3	16p11.2 del	32.26	25.85	0.8	Left: yes Right: yes	No	Syndromic (CAKUT+CS)	Syndromic (CAKUT+CS)
XR330	M	4	16p11.2 del	45.58	36.09	0.79	Left: no Right: suggestive	No	Isolated CS	Isolated CS
XR402	M	13	16p11.2 del	116.96	90.55	0.77	NA	No	Isolated CS	Isolated CS
XR439	F	7	16p11.2 del	61.67	52.13	0.85	Left: no Right: no	No	Isolated CS	Isolated CS
XR468	M	3	16p11.2 del	38.41	32.61	0.85	Left: suggestive Right: suggestive	No	Isolated CS	Isolated CS
XR522	M	8	16p11.2 del	104.28	85.19	0.82	Left: no Right: no	Persistent proteinuria	Isolated CS	Isolated CS
XR529	M	14	16p11.2 del	129.39	129.45	1	NA	No	Isolated CS	Isolated CS
XR636	F	9	16p11.2 del	43.92	53.39	0.82	Left: yes Right: suggestive	No	Syndromic (CAKUT+CS)	Syndromic (CAKUT+CS)
XH122	M	8	c.266dupC (p.V91Gfs*80)	91.09	58.78	0.65	Left: no Right: yes	Bilateral delayed renal tissue tracer transit	Syndromic (CAKUT+CS)	Syndromic (CAKUT+CS)
XH170	M	4	c.704dupG (p.M236Hfs*44)	31.28	40.21	0.78	Left: yes Right: no	No	Syndromic (CAKUT+CS)	Syndromic (CAKUT+CS)
XR341	M	2	c.1179_1180delAG (p.G395Lfs*91)	28.14	27.37	0.97	Left: yes Right: suggestive	No	Syndromic (CAKUT+CS)	Syndromic (CAKUT+CS)
XR625	M	7	c.933C>A (p.C311*)	50.2	59.85	0.84	Left: suggestive Right: no	No	Isolated CS	Isolated CS

Note: The renal phenotypes potentially involved in CAKUT are underlined.

Abbreviations: CAKUT, congenital anomalies of the kidney and urinary tract; CS, congenital scoliosis; del, deletion; F, Female; M, Male; NA, not available; yr, years.

^a Subject age (years) at the time of evaluating the renal volume.

^b The hypomorphic haplotype of human *TBX6* was defined by the mutated alleles of 3 *TBX6* variants: rs2289292, rs3809624, and rs3809627.

^c The left and right kidneys were investigated separately. Differences in kidney volumes of > 2 standard deviations below the means in age-matched and ethnically matched controls were considered to reflect renal hypoplasia.¹⁰ Differences of > 1 standard deviation below the means in control subjects were considered to be suggestive of renal hypoplasia. Please refer to Supplementary Table S1 for details.

^d Genotyping of these subjects was conducted in our previous study.³¹

^e Genotyping of these subjects was conducted in our recent studies.^{2,3}

Table 2]
Renal system evaluation of the 16p11.2/*TBX6*-associated subjects in the BCM cohort

Human subject	Sex	Age at diagnosis	Ancestry	<i>TBX6</i> gene		Urinary tract phenotype	CAKUT	Vertebral malformations
				First null allele	Second hypomorphic allele ^a			
BCM-1	M	2 mo	Hispanic	16p11.2 del	No	Left VUR; bilateral mild increased renal echogenicity	Yes	Hemivertebrae between L2 and L4
BCM-2	M	4 yr	Hispanic	16p11.2 del	Yes	Bilateral VUR and complex cystic structure in left kidney	Yes	NA
BCM-3	F	10 mo	Hispanic	16p11.2 del	Yes	Bilateral kidneys smaller than normal size	Suggestive	Hemivertebrae at T10 and T11
BCM-4	M	6 yr	Caucasian	16p11.2 del	Yes	Bilateral increased renal echogenicity with trace bilateral pelvicalyceal dilation, enlarged right kidney	Yes	NA
BCM-5	M	3 mo	Hispanic	16p11.2 del	No	Right solitary kidney with hydronephrosis with markedly dilated and tortuous right ureter to the level of the pelvis	Yes	Three hemivertebrae at L1, L3, and L5
BCM-6	M	1 yr	Hispanic	c.1018 1019delCT (p.L340Vfs*7)	No	Right kidney agenesis and contralateral compensatory hypertrophy	Yes	No scoliosis
BCM-7	F	2 yr	Hispanic	c.138 141dupGGAT (p.C48Gfs*30)	No	Recurrent urinary tract infections	Suggestive	NA
BCM-8	F	12 yr	Hispanic	c.118+1G>C	No	No obvious abnormality	No	No scoliosis

Note: Renal system evaluation was performed by ultrasound except BCM-5 (by MRI).

Abbreviations: CAKUT, congenital anomalies of the kidney and urinary tract; del, deletion; F, Female; M, Male; L, lumbar; mo, months; NA, not available; T, thoracic; VUR, vesicoureteral reflux; yr, years.

^aThe mild hypomorphic haplotype of human *TBX6* was defined by the mutant alleles of 3 *TBX6* SNPs: rs2289292, rs3809624, and rs3809627.