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Traditional and targeted exome sequencing reveals common, rare and novel functional deleterious variants in RET signaling complex in a cohort of living US patients with urinary tract malformations

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Abstract

Signaling by the glial cell line-derived neurotrophic factor (GDNF)-RET receptor tyrosine kinase and *SPRY1*, a RET repressor, is essential for early urinary tract development. Individual or a combination of *GDNF*, *RET* and *SPRY1* mutant alleles in mice cause renal malformations reminiscent of congenital anomalies of the kidney or urinary tract (CAKUT) in humans and distinct from renal agenesis phenotype in complete *GDNF* or *RET* null mice. We sequenced *GDNF*, *SPRY1* and *RET* in 122 unrelated living CAKUT patients to discover deleterious mutations that cause CAKUT. Novel or rare deleterious mutations in *GDNF* or *RET* were found in 6 unrelated patients. A family with duplicated collecting system had a novel mutation, *RETR831Q*, which showed markedly decreased GDNF dependent MAPK activity. Two patients with *RET-G691S* polymorphism harbored additional rare non-synonymous variants *GDNF-R93W* and *RET-R982C*. The patient with double *RET-G691S/R982C* genotype had multiple defects including renal dysplasia, megaureters and cryptorchidism. Presence of both mutations were necessary to affect RET activity. Targeted whole exome and next-generation sequencing revealed a novel deleterious mutation *G443D* in *GFRa1*, the co-receptor for RET, in this patient. Pedigree analysis indicated that the *GFRa1* mutation was inherited from the unaffected mother and the *RET* mutations from the unaffected father. Our studies indicate that 5% of living CAKUT patients

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SUPPLEMENTAL DATA

Supplemental data includes supplementary methods one figure and four tables.

Statement of competing financial interests

None

harbor deleterious rare variants or novel mutations in GDNF-GFR α 1-RET pathway. We provide evidence for the coexistence of deleterious rare and common variants in genes in the same pathway as a cause of CAKUT and discovered novel phenotypes associated with the RET pathway.

Keywords

RET; CAKUT; kidney; exome; genetics; renal malformations

INTRODUCTION

Congenital anomalies of the kidney or lower urinary tract (CAKUT) are the most common cause of kidney failure in children and a major cause of end stage renal disease in adults (30%) (Pope et al. 1999; Song and Yosypiv). These anomalies include renal agenesis/adysplasia, hypoplasia, dysplasia, ectopic kidneys/ureters, ureteropelvic junction obstruction, megaureter, duplicated collecting system and vesicoureteral reflux (VUR). CAKUT can occur unilaterally or bilaterally and may involve both the upper and the lower urinary tracts. While some of these malformations can be surgically corrected many patients continue to experience significant morbidity and are susceptible to diseases involving other systems including the cardiovascular, skeletal and hematopoietic systems. Despite the anatomic and histological spectrum of CAKUT it is generally believed that many CAKUT originate from gene defects during early urinary tract development.

In mammals three sets of kidneys develop; pronephros, mesonephros and the metanephros. Metanephros is the only structure that becomes the definitive kidney. It begins as an outgrowth, ureteric bud (UB), from the nephric duct at about embryonic day 10.5 (e10.5d) in mice and at about 4 weeks of gestation in humans (Dressler 2006). The UB invades the metanephric mesenchyme and undergoes recursive branching to form the collecting system of the urinary tract. It also induces the mesenchyme to undergo a series of events to produce different cells of the nephron. The signaling pathway consisting of glial cell line-derived neurotrophic factor (GDNF), its co-receptor GFR α 1 and the receptor tyrosine kinase RET is important for early UB induction and lower urinary tract development (Jain et al. 2010). Binding of GDNF-GFR α 1 complex to RET results in its activation and phosphorylation of key docking tyrosine residues that signal through pathways including PLC γ , PI3K and MAPK to elicit cellular responses.

Without RET the UBs fail to initiate or undergo proper induction and branching leading to renal agenesis or aplasia and perinatal lethality. Abnormal expression or absence of Ret also results in failure of distal nephric duct to make proper contact with the developing urinary bladder and causes lower urinary tract defects (Batourina et al. 2002; Yu et al. 2004). RET is a major gene found to be mutated in stillborn fetuses or newborn babies with agenesis (Jeanpierre et al. 2011; Skinner et al. 2005) suggesting conservation of RET pathway and mechanisms between rodents and humans.

We recently characterized urinary tract development in mice with disruption of key Ret-dependent signaling pathways and discovered a spectrum of upper and lower urinary tract malformations highly reminiscent of human CAKUT (Jain 2009; Jain et al. 2006; Jain et al. 2004a). Many of these defects are not present in conventional *Gdnf/Gfra1/Ret* gene knockout mice (Enomoto et al. 1998; Moore et al. 1996; Pichel et al. 1996; Sanchez et al. 1996; Schuchardt et al. 1994) thus suggesting a wider phenotypic role of RET pathway in CAKUT. Some of these defects were also highly identical to mice deficient in sprouty, drosophila, homolog of 1 (Spry1), a repressor of RTKs including RET (Basson et al. 2005).

Studies from others and us showed genetic interaction between *Ret*, *Gdnf* and *Spry1* in mice where different dosage of *Gdnf/Ret* and *Spry1* produce a range of CAKUT (Basson et al. 2005; Michos et al. 2010; Rozen et al. 2009). For example, intermediate dosage of these mutant alleles resulted in unilateral defects, or a subset of CAKUT, or even normal kidney development. These results suggest that genetic interactions between positive and negative regulators has a critical role in determining the type of CAKUT that may develop and may explain the variation observed in phenotypes in living patients. There is only limited information regarding the occurrence of deleterious changes in *GDNF*, *SPRY1* and *RET* in CAKUT in humans other than lethal agenesis phenotype (Jeanpierre et al. 2011; Skinner et al. 2005).

To examine the relevance of studies in disease models involving the GDNF-RET-SPRY1 pathway to human CAKUT and identify deleterious mutations that may provide clues to the mechanism of these malformations, we used traditional and targeted whole exome sequencing in our regional CAKUT cohort. We found that mutations in the GDNF-RET pathway are present in about 5% of living CAKUT patients. The presence of multiple mutations in *RET* or in different genes of the RET pathway suggests that the burden of rare/common deleterious variants in the same developmental pathway influences the expressivity and penetrance of CAKUT. We further identified novel RET pathway mutations that have never been previously associated with CAKUT in humans such as multisystem defects including the reproductive system and CAKUT, VUR and duplicated collecting system. Further, using targeted whole exome sequencing we established a pipeline that helped discover a novel *GFRa1* mutation and delineate genetic mechanism in a child with CAKUT and cryptorchidism.

METHODS

Human studies

Human studies were conducted according to protocols approved by the Washington University human research protection office. Clinical data and specimens were obtained from 122 consented unrelated patients with various CAKUT phenotypes from Washington University George O'Brien Center for Kidney Translational Research Core (see Table 1 and supplemental Table S1). 363 unrelated healthy controls with no known kidney malformations were additionally used to determine the frequency of the discovered mutations. In house exomes of 19 individuals with no known history of renal defects were included for filtering of exome data and served as additional controls. Approval of the ethics committees of the participating center and written informed consent from all individuals (parents in case of minor) were obtained.

DNA isolation and Sanger sequencing

Genomic DNA isolated from peripheral blood leukocytes or buccal cells was obtained from the Washington University George M. O'Brien Center Kidney Translational Research Core. We screened the 20 *RET* exons encoding both protein isoforms (RET9: exons 1 to 19b, RET51: exons 1 to 19a, 20), the four *GDNF* exons (non-coding exon 1 and coding exons 2, 3 and 4), and the two coding *SPRY1* exons (exon 1 and 2). Evolutionary conserved non-coding regions (ECRs) located upstream of *RET* were identified using the UCSC genome browser (<http://genome.ucsc.edu/>) and sequenced. Four regions were identified; -1.284 to -1.125 kb, -4.139 to -3.852 kb, -7.034 to -6.868 kb and -10.241 to -10.047 kb upstream of the transcription start site and analyzed by Sanger sequencing using the primer sets shown in Supplemental Table S2. Finally, as variations in the 3'UTR could potentially disrupt genetic regulation by micro-RNAs, these regions were also analyzed. All primers used are shown in Supplemental Table S2. Sanger sequencing was performed in the tissue

procurement and molecular phenotyping core at the Washington University. The data were analyzed by DNA Star software (<http://www.dnastar.com/>).

Generation of mutant plasmids by site directed mutagenesis

To create vectors expressing mutant *RET* of *GFRα1* forms for biochemical studies site directed mutagenesis was used. Mutant *RET* constructs were made in hRET9 isoform. The wild-type or mutant cDNAs were expressed from pcDNA3.1 plasmid vector. Site-directed mutagenesis to create vectors encoding *RET9-R813Q*, *-G691S* (rs1799939) and *-R982C* (rs17158558) was achieved by a two-stage PCR mutagenesis procedure using the High Fidelity Phusion taq Polymerase (Invitrogen) with the primers shown in Supplemental Table S3. For the *RET9-G691S/R982C* double mutant, pcDNAhRET9_G691S was used as the PCR template and R982C was incorporated in it using site directed PCR mutagenesis. All mutations were confirmed by Sanger sequencing. We used an inactive form of RET, *RET-K758M* (kinase dead, KD) (Jain et al. 2004a), as a negative control, and a constitutively active GDNF-independent (MEN2A) form of RET, *RET-C618F* (Jain et al. 2004b), as a positive control for RET dependent p-MAPK activity.

Cell Culture

Human Kidney cell line HEK293 were maintained in 37 °C and 5% CO₂ atmosphere and DMEM media (Sigma, St. Louis, MO) supplemented with 10% heat inactivated FBS for studies in vitro. The cells were plated at a density of 5×10^5 cells in 6-well plates, and a day later were transfected (80% confluency) with plasmid vectors using Lipofectamin 2000 (Invitrogen, Carlsbad, CA). For each well, 0.1 µg of WT or mutant *RET* and 0.1 µg of *GFRα1* diluted in 250 µL of Opti-MEM (Invitrogen, Carlsbad, CA) were used. Lipofectamine 2000 (3 µL/well) was diluted in 250 µL of Opti-MEM and incubated for 5 min at room temperature. The diluted DNA was slowly added to the diluted Lipofectamine, mixed and incubated for 20 min at room temperature to facilitate the formation of DNA–Lipofectamine (DL) complexes. The DL complexes were added to the cells for 16–18h. The lipofectamine medium was replaced with fresh culture medium and subjected to further treatment. We used the pGL3 plasmid (Promega, Mannheim, Germany) encoding the firefly luciferase as transfection efficiency control. After 36 hours, the cells were lysed with lysis buffer (50mM Tris HCL; pH-7.5, 150mM NaCl, 10% Glycerol, 2% NP-40, 20mM EDTA, 20mM NAF, 1mM Na₃VO₄, 1mM PMSF and 1X Protease inhibitor-Thermo Scientific), and luciferase activity was measured with a luminometer (Spectrafluor Plus, TECAN, Crailsheim, Germany). To measure GDNF-RET pathway activity, 36 hours after transfection cells were serum starved for 4 hours and subsequently stimulated with 100 ng/ml GDNF for 20 min at 37 °C.

Western Blots

A total of 10 µg of each protein sample was resolved on a 10% polyacrylamide gel and transferred to a PVDF (polyvinylidene fluoride) membrane using Bio-Rad semi-dry apparatus. Membranes were blocked in Odyssey Blocking Buffer (927-40000, LI-COR) for one hour at RT followed by incubation with primary antibody. Primary antibodies used were: polyclonal rabbit anti-panRET (1:200) (Tsui-Pierchala et al. 2002), chicken polyclonal β-actin (Abcam, 1:1000), goat polyclonal anti-GFRα1 (Neuromics GT15004, 1:500), rabbit polyclonal pMAPK (Cell Signalling 4370, 1:200). The blots were blocked in Odyssey Blocking Buffer (LI-COR) overnight at 4°C. After washing, the membranes were incubated for 1 hour at RT with IRDye800CW-conjugated goat anti-rabbit IgG (1:2000, green), IRDye800CW-conjugated goat anti-chicken IgG (1:2000, green) and IRDye800-conjugated goat anti-Goat IgG (1:2000, green) secondary antibodies (LI-COR Biosciences). After washing, the respective antigens were visualized on an Odyssey Infrared Imaging System (LI-COR Biosciences) with both 700- and 800-nm channels.

Exome sequencing and SNP phenotyping

Library preparation and target capture was done using gDNA (3 µg) of patient 07-1184-0002 and SureSelect Target Enrichment System 38Mb exome kit (Agilent Technologies) by Cofactor Genomics. Paired-end sequencing was performed on four lanes of Genome Analyzer IIx (Illumina). Raw sequence data in fastq format was aligned to the human reference genome (hg18) using Novoalign™ version 2.05 (Novocraft® Technologies available at <http://www.novocraft.com>). An alignment threshold of 200 was used (-t 200), with adapter stripping (-a AGATCGGAAGAGCG) and quality calibration enabled (-k). Reads with multiple alignments were discarded (-r none, -e 1) and output was in SAM format (-o SAM). SNP calling was done using SAMtools (available at <http://samtools.sourceforge.net>) (Li and Leal 2009). The aligned output was indexed with SAMtools “import” and sorted with SAMtools “sort”. Duplicate reads were then removed using Picard “MarkDuplicates”. The resulting file was sorted again and variants were called with the SAMtools version 0.1.18 mpileup command, using options -AB -ugf and bcftools “view” with settings -bvcg. All variants called with under 99999 reads were retained.

Sequenom validation

The mutations identified from exome sequencing and Sanger sequencing were validated in patients and 90 healthy controls using Sequenom Core facility at Washington University.

Prediction of deleterious mutations: Data filtering, Polyphen/SIFT

The large number of coding region SNPs (cSNPs) were filtered against existing dbSNP129 database and SNPs already discovered in the 1000 genome project. The resultant SNPs were then filtered against 8 HapMap exomes. To reduce the false positive rate of the SNP calls, the data were then further filtered against 19 in-house exomes of patients with no known history of any renal defects. We prioritized screening of the resultant variants on 292 candidate genes. These were selected from genes implicated in the development and function of the embryonic kidney based on their expression patterns in the urinary tract, known biological roles in mutant mice, and from PUBMED, GUDMAP, and published abstracts in national meetings (Supplemental table S4). The effect of amino acid substitution on protein function was predicted with Sorting Intolerant from Tolerant (SIFT, <http://sift.jcvi.org/>) and Polymorphism Phenotyping v2 (PolyPhen-2, <http://genetics.bwh.harvard.edu/pph2/>). PolyPhen-2 is a software tool which predicts possible impact of amino acid substitutions on the structure and function of human proteins by comparing physical and evolutionary characteristics. It uses sequence and structure-based information to predict the effect of variants using a Bayesian approach (Adzhubei et al. 2010). False positive and true positive rates are calculated for the prediction and thresholds applied to give qualitative predictions of benign (least confident), possibly damaging or probably damaging (most confident). The prediction scores are provided in the range of 0–1, whereby 0 is predicted to be completely benign and 1 being the most probable damaging variation.

Statistics

Unpaired student's t test (http://www.physics.csbsju.edu/stats/t-test_NROW_form.html) was performed to determine the statistical significance of western blot results. Armitage trend test, following the guidelines given by Sasieni (Sasieni 1997) as implemented in the program FINETTI, was performed to find the association of *GDNF-R93W* with CAKUT patients. The Armitage trend test based on the genotypes remains valid even if Hardy-Weinberg Equilibrium does not hold. The statistical significance was further confirmed using Fisher exact test (<http://www.quantitativeskills.com/sisa/>).

RESULTS

***GDNF*, *RET* and *SPRY1* sequencing in CAKUT patients**

To discover variants in *GDNF*, *RET* and *SPRY1*, three genes that alone or in combination cause a spectrum of renal malformations in mice, we selected 122 unrelated living US patients with CAKUT (Table 1 and supplemental Table S1). Upstream conserved regions, splice site junctions and coding regions were selected for Sanger sequencing (see methods). Single and multiple non-synonymous (ns) variants were identified in *GDNF* and *RET* in a total of six patients (Figure 1). A common deleterious SNP *RET-G691S* associated with VUR in a French- Canadian cohort (Yang et al. 2008) was found at similar frequency in CAKUT and control patients. However, two patients with *G691S* polymorphism additionally harbored rare deleterious mutations. One in *RET* (R982C), and the other in *GDNF* (R93W, rs36119840). One patient with the double *RET-G691S*, *R982C* mutations had complex renal malformations and extra renal defects (bilateral megaureters, dysplasia and cryptorchidism, Figure 1). The other with the genotype *RET-G691S::GDNF-R93W* had VUR and renal dysplasia. Two novel *RET* mutations occurring singly were also found in two patients. One with the duplicated collecting system had a *RET-R813Q* mutation and the other with multicystic dysplasia had a *RET-A1105V* mutation. The *GDNF-R93W* variant was also detected in two additional patients, one with ectopic kidney and unilateral agenesis and the other with renal dysplasia. To find the association of the *GDNF-R93W* variant which was present in 3 out of 122 CAKUT patients but none of the 363 unrelated control individuals, Armitage trend test was performed. The R93W mutation was found to be associated with CAKUT ($p = 0.002$, $\chi^2 = 8.98$, common OR = 3.12 with 95% confidence interval). Moreover Fisher exact test also predicted the mutation to be deleterious ($p = 0.015$).

All variations were heterozygous and none of them except the known *RET-G691S* was identified among 363 controls. The *RET* variants G691S, R813Q, R982C and A1105 and the *GDNF* variant R93W were all predicted to be “damaging” by PolyPhen-2 software with scores of 0.848, 1.0, 0.996, 0.958 and 0.998, respectively (Table 2). We did not detect any non-synonymous deleterious variant in the *SPRY1*. No novel synonymous SNPs/mutations were detected in the coding or non-coding regions of *RET*, *GDNF* or *SPRY1*.

To further support the hypothesis that the *GDNF* and *RET* mutations found in patients contribute to CAKUT we screened our existing allelic series of *Gdnf* and *Ret* mutant mice (Jain 2009; Jain et al. 2006; Jain et al. 2004a) that phenocopy these defects. In Supplementary Figure 1, we illustrate representative examples of *Ret* pathway mutant mice with features of duplicated collecting system, cystic dysplasia, unilateral agenesis, cryptorchidism and urinary tract defects, and VUR (Supplementary Figure 1A–E).

***RET* mutations exhibit reduced GDNF dependent MAPK activity**

To glean insights into the mechanism of how the *RET* intragenic mutations *RET-G691S*, *R982C*, *RET-R813Q* and *RET-A1105V* lead to CAKUT we performed biochemical analysis *in vitro*. Wild-type *RET* and the mutant *RET* were created by site-directed mutagenesis and assayed for GDNF-dependent activation of MAPK, a bona fide downstream effector of *RET* activity critical for kidney formation (Baloh et al. 2000; Jain et al. 2006). While wild-type *RET* showed GDNF-dependent MAPK phosphorylation, *RET-R813Q* showed complete abrogation of MAPK activity (Figure 2A). The double *RET-G691S*, *R982C* mutant also showed near complete loss of GDNF-dependent MAPK phosphorylation and it required the presence of both mutations as *RET-G691S* and the *RET-R982C* variants individually did not affect MAPK activity (Figure 2A, B). These results further support that the mechanism of CAKUT pathogenesis in patients with these *RET* mutations (megaureters, dysplasia and

cryptorchidism, and duplication) is at least through modulation of RET-activated MAPK activity. In contrast, *RET-A1105V* did not significantly alter the GDNF-dependent MAPK activity compared to the wild-type RET suggesting that this mutation by itself may not be causative (Figure 2B). As expected the constitutively active *RET^{MEN2A}* mutant showed pMAPK activity irrespective of GDNF stimulation, while an inactive form of RET (*RET-KD*) shows no GDNF-dependent pMAPK activity (Figure 2C).

***RET-R813Q* mutation is present in an affected relative with duplicated collecting system**

On surveying our CAKUT database we found a child with duplicated collecting system who was recently diagnosed with duplicated collecting system and is related (great-granddaughter) to the index case 1184-00391 with the *RET-813Q* mutation (Figure 3). No other family member from this pedigree were reported to harbor any renal malformations and we have classified their phenotypes as unknown since no radiographic imaging was available from them. To further gain support for *RET-R813Q* mutation as a likely cause of duplication we enrolled this child (1184-00371) and sequenced for *R813Q* mutation. Results showed that the great-granddaughter also harbored the *RET-R813Q* mutation. Thus, presence of duplicated collecting system in *Ret*-mutant mice, reduction in GDNF-dependent RET-MAPK activity in *RET-R813Q* mutant and presence of this mutation in affected relatives strongly support that aberrant RET pathway activation as one of the causes of abnormalities in duplicated collecting system in humans.

Targeted whole exome sequencing reveals a novel deleterious *GFR α 1* mutation in the patient with multiple renal anomalies and cryptorchidism

We next examined the genetic mechanism leading to the complex CAKUT and cryptorchidism phenotype in the patient with the *G691S* and *R982C* mutations (07-1184-00092). To determine if these mutations occurred *de novo*, or were transmitted by each parent (recessive) or from the same parent (dominant), we enrolled the parents and sequenced their genomic DNA to detect *G691S* and *R982C* mutations. We observed that both the *RET* mutations were inherited from the father and therefore most likely on the same chromosome (Figure 4A and data not shown). However, the father is unaffected and has no known renal disease. While it is possible that the double *RET* mutations in the unaffected father may be incompletely penetrant, it is also likely that another deleterious variant of moderate to mild potency may be present in this patient occurring *de novo* or inherited from the mother.

To this end we performed targeted capture in conjunction with whole exome next-generation sequencing on genomic DNA from patient 07-1184-00092. The performance metrics of the whole exome revealed that greater than 95% of the targets were covered at 8X read depth, and a total of 17,402 variants were present, consistent with other studies (Figure 4B) (Ng et al. 2010; Ng et al. 2009). Robustness of the capture and exome sequencing was further confirmed independently on a SNP array (Affymetrix 6.0) where greater than 98% of coding region SNPs were present in both the SNP array and the exome array (not shown). Further, both deleterious *RET* variants (*G691S* and *R982C*) and four synonymous DNA changes that were identified by traditional sequencing also were detected by the exome approach. We next developed a filtering strategy to identify the most likely variants that may contribute to the phenotype seen in this patient with genitourinary defects (Figure 4B). We filtered the variant list against known information in public databases (dbSNP129, HapMap, 1000 genome), in-house exomes from patients with no renal malformations. We then prioritized a candidate list of 292 genes that are most likely to be involved in kidney development based on their expression and roles in mice with kidney defects (see methods and Figure 4). From the 27 novel non-synonymous variants, 11 were predicted to be damaging by the PolyPhen-2 and SIFT softwares. After validation by Sequenom, traditional Sanger

sequencing and filtering against an additional 90 in-house controls, two novel variants were short listed, *GFRa1-G443D* and *LAMC1-P321S*. These mutations were further found to be absent in 273 unrelated control individuals screened using Nextgen sequencing.

The novel non-synonymous *GFRa1-G443D* had a PolyPhen-2 damage prediction score of 1.0 and was particularly intriguing as GFRa1 is the co-receptor for RET and hence in the same pathway. The *GFRa1-G443D* mutation was present in exon 11 near the hydrophilic domain and the GPI-anchoring domain suggesting it may be important in GPI-anchorage and RET interaction (Figure 5A, B). Comparison *in-silico* revealed that the G443 residue was highly conserved in multiple mammals (Figure 5C), and the Aspartic-Acid change was not found in the 10 species examined (data not shown). These observations suggest that *GFRa1-G443D* mutation may further affect RET signaling in conjunction with already present double *RET* mutation in this patient. In biochemical experiments *GFRa1-G443D* alone did not show any effect on MAPK activity and when present with the double *RET* mutations maintained the already decreased MAPK activity; further decrease was difficult to appreciate as the activity was already reduced to basal levels (data not shown). To determine if *GFRa1-G443D* mutation occurred *de novo* or was inherited from one of the parents, we screened the parents and siblings for the presence of this mutation. Sequencing results show that *GFRa1-G443D* was inherited from the unaffected mother (Figure 5D).

Genetic mechanism of inheritance of *GFRa1* and *RET* mutations in patient 07-1184-00092

Pedigree analysis of the patient 07-1184-00092 with CAKUT and cryptorchidism showed that *GFRa1-G443D*; *RET-G691S*, *R982C* mutations are inherited in a compound heterozygous manner (Figure 6A); *GFRa1* from the mother and *RET* from the father. An unaffected male sibling did not inherit the deleterious *RET* variants. Interestingly, the patient's toddler sister whose renal disease status is unknown shares all three variants with the affected patient. While she cannot develop cryptorchidism, whether she will manifest any renal abnormalities remains to be seen. These analyses together suggest that the CAKUT and cryptorchidism phenotypes in the patient 07-1184-00092 are due to a combination of rare, novel and common deleterious variants affecting the RET pathway (Figure 6B).

DISCUSSION

RET has been implicated in renal agenesis and survival for more than a decade from studies in *Ret*-null mice (Schuchardt et al. 1994). *RET* mutations were only recently identified in fetuses with renal agenesis (Jeanpierre et al. 2011; Skinner et al. 2008). It was only through studies of specific *Ret*-signaling mutant mice by us and others that it was realized that RET may regulate much broader aspects of urinary system defects including dysplasia, duplications, megaureters, reflux and gonadal abnormalities (Basson et al. 2005; Jain et al. 2006; Jain et al. 2010; Jain et al. 2004a; Rozen et al. 2009). Because many of these defects don't cause neonatal lethality as seen in bilateral agenesis, the relevance of this pathway in living humans with CAKUT and the molecular and genetic mechanisms of how RET pathway may result in CAKUT had not been examined. In the present study we discovered that non-synonymous deleterious germline variants in *GDNF-RET* are present in approximately 5% of our cohort of living patients in the USA with CAKUT. Presence of similar phenotypes in our previous reports and current study in *Gdnf-Ret* mutant mice with urinary tract defects provide strong support for these mutations to be pathogenic and provide novel associations of the GDNF-RET pathway mutations in conditions such as duplication, VUR, megaureters, dysplasia and renal anomalies with cryptorchidism in humans. Considering that disease model studies and gene expression analysis implicate several hundred genes in CAKUT, GDNF-RET pathway mutations in 6 of 122 patients suggests it to be a significant factor in CAKUT pathogenesis. The identification of novel mutations,

rare and common deleterious variants in the same genes and in different genes of the same pathway support the idea that presence of intra and intergenic variations partly modulate the phenotypic outcome of CAKUT. Our results also show that the phenotypes associated with the observed mutations in this single growth factor/ligand pathway are themselves diverse suggesting careful genotype/phenotype/functional analysis may ultimately allow clinicians to dissect CAKUT into distinct subtypes more readily.

Studies in mice with disrupted RET signaling have been highly relevant in understanding the mechanisms of RET associated diseases such as MEN2 syndromes and HSCR. In HSCR, *RET* mutations are found throughout the coding region and affect extracellular, and intracellular domains (Heanue and Pachnis 2007). In renal agenesis *RET* mutations are also present throughout its coding region (Table 2) (Jeanpierre et al. 2011; Skinner et al. 2008). In our CAKUT patients with phenotypes other than agenesis, we detected common, rare and novel mutations in the *RET* intracellular domain. We found that a subset of these affected MAPK activity. MAPK is a downstream effector of RET that can be directly or indirectly modulated by signaling through one of the key RET docking tyrosine adaptor sites for PLC γ (Y1015), SRC (Y981) and SHC (Y1062) (Encinas et al. 2008; Gustin et al. 2007). In mice, altered RET-MAPK leads to HSCR or CAKUT. Therefore as in mice, MAPK signaling in humans is an important pathway in renal malformations. In Hirschsprung Disease (HSCR), reduced MAPK is generally thought to affect proliferation or migration of enteric neural crest precursors and cause aganglionosis. Altered MAPK activity observed in CAKUT patients with RET mutations (Skinner et al. 2008) and our study may also be due to defects in proliferation or migration. Studies in mice with agenesis, dysplasia or megaureters suggest that these defects may be partly due to abrogated MAPK signaling. However, it is not clear how increased MAPK activity as seen by Skinner et al can lead to agenesis, or reduced MAPK activity causes duplication as in the family with *RET-R813Q* mutation in our study. The RET mutations *R813Q* and *R982C* are in the kinase domain and the *R691S* in the juxtamembrane domain. While we focused the effect of these mutations on the MAPK pathway it is possible that these can also influence interaction with intracellular adaptor SRC via Tyr981, PLC γ via Tyr1015 or PI3K via Tyr1062 pathway that were not evaluated in this study.

RET-G691S is a common polymorphism with a reported allele frequency of 20% (dbSNP database). Role of G691S by itself in renal disease is less clear. In a French-Canadian cohort, *G691S* was more common in patients with VUR (Yang et al. 2008), whereas in an Irish cohort there was no difference between the frequencies of G691S in controls and VUR patients (Darlow et al. 2009). In our study we also did not find any difference between the frequencies of *G691S* in controls and CAKUT patients although our patients included VUR and other renal anomalies. However, there were two patients with *RET-G691S* who had additional mutations, one with *RET-R982C* and the other with *GDNF-R93W*. The *RET-G691S* polymorphism likely modulates the penetrance of these other RET pathway mutations particularly that alone are of mild or moderate severity. This idea is supported by double mutations of *RET-691/666* residues in MEN2 tumors (Borrello et al.), and the complex renal malformation seen in our patient with the double *RET-G691S/R982C* mutation with reduced RET-MAPK activity. Our biochemical studies indicated that presence of both mutations was necessary to affect RET activity. In addition to the biochemical studies, genetic analysis of the patient with CAKUT and cryptorchidism showed that the double *RET* mutations were inherited from the father while a conserved, novel *GFR α 1-G443D* mutation that was predicted to be damaging from the mother. These observations suggest that the presence of mutant *GFR α 1* and *RET* alleles in this patient severely affects the presence of functional wild-type RET complexes and affect downstream signaling (Figure 6B) resulting in genitourinary malformations. These mutations likely act in a dominant manner by producing signaling complexes with reduced RET activity as

suggested by our studies with a Ret dominant negative mouse with a double *Ret985/1062* mutations with reduced Mapk activity (Jain et al. 2004a). We therefore propose that genes in the RET pathway should be sequenced for additional mutations in patients with *RET-G691S* polymorphism. The father has no kidney disease but harbors both the *RET* mutations present in this patient suggests incomplete penetrance and presence of other modifiers of RET signaling that result in the disease phenotype. The sister of the patient carries all the three mutations but her disease status is unknown. It is possible that the *LAMC1-P321S* mutation found in the patient may also contribute to the CAKUT phenotype.

In three CAKUT patients we observed a rare GDNF variant, *GDNF-R93W* (rs36119840), that is highly conserved among species. This variant has been suggested previously to represent a low penetrance susceptibility gene that acts by modulating the disease phenotype through interactions with other major susceptibility loci like *RET* in Hirschsprung disease (Salomon et al. 1996) and Ondine's curse (congenital central hypoventilation syndrome) and never been reported in healthy individuals (Amiel et al. 1998). In our study, out of the three patients one harbored both the *GDNF-R93W* and the common *RET-G691S* polymorphism. Because *R93W* is in the protein interaction domain and the *RET-G691S* alters MAPK activity when present with other mutations (above), presence of *GDNF-R93W* and *RET-G691S* mutations together also likely influences overall RET activity and early UB induction. In this regard unilateral agenesis is observed in about 30% of mice with *Gdnf* haploinsufficiency indicating that reduced Ret signaling as an etiological factor. The *GDNF-R93W* variant was not present in the 1000 Genome or 363 patients with no renal disease at our institution and has not been identified in any control subjects studied by others including more than 163 healthy Caucasian infants (Zhang et al. 2009). Moreover the *GDNF-R93W* variant was found to be significantly associated with CAKUT in our study population. Therefore, we suggest that the *GDNF-R93W* variant may be a clinically significant modifying variant in a variety of renal anomalies and RET related disorders.

We applied and validated exome sequencing approach with a customized pipeline we developed to identify deleterious rare and novel variants that are most likely to be causative in CAKUT. Because one of the challenges of NGS data is to interpret and delineate the biologically most relevant changes, our approach was to first examine variants in genes we suspected to be highly causative based on their roles in the development of the urinary tract. We therefore shortlisted candidate list of 292 genes that we suspected most likely to be involved in kidney defects. Similar approaches have been used recently to analyze 100 genes from known immunologic pathways in a family with autosomal dominant inherited chronic mucocutaneous candidiasis revealing mutations in the *STAT1* gene (Liu et al. 2011), and to analyze exons of 395 human genes, known or predicted to be associated with primary immunodeficiency and immune regulation (Ghosh et al. 2012). In our exome pipeline we minimized overfiltering by using an older version of dbSNP database (db129) as many of the variants reported in the more recent releases may overfilter rare variants. Using our approach we confirmed all the *RET* mutations we had identified in the patient with CAKUT and cryptorchidism with traditional sequencing, and identified a novel *GFRa1* mutation that also belongs to the RET pathway. The presence of *RET* and *GFRa1* mutations in the same patient strongly suggests that disruption of this pathway as the likely molecular mechanism (confirmed by biochemical analysis) of the complex phenotype in this patient. The presence of similar defects in *Ret*-mutant mice provides further credence to these mutations being causative. We also were able to use this information and show that this rare phenotype is compound heterozygous as the *GFRa1* mutation was inherited from the mother and the *RET* mutations from the father. The same mutations were not found in 7 additional patients with renal agenesis and cryptorchidism or 10 patients with Prune Belly Syndrome (triad of CAKUT, cryptorchidism and abdominal muscle defects, data not shown) suggesting that the CAKUT phenotype in the index case is distinct from these diseases or

other mutations may contribute to these other defects. Nevertheless, we show the feasibility of a customized targeted approach combining biological and molecular insights with whole exome sequencing in identifying most likely disease causing variants in CAKUT in a single patient. Other pathways such as p-PLC γ and p-AKT are also activated by RET that can be affected by the identified mutations. However we focused on MAPK activity for several reasons: 1) Plc γ interacts with Spry1 (repressor of MAPK) (Akbulut et al. 2010), 2) Shc docking site Y1062 activates MAPK, and 3) normal kidney budding in RetTyr1062-Spry1 double mutant (Rozen et al. 2009).

Our studies for the first time provide evidence for presence of nonsynonymous deleterious variants in the GDNF-GFR α 1-RET pathway in living patients with CAKUT. Our results also establish for the first time a significant association of the *GDNF* gene with CAKUT. The variants in the GDNF-GFR α 1-RET pathway are likely of mild to moderate effect as more severe ones would not be viable as seen in patients with bilateral renal agenesis. We report novel associations of RET with CAKUT phenotypes such as duplication and reflux, and also with complex phenotypes involving both the urinary and the reproductive system. Our results thus support the idea that increased deleterious rare variant burden plays a role in pathogenesis of CAKUT as multiple mutations in different genes of the same pathway were found in several individuals. Our study supports 5% CAKUT are due to mutations in either GDNF/RET/GFR α 1. Since there are hundreds of genes implicated in urinary system development, targeted approaches should be undertaken to reveal variants in these genes in CAKUT patients. Additionally, non-genetic factors such as environmental exposures, posttranslational modifications and epigenetics may also cause or further contribute to development of CAKUT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Identification of novel and rare variants in RET and GDNF
(A) Gene map of human *RET*, *GDNF* and *SPRY1* depicting non-synonymous variants (arrows) identified in our CAKUT patient population (boxes represent exons). (B–F) Sequencing traces of the nonsynonymous variants in the *RET* and *GDNF* genes; the position of the nucleotide and the amino acid changes are indicated (arrows). Common/rare/novel represents the prevalence of the mutations in our patient population. (G) Description of the variants found in *RET* and *GDNF* along with the patient description and disease phenotype. Values in parenthesis indicate the variation frequency in 363 unrelated controls. Asterisk (*) indicates novel variant.

Figure 2. Malformations in patients with *RET* mutations have reduced MAPK activity
 Biochemical analysis of *RET* mutants. Anti-RET, anti-GFRα1 and anti-phospho MAPK (pMAPK) immunoblot of total cell lysates from HEK293 cells transfected with plasmids expressing wild-type *GFRα1* and different *RET* mutant constructs: (A) Wild type RET, *RET*R813Q and *RET*-G691S, R982C double mutant (DM), (B) *RET*-G691S, *RET*-R982C and *RET*A1105V and (C) *RET*-C618F (*MEN2A*) and *RET*-K758M (kinase dead, KD) as positive and negative controls of RET-MAPK activity, respectively. RET pathway activation was assessed by increase in pMAPK (doublet) on exposure with GDNF. GDNF-dependent increase in pMAPK is clearly seen in cells transfected with RET-WT (positive control). Note severe reduction in GDNF-dependent pMAPK activation in *RET*-R813Q and the *RET*-DM. The bar graphs quantifies the reduction in pMAPK activity in the mutants

determined by the ratio of integrated density (ID) between phospho-MAPK (pMAPK) and total RET (doublet at 150 and 170kD), to normalize expression of transiently transfected plasmids (mean $\pm$ SE, n = 4, * = p < 0.05). β -actin is shown as the loading control.

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Figure 3. The novel *RET-R813Q* mutation is detected in 2 affected relatives and associated with duplication

(A) Pedigree featuring the family of the patient 07-1184-00391. Heterozygous *G42368A* missense mutation in *RET* leads to the deleterious R813Q change in patient 07-1184-00391. The same mutation is also present in the affected great granddaughter (07-1184-00371). (B) Ultrasonograph of patient 07-1184-00371 showing two distinct poles of the left kidney indicative of duplication. (C) Sequencing traces confirm *RET-R813Q* mutation (arrow) in the great granddaughter of patient 07-1184-00391 with both the forward and reverse primers.

Figure 4. The *RET*-R982C variant in patient 07-1184-00092 with the double *RET*-G691S, R982C genotype is inherited from the father and exome sequencing pipeline identifies a novel *GFR α 1* mutation

(A) Sequencing traces depict that the rare non-synonymous variants G691S (2 traces on the left) and R982C (2 traces on the right) in the *RET* gene is present in father of patient 07-1184-00092 but absent in the mother. Arrows point to the position of the C/T nucleotide change. (B) Whole-exome Capture Sequencing Metrics from the CAKUT patient 07-1184-00092 and filtering approach used to identify two novel mutations *LAMA5P321S* and *GFR α 1G443D*.

Figure 6. Pedigree shows compound heterozygosity as a mechanism of inheritance of RET pathway mutation in the patient 07-1184-00092 with CAKUT and cryptorchidism and model summary of mechanism of CAKUT

(A) Pedigree depicting the family of patient 07-1184-00092 whereby both the *RET* mutations, *RET-G691S* and *RET-R982C* are inherited from the unaffected father while the *GFRa1-G443D* mutation is inherited from the unaffected mother. The brother does not have any *RET* mutations, while the sister inherited all the three mutations but her disease status is unknown (?). (B) Diagrammatic representation of proposed mechanism by which the various *RET* and *GFRa1* mutations lead to genitourinary defects in the patient 07-1184-00092. The presence of the *GFRa1-G443D* and *RET-G691S*, *R982C* variations in the same patient results in GDNF-GFRa1-RET heterodimeric complexes with disrupted MAPK signaling thus reducing the availability of normal RET signaling complexes, similar

to previously seen in mutant mice and observed in vitro (Figure 2). The reduced RET signaling leads to developmental defects including megaureters (ultrasonograph image of patient 07-1184-00092) and cryptorchidism as seen in this patient.

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Table 1

Clinical characteristics of study subjects

Characteristics	Values
Total Patient Number	122
Gender ratio (Male:Female)	1.03:1
Single anomaly	
Duplicated collecting system	11
Dysplasia	17
Hypoplasia	11
Megaureter	8
Solitary kidney/Unilateral agenesis	16
UPJO	8
VUR	7
Horseshoe kidney	2
Prune Belly Syndrome	1
Ectopic kidney	1
Multiple anomalies	
Duplicated collecting system and other renal defects	8
Renal dysplasia and other renal defects	14
Unilateral agenesis and other renal defects	15
VUR and other renal defects	2
Malrotation of kidney and Turner's	1

Table 2

Summary of RET pathway mutations in CAKUT studies

Study	Total number of patients	Patient Cohort	Mutations observed	Phenotypes/Affected Individuals	PolyPhen Prediction
Skinner et. al (Skinner et al. 2005)	33 stillborn fetuses	USA	RET_P198T	Bilateral Agenesis/1	Possibly Damaging
			RET_V376A	Bilateral Agenesis/1	Damaging
			RET_N394H	Bilateral Agenesis/1	Damaging
			RET_Q421X	Bilateral Agenesis/1	Nonsense
			RET_V778I	Bilateral Agenesis/2	Benign
			RET_G894S	Bilateral Agenesis/1	Damaging
			RET_M918T	Bilateral Agenesis/1	Damaging
			RET_P1049L	Unilateral Agenesis/1	Damaging
Jeanpierre et. al (Jeanpierre et al. 2011)	105 fetuses with bilateral defects	France	RET_P1067S	Unilateral Agenesis/1	Damaging
			GDNF_A98T	Unilateral Agenesis/1	Benign
			RET_L56M	Bilateral Agenesis and uterine agenesis/1	Damaging
			RET_R57Q	Multicystic dysplasia/1	Benign
Our Study	122 Live CAKUT Patients	USA	RET_D567N	Bilateral Agenesis/1	Damaging
			RET_V787I	Unilateral Agenesis/Right hypo dysplasia/1	Damaging
			RET_W1056X	Bilateral Agenesis/1	Nonsense
			RET_R813Q *	Duplicated collecting system/1	Damaging
			RET_A1105V	Renal Dysplasia/1	Damaging **
			RET_G691S, R982C, GFRa1_G443D	Right cystic-dysplasia, left dysplasia, Bilateral megaureter, cryptorchidism/1	Damaging
			RET_R691S, GDNF_R93W	Bilateral VUR/1	Damaging
			GDNF_R93W	Left VUR, Right ectopic hydronephrotic kidney, or Unilateral Agenesis, Right ectopic kidney/2	Damaging

* denotes mutation seen in 2 affected relatives

** based on biochemical analysis this mutation is unlikely to be causative on its own