ETV4 Mutation in a Patient with Congenital Anomalies of the Kidney and Urinary Tract

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Abstract: Congenital anomalies of the kidney and urinary tract (CAKUT) are the most common reason for chronic kidney disease in children. Although more than 30 monogenic causes have been implicated in isolated forms of human CAKUT so far, the vast majority remains elusive. To identify novel monogenic causes of CAKUT we applied homozygosity mapping, together with whole exome sequencing, in a patient from consanguineous descent with isolated CAKUT. We identified a homozygous missense mutation (p.Arg415His) of the Ets Translocation Variant Gene 4 (ETV4). The transcription factor ETV4 is a downstream target of the GDNF/RET signaling pathway that plays a crucial role in kidney development. We show by means of electrophoretic mobility shift assay that the Arg415His mutant causes loss of the DNA binding affinity of ETV4 and fails to activate transcription in a cell-based luciferase reporter assay. We furthermore investigated the impact of the mutant protein on cell migration rate. Unlike wildtype ETV4, the Arg415His mutant failed to rescue cell migration defects observed in two ETV4 knock-down cell-lines. We therefore identified and functionally characterized a recessive mutation in ETV4 in a human patient with CAKUT. We hypothesize that the pathomechanism of this mutation could be via loss of the transcriptional function of ETV4, and a resulting abrogation of GDNF/RET/ETV4 signaling pathway.

Keywords: CAKUT, cell migration, DNA binding, ETS domain.

1. INTRODUCTION

Congenital anomalies of the kidney and urinary tract (CAKUT) encompass a wide spectrum of urinary tract malformations involving the kidney (e.g. renal agenesis, hypoplasia) and the urinary tract (e.g. vesicoureteral reflux (VUR), ureteropelvic junction obstruction (UPJO)) [1]. These disorders belong to the most common birth defects and constitute the main cause of chronic kidney disease in children (~50%) [2-4].
It has been hypothesized that single-gene mutations may cause CAKUT in humans [5]. To date, over 30 monogenic causes have been described to lead to CAKUT [6]. Many of these genes show a dominant mode of inheritance [6], yet a significant proportion of recessive genes has been previously implied to cause CAKUT as well (e.g., ITGA8 and FGF20) [7, 8]. In >80% of cases, the causative gene remains to be elucidated [6, 9-12]. Genetic research in CAKUT is complicated by a distinct heterogeneity (>30 genes explain less than 20% of cases), incomplete penetrance and variable expressivity of the phenotype. Mouse models have been helpful in the past for improving the knowledge about the pathogenesis and genetics underlying CAKUT. Screenings for mutations in mouse CAKUT genes have revealed that a significant proportion can be identified in human CAKUT patients [7, 11].

We obtained blood samples, pedigree, and clinical information after receiving informed consent (http://renalgenes.org) from the respective proband with CAKUT. The diagnosis of CAKUT was made by (pediatric) nephrologists and / or urologists based on published clinical criteria. Approval for experiments on CAKUT patients [7, 11].

Here, we identify by whole-exome sequencing (WES) a recessive missense mutation in the ETS translocation variant 4 (ETV4; also known as PEA3 or E1AF) in a consanguineous patient with CAKUT. ETV4 belongs to the E26 transformation-specific (ETS) transcription factor family [13]. ETV4 has been identified to act downstream of the GDNF/RET signaling pathway, which plays essential roles in kidney development [14]. ETV4 has previously been shown to be important for migration of progenitor cells during renal branching morphogenesis [15] and is a known cause of CAKUT in mice and zebrafish [14, 16].

2. MATERIALS AND METHODS

2.1. Human Subjects

We obtained blood samples, pedigree, and clinical information after receiving informed consent (http://renalgenes.org) from the respective proband with CAKUT. The diagnosis of CAKUT was made by (pediatric) nephrologists and / or urologists based on published clinical criteria. Approval for experiments on humans was obtained from the Institutional Review Boards of Boston Children’s Hospital, and local IRBs according to the Declaration of Helsinki.

2.2. Whole-Exome Sequencing, Homozygosity Mapping and Mutation Calling

Whole-exome sequencing (WES) and a variant burden analysis were performed as described previously [17]. Briefly, genomic DNA was isolated from blood lymphocytes and subjected to exome capture using Agilent Sure Select™ human exome capture arrays (Life Technologies) followed by next generation sequencing on the HiSeq Illumina sequencing platform. Homozygosity mapping was generated from VCF files using Homozygosity Mapper [18]. Sequence reads were mapped against the human reference genome (NCBI build 37/hg19) using CLC Genomics Workbench (version 6.5.1) software (CLC bio). Variants with minor allele frequencies <1% in the dbSNP (Version 142) database were selected and annotated for impact on the encoded protein and for conservation of the reference base and amino acid among orthologs across phylogeny. Variants were filtered on the basis of an autosomal recessive mode of inheritance. Identified mutations were confirmed by Sanger sequencing of genomic DNA.

2.3. High-Throughput ETV4 Mutation Analysis

Target sequencing of all coding exons and adjacent splice sites of ETV4, as well as its 2 interaction partners (STK11 and SMAD2) [19, 20], was performed in 864 individuals from 589 different families with CAKUT from different pediatric nephrology centers worldwide using microfluidic technology (Fluidigm) with subsequent next-generation sequencing as described previously [21]. PCR primer sequences are available upon request. Variants were confirmed by Sanger sequencing, and tested for segregation with the CAKUT phenotype.

2.4. cDNA Cloning

Human full-length ETV4 cDNA was subcloned from human full-length cDNA clone (NM_001986.2, clone ID: HsCD00326736) (DF/HCC DNA Resource Core, Boston, MA) and the resultant plasmid was used for the generation of the ETV4 mutant (p.Arg415His) by Quick change II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). The sequence was verified via Sanger sequencing. The cDNAs for human wild-type and mutant ETV4 were cloned into pRK5-N-Myc expression vector using LR clonase (Invitrogen, Carlsbad, CA) following the manufacturer’s instruction.

2.5. Cell Culture

All cells used in this study were purchased from the ATCC biological resource center. HEK293T cells and U-2 OS cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM). PC3 cells were grown in Roswell Park Memorial Institute medium (RPMI-1640). Media were supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin 100 U/ml and streptomycin 100µg/ml). PC3 and U-2 OS cell lines
with shRNA-mediated stable ETV4 knock-down were selected and maintained with 4µg/ml puromycin (Life Technologies, Grand Island, NY).

2.6. Electrophoretic Mobility Shift Assay

The dsDNA probe contained the single consensus Ets-binding site (underlined), with oligonucleotides forward (5’- ATCTCACCGGAGTGTAGCA -3’) and reverse (5’- TGCTACTCCCGTGAGAT -3’). Substrates were prepared by 32P-5’-end-labeling one oligonucleotide, annealing to the complementary strand in 10mM Tris-HCl, pH 7.5, 50mM NaCl, prior to purification with Micro Bio-Spin® P-6 columns. Proteins were incubated 30min at room temperature in 50mM Tris-HCl, pH 7.5, 50mM arginine-HCl, pH 7.5, 25mM NaCl, 5% glycerol, 0.01% tween-20 with 0.02nM final 32P-labeled probe and protein concentrations ranging from 0.025 to 12nM. DNA-protein complexes were separated on 12% polyacrylamide gel with 0.5xTBE. Images were obtained and quantitated using the BioRad Personal Molecular Imaging system (Bio-Rad Laboratories, Hercules, CA) and the binding isotherm was calculated using Graph Pad Prism.

2.7. Luciferase Reporter Assays

The pGL3-5xETV4-Luc reporter plasmid (kindly provided by Andrew Sharrocks [22]), contains 5 copies of ETV4/ETS binding sites and AdML promoter, and has been described previously [23]. HEK293T cells were seeded into 24-well plates with density of 50,000 cells per well. 24h after plating, at about 75% confluence the cell were co-transfected with 300ng wild-type or mutant pRK5-N-Myc-ETV4, 200ng pGL3-5xETV4-Luc, and 40ng pCS2 Renilla luciferase (Promega, Madison, WI). Plasmid transfections for HEK293 cells were performed with Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Cells were harvested 48h after transfection. Firefly and renilla luciferase levels were assayed with the dual-luciferase reporter assay system (Promega, Madison, WI) using a GloMax microplate luminometer with dual injectors (Promega, Madison, WI). The activity of firefly luciferase was normalized to that of renilla luciferase. After normalization, the mean luciferase activities and standard deviations were plotted as “fold activation” when compared with the empty expression plasmid. All transfactions were performed in triplicate, and individual experiments were repeated in duplicate. Statistical analysis was performed with a two-tailed Student t-test using GraphPad Prism®. P < 0.05 was considered statistically significant.

2.8. Retroviral Infections and Stable Selection

ShRNA against human ETV4 was subcloned into pSIREN-RetroQ for retroviral transduction using HEK293T cells. 48 hours after transduction puromycin at a final concentration of 4µg/ml was added to the medium for selection of transduced PC3 or U-2 OS cells. Target sequence of ETV4 shRNA is GCTGCCCCTGTTGACATATAAA (Region: 3′UTR). See Figure 3a-d for knockdown efficiency.

2.9. Western Blot Analysis

For the Western blot assay, whole cell protein was extracted from PC3 cells with RIPA buffer supplemented with protease inhibitors and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL); nuclear protein was isolated extracted from U-2 OS cells using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL). Protein concentration was determined by the DC protein assay (Bio-Rad Laboratories, Hercules, CA) and equivalent amounts of protein were electrophoresed on NuPAGE™ Novex™4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA). Prestained Protein Ladder was used to determine relative molecular weights. The gels were electro blotted onto PVDF membranes using iBlot® Dry Blotting System (Invitrogen, Carlsbad, CA). After blocking with 5% defatted milk powder, membranes were incubated with the following anti-human antibodies: mouse monoclonal antibody (mAb) anti-ETV4 (clone 3G9-1B9, Abnova, Taipei, Taiwan), mAb anti-beta-actin (ab20272, Abcam, Cambridge, MA), mAb anti-PCNA (ab18390, Abcam, Cambridge, MA). Except for mAb anti-beta-actin, primary antibodies were detected with donkey anti-mouse sera conjugated to horseradish peroxidase (sc-2314, Santa Cruz Biotechnology, Dallas, TX). Chemiluminescent signals were developed using Super Signal West Pico substrate or SuperSignal West Dura substrate (Thermo Scientific, Rockford, IL). The images were acquired with ChemiDocTM MP Imaging System (Bio-Rad Laboratories, Hercules, CA).

2.10. Quantitative Polymerase-Chain Reaction

RNAs of U-2 OS and PC3 cells were isolated using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA) following the manufacturer’s instructions. RNA was subsequently converted into cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA).

Quantitative real time PCR was performed using TaqMan® reagents (Applied Biosystem, Foster City,
CA) following the manufacturer’s instructions. Expression values of human ETV4 were normalized against expression levels of human GAPDH. Results are plotted as relative mRNA levels. Error bars indicate the standard deviation of the mean. Each experiment was performed in triplicate.

2.11. Migration Assay

Real-time migration assays were performed using the Incucyte System (Essen Instruments, Ann Arbor, MI). Immortalized human osteosarcoma (U-2 OS) and prostate cancer (PC3) cells with shRNA-mediated knockdown of ETV4 were transfected with MOCK-Myc, N-myc ETV4-WT or N-myc ETV4-Arg415His. Mirus IT-LT (Mirus Bio LLC, Madison, WI) (U-2 OS) and Lipofectamine 2000 (PC3) were used as transfection reagents following the manufacturer’s instructions. 48 hours after the transfection, 30,000 cells (U-2 OS) / 35,000 cells (PC3) were seeded onto a 96-well Image Lock Plate. After 6-12 hours, 100% confluent cells were wounded using a semi-manual wound-maker tool. Cells were subsequently cultured in cell-specific media supplemented with 15% FBS. Cells with non-target control shRNA (unspecific knock-down) served as control. Serum-free media was added to a subset of wells with non-target control shRNA as negative control.

Images and measurements of the created wounds were taken every hour for up to 20 hours applying around-the-clock kinetic imaging. Changes in wound confluence were calculated using the Incucyte System (Essen Instruments). Results are plotted as wound width (%) over time (h). Each condition was performed in triplicate, experiments were repeated two times independently. Changes in wound width are presented as means with ± SD.

3. RESULTS

3.1. Identification of the Arg415His ETV4 Mutation in a CAKUT Patient

To identify additional CAKUT-causing genes, we performed WES in an individual of Kuwaiti ancestry with isolated CAKUT (Figure 1a and 1d). Homozygosity mapping revealed 173 Mb of total homozygosity indicating a close relationship of both parents, potentially to the degree of first cousins. Given the parent’s consanguinity as well as the unaffected status in regards to CAKUT, we hypothesized a recessive mode of inheritance. Among the homozygous missense variants identified (filtering strategy outlined in methods section and Table 1), five were successfully confirmed by Sanger sequencing of the patient’s DNA and were shown to be heterozygous in the maternal DNA. ETV4 was subsequently prioritized due to its known involvement in the development of the kidney and urinary tract and corresponding animal models with CAKUT [14, 15, 24].

The index patient had right-sided grade II vesicoureteral reflux (VUR) (Figure 1b) for which she required a subureteral injection of Teflon at the age of 5 years. This treatment resulted in a complete resolution of VUR as shown by a subsequent voiding cystourethrogram (Figure 1c). In the patient, we detected a homozygous missense mutation in position chr17: 41606098; c.1244G>A in exon 13 of the ETV4 gene (NM_001079675.2). This homozygous missense mutation (rs373515634) results in the amino-acid change p. Arg415His. The healthy mother was heterozygous for the p. Arg415His mutation confirming her carrier status (Figure 1e).

Arginine 415 is an ETV4 encoded residue that is well conserved across species including C. elegans and of all H. sapiens ETS paralogous proteins (Figure 1f and 1g) which is also reflected by conservation scores of 1.00 and 6.42 in phastCons and phyloP, respectively. The ETV4 mutation was predicted to be ‘disease causing’ by the Mutation Taster software, ‘deleterious’ in SIFT (see URLs) and to date has never been reported homozygously in the healthy population (in regards to CAKUT) in the Exome Variant Server, the 1 000 Genomes Project and the Exome Aggregation Consortium (see URLs).

The patients DNA was additionally screened for mutations in 36 established CAKUT causing genes including RET, PAX2, HNF1β, EYA1, SIX1, and copy number variations in HNF1β without results [6].

We performed targeted sequencing of all exons of ETV4 and its 2 interaction partners (STK11 and SMAD2) in a worldwide cohort of 864 individuals from 589 different families with CAKUT, but were not able to detect any additional CAKUT families with mutations in either of these 3 genes [19, 20].

3.2. The Arg415His ETV4 Mutant Abrogates DNA Binding Affinity of ETV4

ETV4 encodes the ETS family transcription factor Ets variant 4. It belongs to the ETS gene family, a large group of transcription factors involved in diverse aspects of embryonic development. The ETV4 protein
Figure 1: Identification of a recessive mutation in ETV4 in family B24 with CAKUT. (a) Pedigree of index family B24. Squares represent males, circles females, black symbol indicates the affected patient and white symbols the unaffected parents. Double horizontal lines denote consanguineous marriage. The black arrow points to the proband B24-21. (b) Voiding cystourethrography of patient B24-21 with reported right grade II vesicoureteral reflux (VUR). The image shows reflux into the ureter (white arrowhead). Images demonstrating a reflux into the kidney could not be obtained. (c) Follow-up voiding cystourethrography shows complete resolution after subureteral transurethral injection (STING). (d) Homozygosity mapping identifies recessive candidate loci (red peaks) in family B24 with CAKUT, and whole-exome sequencing identifies a homozygous mutation in ETV4 (positioned at blue arrowhead). (e) The exon (black and white) and protein domain (blue) structures of human ETV4 cDNA. Positions of start codon (ATG) and of stop codon (TAG) are indicated. The arrow indicates the position of ETV4 mutation in relation to exons and protein domain. Chromatograms obtained by direct sequencing of PCR products reveal a homozygous substitution of A for G in exon 13 of the ETV4 gene in the patient, and heterozygous mutation in the patient’s mother. (f) Amino acid sequence conservation among species of p.Arg415His that was altered in ETV4 of patient B24-21 with CAKUT. The arginine residue in this position is well conserved throughout evolution. (g) Amino acid residue Arg415 is highly conserved in 11 of all 12 different human ETV4 paralogs. In ETV2 it is replaced by another positively charged residue, K.
Table 1: Filtering Process for Variants from hg19 Reference Sequence Following WES in Individual from Consanguineous Descent and Isolated CAKUT

<table>
<thead>
<tr>
<th>Family</th>
<th>B24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Affected Individual Sent for WES</td>
<td>B24-21</td>
</tr>
<tr>
<td>Consanguinity</td>
<td>Yes</td>
</tr>
<tr>
<td>*# of homozygosity peaks</td>
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</tr>
<tr>
<td>Cumulative Homozygosity by descent [Mb]</td>
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</tr>
<tr>
<td>Hypothesis from mapping: homozygous (H), heterozygous (h)</td>
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</tr>
<tr>
<td>Total sequence reads (Mill.)</td>
<td>75</td>
</tr>
<tr>
<td>Matched Reads</td>
<td>73</td>
</tr>
<tr>
<td>total # of variants detected</td>
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</tr>
<tr>
<td>Reject common dbSNP142, MAF &gt;1%</td>
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</tr>
<tr>
<td>Keep VF&gt;55% AND Cov &gt;=2</td>
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<tr>
<td>Non-synonymous and Splice</td>
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<tr>
<td>Located in hom peaks</td>
<td>83</td>
</tr>
<tr>
<td>(Located within splice site)</td>
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</tr>
<tr>
<td>(Deletion/Insertion)</td>
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</tr>
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<td>(Stop gained/stop lost)</td>
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<tr>
<td>(Missenses)</td>
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</tr>
<tr>
<td>Surviving variants after inspection</td>
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<td>Sanger confirmation/Segregation</td>
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<td>Causative gene</td>
<td>ETV4</td>
</tr>
<tr>
<td>Mutation effect on gene product</td>
<td>R415H (H)</td>
</tr>
</tbody>
</table>

*aSee Figure 1.

*bEvaluation for homozygous variants was done in regions of homozygosity by descent.

WES: Whole exome sequencing; CAKUT: Congenital anomalies of the kidney and urinary tract; SNP: Single nucleotide polymorphism; MAF: Minor allele frequency.

contains an evolutionarily conserved ETS DNA-binding domain shared by all ETS family proteins. Interestingly, the amino acid residue Arg415, which is altered in our patient, resides in the ETS DNA-binding domain. The proximity of the Arg415 residue to the DNA in our molecular modeling suggests that the mutation may interfere with the DNA binding of ETV4 (Figure 2a). In addition, it has been previously shown that the charged residue Arg409 of ETV1, the equivalent of ETV4 Arg415, could be involved in DNA binding through a salt bridge [25].

Thus, we investigated the effect of the Arg415His ETV4 variant on DNA binding by electrophoretic mobility shift assay (EMSA) using a DNA probe containing a consensus ETS binding site. A shift in mobility of the labeled DNA probe was observed with 0.1nM of the wild-type ETV4 (Kd = 0.35 ± 0.04nM), whereas no shift was detected after the addition of up to 12nM of the Arg415His ETV4 mutant, suggesting that the p.Arg415His alteration fails to bind to its DNA sequence (Figure 2b).

To further test the DNA binding properties of the ETV4 mutant, we performed a dual-luciferase reporter assay in HEK293T cells. As shown in Figure 2c, the induced transcriptional activation of the Arg415His ETV4 mutation was strongly reduced when compared to the wild-type ETV4 (P < 0.0001), and did not differ significantly to that of the mock plasmid (P = 0.8527). The results therefore demonstrate an impairment of the Arg415His ETV4 mutant in activating the transcription of a reporter gene. This most likely results from the loss of the DNA-binding activity and supports a loss-of-function hypothesis for the mutant ETV4 (Figure 2b).

3.3. Wild-Type ETV4 but not Arg415His Mutant Rescues Migration Defects in ETV4 Knock-Down Cells

ETV4 has previously been linked to a wide variety of functions including the regulation of cell proliferation, cell differentiation, and cell migration in various tissues [15, 24]. In the developing kidney, ETV4 is known as a downstream target of Ret and is required for normal development of the ureteric bud and the nephric duct [14, 17, 26]. Recent studies more specifically imply a role of ETV4 in ureteric bud cell movement, and promotion of ureteric bud growth and branching.
Deficiencies in either of these processes constitute known causes of CAKUT. We therefore performed migration assays to assess a possible impact of the Arg415His ETV4 variant on cell migration. Human PC3 and U-2 OS cells were chosen here, because cell lines generated from the human embryonic urinary system (e.g., HEK293T) were not considered suitable given the experimental setup, whereas these two cell lines have successfully been shown to exhibit ETV4 dependent cell migration rates in the literature and were suitable for the scratch-wound migration assay [26].

The envisaged experimental setup of initial knock-down and subsequent visualization of a rescue vs. non-rescue effect via transfection of the wild type vs. mutant ETV4 construct, respectively, made a stable ETV4 knock-down cell-line preferable. Stable knock-down cells appeared to tolerate the following transfection of constructs better, with less impact on cell viability, and therefore had a more reliable and reproducible performance in the migration assay. However, this setup made the use of primary cell lines (e.g., ureteric bud cells) less feasible.
Figure 3: *ETV4* mutant p.Arg415His fails to rescue cell migration rate reduced by *ETV4* knock-down. All experiments were performed in the Incucyte system in two different cell lines, PC3 cells (up lay, a, b, e, g) and U-2 OS cells (down lay, c, d, f, h). (a-d) Efficient knock down of *ETV4* mRNA level, (a, c) and protein level, (b, d) in PC3 (a, b) and U-2 OS (c, d) cell lines, respectively. Cells were stably transduced with retroviral shRNA expression vectors encoding either a non-target control (scrambled) or *ETV4* shRNA. *ETV4* mRNA level (normalized to housekeeping gene *GAPDH*) was tested by quantitative real-time (qRT)-PCR. *ETV4*, β-actin, and PCNA protein levels were analyzed by Western blot. (e and f) Confluent monolayers were wounded using a semi-manual wound maker tool. Migration of cells was quantified based on images that were taken every hour for up to 20 hours. In PC3 (e) and U-2 OS (f) cells, knockdown of *ETV4* by using *ETV4* shRNA reduces cell migration (yellow versus black). Base line migration rate (dashed lines) is strongly increased upon addition of serum (solid black lines). The decrease in cell migration is partially rescued by transfection with full-length human *ETV4* cDNA (green lines), but not rescued by transfection with *ETV4* Arg415His mutant vector (red lines) or mock transfection (yellow lines). (g and h) Representative images are shown in PC3 cells (g) and U-2 OS cells (h).

An efficient knock-down of *ETV4* on both the mRNA and protein level was obtained via retroviral infection of the target cell lines with an shRNA expression construct (Figure 3a-d). Human PC3 and U-2 OS cells
with stable depletion of endogenous human ETV4 demonstrated a slower migration rate in comparison to non-target control cells (Figure 3e-h). This effect was rescued by a transfection with human N-myc ETV4-WT. However, stable ETV4 knockout cells transfected with cDNA construct N-myc-ETV4-Arg415His, that was present homozygously in patient B24-21 with CAKUT, remained to exhibit migratory deficiencies (Figure 3e-h).

This loss-of-function effect in the migration assay together with the insights gained from the EMSA and the luciferase assay give reason to speculate that the ETV4 Arg415His mutation eliminates the expression of effector genes that participate in cell movement. Candidate genes include the chemokine receptor Cxcr4 and the matrix metalloproteinase Mmp14 [14]. Furthermore, no extra-renal phenotypes, such as nervous system or limb malformations were seen, which have been described in ETV4 null mice, and most ETV4; ETV5 compound mutant mice [14]. Furthermore, no extra-renal phenotypes, such as nervous system or limb malformations were seen, which have been described in ETV4 [27] or ETV4 [28]; ETV5 compound knock-out mice, respectively. This discrepancy may be explained by a redundancy of ETV4 with ETV5 or other ETS proteins and/or alternative mechanisms of ETV4 that work independently of the DNA-binding property [26, 29].

However, in view of the convincing in vivo data in the literature and the functional data presented here we hypothesize that the recessive mutation identified in ETV4 causes the CAKUT phenotype seen in our patient, the most likely mechanism here by being an impairment of the GDNF/RET/ETV4 signaling pathway.

4. DISCUSSION

In this study, we identified a homozygous missense mutation in ETV4 in a consanguineous individual with isolated CAKUT. The discovered variant causes an amino acid substitution in the highly conserved Ets DNA-binding domain and has never been reported homozygously in population databases. At the biochemical level we demonstrate that the ETV4 mutant lost its DNA binding property. It furthermore exhibits a loss-of-function effect in rescuing migration defects in two ETV4 knock-down cell lines. Hence, we hypothesize that the detected homozygous missense variant in the ETV4 gene causes the CAKUT phenotype in the patient of the present study.

The phenotype of the patient with the recessive ETV4 missense mutation resembles the murine CAKUT phenotype, although much milder when compared to the severe renal phenotypes seen in a proportion of ETV4 null mice, and most ETV4; ETV5 compound mutant mice [14]. Furthermore, no extra-renal phenotypes, such as nervous system or limb malformations were seen, which have been described in ETV4 [27] or ETV4 [28]; ETV5 compound knock-out mice, respectively. This discrepancy may be explained by a redundancy of ETV4 with ETV5 or other ETS proteins and/or alternative mechanisms of ETV4 that work independently of the DNA-binding property [26, 29].

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**AUTHOR CONTRIBUTION**

#These authors contributed equally to this work.

**REFERENCES**


[28] Zhang Z, Verheyden JM, Hassell JA and Sun X. FGF-regulated Etv genes are essential for repressing Shh
https://doi.org/10.1016/j.devcel.2009.02.008

https://doi.org/10.1146/annurev.biochem.79.081507.103945