



# DCHSI DNA copy number loss associated with pediatric urinary tract infection risk

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## Abstract

Urinary tract infections (UTI), associated with vesicoureteral reflux (VUR), can lead to chronic kidney disease. Genetic alterations in the innate immune defenses contribute to UTI risk. We investigated a novel gene, Dachshous Cadherin-Related 1 (*DCHSI*), in children with UTI. We determined absolute DNA copy number (CN) of *DCHSI* in children with UTI. In this case-control study, we utilized multiple complementary methods to determine the genomic CN of *DCHSI*. Children with ( $n = 370$ ) and without ( $n = 71$ ) VUR from two well-phenotyped clinical trials of UTI were copy-typed and compared to 491 healthy controls with no known history of VUR or UTI. Less than 1% of controls had a single copy of *DCHSI*, while 31% of children with UTI and no VUR and 7% of children with UTI and VUR had a single copy of the *DCHSI* gene. Using immunostaining, we localized expression postnatally to the bladder and renal epithelia. Mice were also challenged with two uropathogenic *Escherichia coli* strains, and *Dchs1* mRNA was quantified. This study represents the first report of *DCHSI* in association with pediatric UTI. We hypothesize that its role in innate immunity is critical to lower urinary tract defense. Further investigation is required to determine the role of *DCHSI* in innate immunity.

## Keywords

DNA CN variations, pediatrics, urinary tract infections, vesicoureteral reflux

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## Introduction

Urinary tract infection (UTI) is one of the most common childhood infections and is caused by uropathogenic *Escherichia coli* (UPEC) in 80% of cases.<sup>1</sup> Acute pyelonephritis has variable sequelae with or without renal scars, depending on the virulence of the organism and host defense mechanisms.<sup>2,3</sup> Children with congenital anomalies of the kidney and urinary tract, such as vesicoureteral reflux (VUR), are at increased risk for UTI-associated sequelae such as chronic kidney disease and renal scarring.<sup>4</sup>

The innate immune system plays a critical role in protecting the urinary tract from uropathogens.<sup>5,6</sup> Innate immune responses are rapid and encompass a variety of components, including epithelial extracellular barriers and intracellular tight junctions, antimicrobial peptides, PRRs, inflammatory mediators, and phagocytes.<sup>6–8</sup> PRRs are protein receptors on the

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surface of phagocytes, dendrites, and epithelial cells, which recognize microbial molecules and are capable of initiating immune responses called PAMPs.<sup>9</sup>

A number of studies implicate a heritable component to UTI susceptibility.<sup>10,11</sup> A potential mechanism for UTI heritability is genetic alterations in innate immune defenses.<sup>12</sup> For example, DNA copy number (CN) variations (CNVs) in the gene that encodes  $\alpha$ -defensin 1 have been shown to be associated with variable susceptibility to UTIs and response to antibiotic prophylaxis.<sup>13</sup> CNVs occur when DNA sections between a few hundred and several million bp are deleted or duplicated and influence disease by a range of mechanisms, including gene dosage.<sup>14,15</sup> We performed high-resolution genome-wide array comparative genomic hybridization (aCGH) comparing children with VUR and UTI to healthy controls. We identified a relative CNV in the gene *Dachsous Cadherin-Related 1 (DCHSI)* that segregated to patients with VUR.<sup>16</sup> A limitation of aCGH is that it only determines if DNA CN is relatively increased or decreased compared to a reference sample and does not define the absolute number of gene copies that are present.<sup>13,16</sup> Thus, validation methods to determine true CNs are needed to rule out false-positives.<sup>13</sup>

*DCHSI* encodes for a calcium-dependent adhesion molecule, which has a role in kidney development and planar cell polarity.<sup>16,18</sup> The gene has not been previously investigated in the context of UTI. The purposes of this study were to determine absolute *DCHSI* CN in two pediatric clinical trial cohorts of UTI and to investigate its postnatal role in innate immune defenses.

## Methods

### Safety approval of human subjects

Approval of human subjects was obtained through the University of Tennessee Health Science Center Institutional Review Board Protocol 14-03325-XP. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki.

### Cohorts

We utilized genetic samples from three clinical cohorts of children and adults with and without a history of UTI. All genomic DNA samples were obtained from immortalized lymphoblastic cell lines. Each is described below.

### Cohort of subjects with VUR and UTI

The Randomized Intervention for Children with Vesicoureteral Reflux (RIVUR) Clinical Trial

(ClinicalTrials.gov Identifier NCT00405704) enrolled patients aged 2–71 mo with one to two UTI who were diagnosed with grade 1–4 VUR.<sup>19</sup> Patients were randomized to receive antibiotic prophylaxis or placebo and were followed for 2 yr for recurrent UTI. UTI was defined as a positive culture with a single organism of 50,000 CFU/ml on a catheter specimen or 100,000 CFU/ml on a clean catch specimen with evidence of pyuria on the urinalysis, as per guidelines by American Academy of Pediatrics (AAP) at the time of collection.<sup>20</sup> We utilized DNA obtained from 320 non-Hispanic Caucasian girls from the RIVUR study.

### Cohort of subjects with UTI without evidence of VUR

The Careful Urinary Tract Infection Evaluation (CUTIE) Study observed 2–71 mo old pediatric patients who had one to two UTI without VUR for a 2 yr period.<sup>21</sup> The CUTIE Study was concomitant with the RIVUR study. We utilized 71 DNA samples from non-Hispanic Caucasian girls enrolled in the CUTIE Study.

### Control cohort without VUR and UTI

We utilized 491 DNA samples of healthy, non-Hispanic Caucasian adult women with no known history of VUR and UTI.

### Mice

*In vivo* murine studies were approved by Indiana University Institutional Animal Care and Use Committee (IACUC) under protocol # 11333. Wild-type C57BL/6J female mice (Jackson Laboratory, Bar Harbor, ME) were used for induction of pyelonephritis and cystitis in infection studies.

### Absolute CN determination

Absolute CNs of *DCHSI* were determined by digital PCR (dPCR) and were confirmed with Short Multiplex Aggregated Sequence Homologies (SMASH) methods for seven reference individuals who were used on each plate as calibrators for relative quantitation for quantitative RT-PCR (qRT-PCR).

### dPCR

To determine absolute *DCHSI* CN, quantitative dPCR was performed on seven reference individuals at the Molecular Resource Center at the University of Tennessee Health Science Center on a Fluidigm BioMark System (San Francisco, CA). For complete methodology, please refer to the manufacturer's protocol (Fluidigm PN 100-6896 B1). Briefly, we used dPCR 37 K integrated fluidic circuits (IFC). After filling IFC

with standard control line fluid, we primed the IFC in Fluidigm MX. A pre-mix sample mixture, which included TaqMan gene expression master mix (Applied Biosystems, Foster City, CA), 20× GE Sample Loading Reagent, and TaqMan copy-typing primer/probe mixtures (Applied Biosystems), was used.

Specifically, *RNASEP* VIC-labeled probes were used as an internal reference for diploid genes and *DCHS1*-FAM labeled probes for our target gene (Applied Biosystems). We added 1.8 µl sample DNA (20 ng/µl) to 4.2 µl above mixture in each pre-mix well. After priming, IFC 37 K was loaded with 4.0 µl from each sample. Each sample was genotyped in triplicate. The data were analyzed with Fluidigm Digital PCR Analysis Software v4.1.2.

### SMASH

We performed a next-generation sequencing-based method for CNV analysis by SMASH, as previously described.<sup>22</sup>

### qRT-PCR

Multiplex qRT-PCR was performed on all subjects and controls to determine the relative CN of the *DCHS1* gene, as previously described.<sup>13</sup> Briefly, individuals with known CNs from dPCR and SMASH sequencing served as calibrators to determine CN for unknown samples using a standard curve for  $\Delta\Delta Ct$ . TaqMan copy-typing primer/probe mixtures (Applied Biosystems) were used. *RNASEP*-VIC-labeled probes were used as an internal reference for diploid gene, and *DCHS1*-FAM labeled probes for the target gene. Each sample was performed in triplicate. Experiments were performed on a Light Cycler 480 (Roche, Basel, Switzerland) Real-Time PCR System. Primers/probe sequences were as follows: *DCHS1\_F* 5'-CGG TAA GGC AGG TTG GTG-3'; Probe FAM-AGGTGCTGATGTGGAGTGTGGGG-MB G; and *DCHS1\_R* 5'-AGT TAG GTC TGG GAG TGA GG-3'.

The comparative cycle threshold (Ct) difference method was used to determine the CN for the *DCHS1* gene for each patient.<sup>23</sup> This was done using LCS480 v1.5.1.62 software and Microsoft Excel 2010. The method measured the  $\Delta Ct$  by subtracting the average Ct of *DCHS1* as the target and the average Ct *RNASEP* as the reference and then calculating the  $\Delta\Delta Ct$  value by comparing the average  $\Delta Ct$  values of samples with the average  $\Delta Ct$  of the calibrator sample known to have two copies of the target sequence.<sup>24</sup> Known calibrators with two CNs of *DCHS1* gene per diploid genome as determined by dPCR and SMASH were included on each plate to create a standard curve

for CN in unknown. The amount of target normalized to endogenous reference and relative to calibrator is calculated by  $2^{-\Delta\Delta Ct}$ .

### Immunofluorescence staining

Paraffin embedded human kidney and bladder sections (7 µm thick) were stained with polyclonal rabbit anti-DCHS1 Ab (cat. no. Ab203690; Abcam, Cambridge, MA) to locate DCSH1-positive cells in the kidney and bladder and V-ATPase E1 (cat. no. GW22284F; Sigma-Aldrich, St. Louis, MO) in conjunction with DCHS1 to locate intercalated cells positive for DCHS1 in the kidney. Human kidney samples with and without pyelonephritis were obtained from the Cooperative Human Tissue Network (Westerville, OH), as previously described.<sup>25</sup> DCHS1 primary Ab was added at a 1:50 dilution, and V-ATPase E1 was added at 1:300 dilution and incubated overnight at 4°C. To visualize, DCHS1 and anti-Rabbit Cy3 (1:600 final dilution) secondary Ab and to locate intercalated cells, anti-Chicken AF488 (1:600 final dilution) was used (Jackson ImmunoResearch, West Grove, PA) for 30 min at 20°C. Slides were mounted using DAPI containing a hard-set solution to visualize nuclear staining (Vector Laboratories, Burlingame, CA). Sections were visualized using a Keyence BZ9000 microscope (Keyence Corporation, Osaka, Japan).

### Murine bacterial challenge experiments

To determine the mRNA in mouse models with UTI compared to control, *E. coli* pyelonephritis strain (UPEC; CFT073) and cystitis strain (UPEC; UTI89) were grown overnight on Luria-Bertani (LB) media at 37°C in a 20xg shaker and static incubator, respectively. OD<sub>600</sub> measurements were recorded using the Eppendorf Biophotometer Spectrophotometer UV/Vis Reader (Eppendorf North America, Hauppauge, NY) to adjust the bacterial inoculum concentration to  $1 \times 10^8$  CFU/ml per mouse. Bacterial cultures were made into two 1 ml aliquots and centrifuged at 5,500g for 1 min. Supernatant was discarded without disturbing the pellet. Sterile PBS (1 ml) was then added to each tube. Tubes were vortexed several times in order to suspend the bacteria pellet in sterile PBS. The goal optical density of the bacterial solution was 0.073 OD<sub>600</sub>, which correlated to a concentration of  $1 \times 10^8$  CFU/ml. Specifically, a mixture of 800 µl sterile PBS and 20 µl bacterial solution were combined and placed in a cuvette to be measured in the OD<sub>600</sub> reader. From this baseline measurement, the bacterial/PBS suspension was added from the stock in 20 µl increments until an OD<sub>600</sub> of 0.073 was reached. The mice were anesthetized with 2.5% isoflurane and then placed

in the supine position. Bladders were palpated, and urine was expelled before transurethral inoculation was performed. Then, a sterile 1 cc syringe (Becton Dickinson, Franklin Lakes, NJ) with lubricated polyethylene-coated needle (Becton Dickinson, Franklin Lakes, NJ) was used to administer 50  $\mu$ l of bacterial suspension transurethrally. The same volume amount of sterile PBS was inoculated transurethrally as a negative control. Mice were euthanized 6 h after inoculation of bacteria by CO<sub>2</sub> inhalation for organ harvesting.

### mRNA extraction and quantification

For quantifying the mRNA in mice, murine kidneys and bladders were placed into zirconium bead/PBS vials for homogenization in the Bullet Blender Storm 24 (Next Advance, Troy, NY) for 10 min at maximum speed. Following tissue homogenization, mRNA was extracted using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA) and reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as per the manufacturer's protocol. qRT-PCR was performed using gene expression *Dchs1* primers (Sigma–Aldrich) and *Gapdh* (Real Time Primers, LLC, Elkins Park, PA) as the housekeeping gene primer. The forward and reverse primer sequences for *Dchs1* were 5'-CTATACACCCTAACTGGTCC-3' and 5'-AGAGTTAGTACATAGTGAGGC-3', respectively. QuantiFast SYBR Green real-time PCR Mastermix (Qiagen) was used for qRT-PCR assays on the CFX Connect 96 well real-time system (Bio-Rad Laboratories, Hercules, CA). *Dchs1* mRNA expression was quantified using the  $2^{-\Delta\Delta C_t}$  method and normalized values to the gene expression of *Gapdh*.

### Statistical analyses

In genetics, the results were analyzed using SAS v9.4 (SAS Institute, Cary, NC). Categorical variables were compared using the chi-square test or Fisher's exact test, depending upon the variable number in the box. The level of significance was defined as a *P* value of <0.05. In mRNA comparisons, normalized *Dchs1* mRNA expression data are presented as the mean  $\pm$  SD. Statistical analysis by unpaired two-tailed *t*-test was used to compare the response between UPEC to saline control groups and plotted in GraphPad

Prism 8 (GraphPad Software, La Jolla, CA). A *P* value of <0.05 was considered statistically significant.

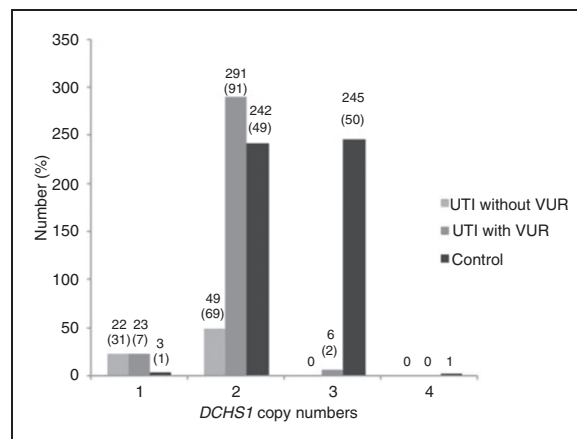
## Results

### Subject and control cohort characteristics

We determined *DCHS1* gene CN per diploid genome using qRT-PCR in RIVUR (UTI and VUR; *n* = 320) subjects, CUTIE (UTI and no VUR; *n* = 71) subjects and controls (no UTI and no VUR; *n* = 491). All cohorts comprised non-Hispanic Caucasian females. The clinical characteristics of the groups are given in Table 1.

### Children with VUR and UTI had decreased CN of *DCHS1* compared to controls

*DCHS1* CN in children with RIVUR varied from 1 to 3, with a mean CN of  $1.95 \pm 0.30$ . *DCHS1* CN for controls ranged between 1 and 4, with a mean CN of  $2.49 \pm 0.39$ . The distribution of CNs is shown in Figure 1. More RIVUR subjects (*n* = 23; 7%) had one copy of *DCHS1* compared to controls (*n* = 3; 0.6%). In the RIVUR cohort, one copy individuals were 13 times more prevalent (odds ratio (OR) = 12.59; 95% confidence interval (CI) 3.75–42.32;

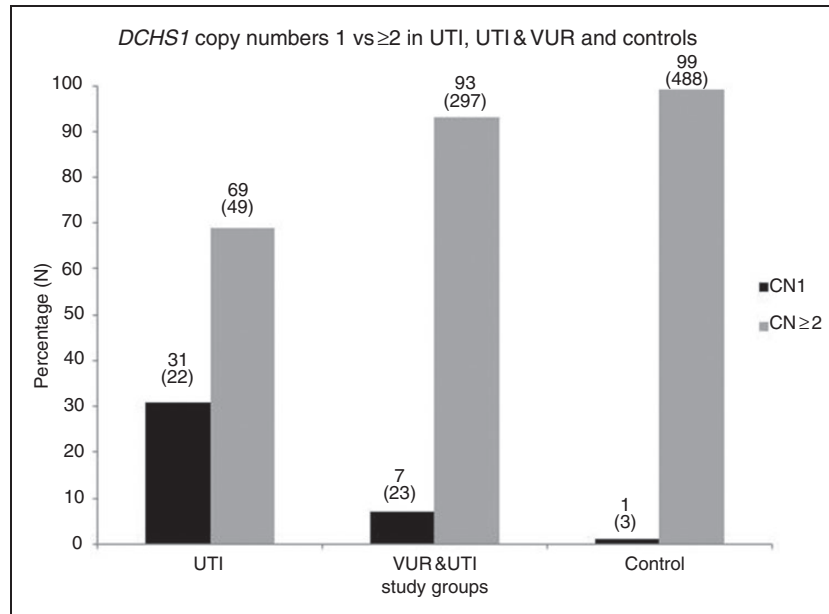


**Figure 1.** *DCHS1* DNA copy number (CN) in subjects with UTI without vesicoureteral reflux (VUR), UTI with VUR, and controls. *DCHS1* CN distribution and percentages in subjects with UTI without VUR (*n* = 71), UTI with VUR (*n* = 320), and controls (*n* = 491).

**Table 1.** Clinical characteristics of subjects and controls.

	RIVUR, <i>n</i> = 20	CUTIE, <i>n</i> = 71	Control, <i>n</i> = 491
Disease profile	UTI + VUR	UTI	No UTI and no VUR
Mean age, mo (range)	22.7 (2–71)	22.7 (2–71)	>18 yr
Total subject samples, <i>n</i>	320	71	491





**Figure 2.** *DCHS1* DNA CNs 1 vs.  $\geq 2$  in subjects with UTI without VUR, UTI with VUR, and controls. Percentage and distribution of 1 CN vs.  $\geq 2$  CN of *DCHS1* in subjects with UTI without VUR ( $n = 71$ ), UTI with VUR ( $n = 320$ ), and controls ( $n = 491$ ). CN = DNA CN of *DCHS1*.

$P < 0.0001$ ). Distribution of 1 versus  $\geq 2$  CNs in groups is given in Figure 2.

### Children with UTI and without VUR had a higher incidence of low DNA CN of *DCHS1* compared to controls, as well as children with VUR

The *DCHS1* CN in children with UTI and no VUR (CUTIE) varied from 1 to 2, with a mean CN of  $1.69 \pm 0.47$ . A statistically significant number of children with UTI ( $n = 22$ ; 31%) had one copy of *DCHS1* compared to controls ( $n = 3$ ; 0.6%). Individuals with one copy of *DCHS1* compared to all other genotypes were 73 times more prevalent in the CUTIE cohort compared to controls (OR = 73.03; 95% CI 21.10–252.76;  $P < 0.0001$ ). When comparing the two UTI cohorts, the CUTIE cohort (UTI and no VUR) had a statistically significant larger percentage of children with one copy of *DCHS1* compared with RIVUR (UTI and VUR; 31% vs. 7%; OR = 5.8 95% CI 3.00–11.19;  $P < 0.0001$ ).

### *DCHS1* protein is expressed in bladder epithelial and renal intercalated cells

*DCHS1*'s expression and location in postnatal kidney and bladder tissue has not been previously described. Because we discovered a significant genetic variation in *DCHS1* in patients with UTIs, we aimed to localize *DCHS1* protein expression using human tissue. In the

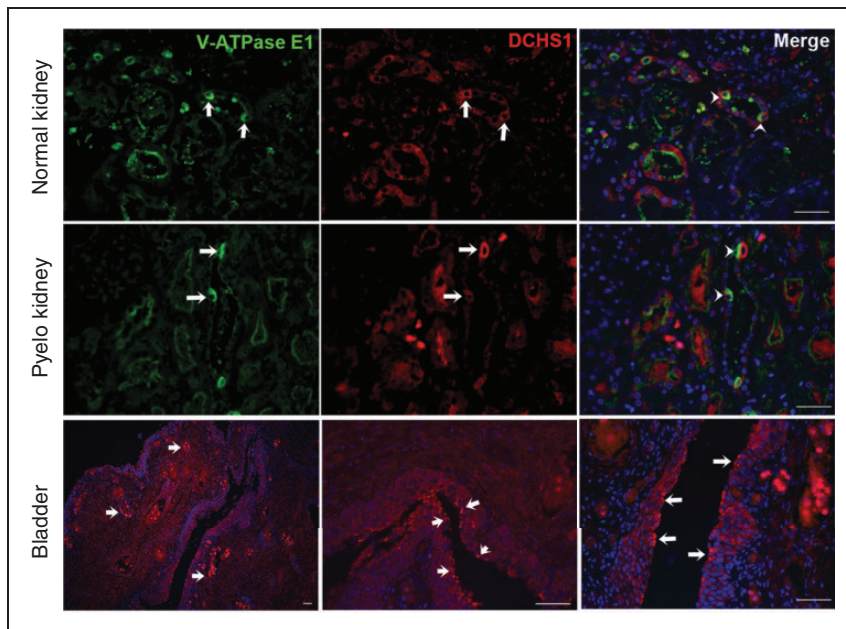
normal human kidney and the kidney from a patient with pyelonephritis, we demonstrated *DCHS1* was expressed by intercalated cells of renal-collecting duct epithelia. As many of the innate immune proteins are expressed in the kidney, *DCHS1* localization in intercalated cells highlights its likely role in innate immunity. In the bladder, we demonstrated strong immunostaining in the bladder epithelia. All tissue immunostaining is demonstrated in Figure 3.

### *Dchs1* expression does not significantly increase in response to murine uropathogen challenge

Following a UPEC challenge in mice ( $n = 15$  UTI89,  $n = 16$  CFT073), we quantified the *Dchs1* mRNA amount in both the bladder and kidney (Figure 4). The cystitis UPEC strain (UTI89) produced a larger increase in *Dchs1* mRNA than the pyelonephritis UPEC strain did (CFT073). Compared to saline controls, neither kidney nor bladder *Dchs1* mRNA transcripts were significantly increased. The bladder demonstrated the largest increase in UTI89-challenged mice but missed significance ( $P = 0.11$ ).

## Discussion

This study used well-characterized pediatric cohorts from the previously published RIVUR and CUTIE studies.<sup>19,21</sup> In a genome-wide study focused on a subset of UTI patients, our research group identified



**Figure 3.** Immunolocalization of *DCHS1* in human kidney (upper and middle panel) and bladder (lower panel). Upper-left panel: kidney section showing V-ATPase E1-positive intercalated cell in collecting duct (green, white arrow); middle panel: *DCHS1*-positive cells (red, white arrow); and right-panel: merged image (white arrowheads) showing *DCHS1*-positive intercalated cells. Lower panel: bladder staining for *DCHS1* indicated by arrows; left panel: 10 $\times$  magnification showing islands of *DCHS1* positive infiltrating cells; middle and right panel: 40 $\times$  magnification, showing *DCHS1*-positive bladder urothelium. Blue nucleus staining DAPI, scale bar 50  $\mu$ m.

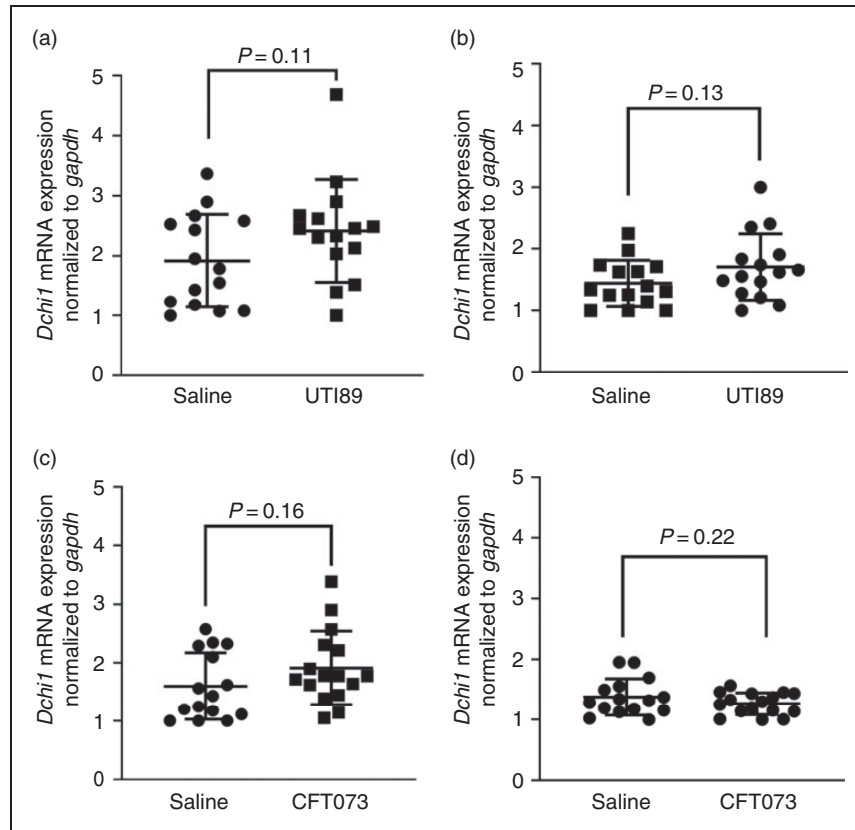
a novel association of *DCHS1* relative gene CNV with UTI, which has not been reported previously.<sup>16</sup> In this report, we determined the absolute DNA CN of *DCHS1* in two clinical cohorts of pediatric UTI to expand on our genome-wide screening study. We found that individuals with low *DCHS1* CN (one copy) were 73 times more prevalent in the CUTIE (UTI without VUR) cohort compared to controls. In the RIVUR cohort (UTI with VUR), one copy individuals were 13 times more prevalent. Even in the absence of VUR, children with UTIs can develop renal scarring, thus showing that UTI-associated genes are important for children at risk.<sup>21</sup>

*DCHS1* encodes for atypical cadherin, which belongs to the cadherin superfamily and is a calcium-dependent cell–cell adhesion molecule. The encoded protein has 27 extracellular cadherin repeat domains, transmembrane domain, and a unique cytoplasmic region.<sup>26</sup> The *Dchs1* gene was found to have role in kidney development in a murine transgenic model.<sup>17</sup> *Dchs1* also has been shown to have a role in cell adhesion, growth, tissue patterning, and planar cell polarity.<sup>18</sup> To the best of our knowledge, the role of *DCHS1* in the postnatal kidney has not been investigated.

This report serves as the first study to demonstrate the association of *DCHS1* gene loss in patients with pediatric UTIs. Our immunostaining demonstrates a potential postnatal role of *DCHS1* in the bladder and

intercalated cells of the kidneys. Many key components of the innate immune system demonstrate a similar expression pattern. Thus, this finding supports the notion that it is critical in innate defenses.<sup>25,27</sup> Because *Dchs1* expression did not significantly increase following UPEC challenge, this gene is likely important in basal defenses and not inducible. Conversely, UPEC may subvert the epithelial response to invasion, although our experiments missed this possible role. Additional murine experiments are needed to dissect the pathways up- and downstream of *DCHS1*. Additionally, transgenic murine studies with bladder and renal collecting duct deletion will help to determine which tissue is key in innate defenses and if intercalated cell dysfunction exists with gene loss.

We can only hypothesize the precise mechanisms of UTI susceptibility with a single copy of *DCHS1*, and further studies are needed. Because *DCHS1* is expressed on the apical surface of epithelia, *DCHS1* may have a role in pattern recognition and/or inflammatory cascade signaling. Our research group and others have demonstrated the role of intercalated cells in the innate defense of the kidney and urinary tract. Because cell polarity is critical in epithelial cells such as intercalated cells of the collecting duct, we postulate that *DCHS1* single-copy individuals may have subtle intercalated cell innate immune dysfunction leading to UTI susceptibility.<sup>28</sup>



**Figure 4.** *Dchs1* mRNA expression of murine bladder and kidneys in response to different uropathogenic *E. coli* strains. A and B graphs correspond to mRNA upregulation after 6 h on mouse bladder and kidneys infected with UTI89 compared to saline controls ( $n = 15$  UTI89 and  $n = 15$  saline). C and D graphs show mRNA transcripts of mouse bladder and kidneys upon inoculation with CFT073 compared to controls ( $n = 16$  CFT073 and  $n = 15$  saline), respectively. Long horizontal bars represent the mean, with upper and lower smaller horizontal bars representing  $\pm 1$  standard deviation.

Because we observed the highest percentage of subjects in the UTI cohort without VUR (CUTIE Study cohort) with a single copy of *DCHS1*, we postulate that *DCHS1* CNV is more relevant to infection and immunity than abnormal development of the kidney and urinary tract. Additionally, because genetic variations in *DCHS1* were more prevalent in those with normal urinary tracts and an intact anti-reflux anatomy, the *DCHS1* gene is likely more relevant to bladder as opposed to kidney innate immunity. The gene product may also prevent attachment in the bladder and/or urethra. Thus, in the context of VUR, bacteria may be able to ascend the urinary tract to the kidneys quickly, and the effect of DNA CNVs is blunted if the primary site of action postnatally is the bladder. If this speculation is true, the association of DNA CNVs in patients without VUR would be more consistent with the results of this study.

We do acknowledge some limitations in this study. First, our study was limited to Caucasian girls. So, results may not apply to other cohorts. Further investigations are needed on diverse cohorts to confirm these

findings are consistent across study populations. Additionally, we utilized an adult cohort of women for controls. We utilized this cohort for two reasons. First, large control cohorts of children with no history of UTI are not publicly available. Second, an adult control cohort with no history of UTI should actually have a higher risk of UTI than a pediatric cohort, given the high incidence of UTI in adult women compared to children. The impact of this study may need to be tempered until we can perform additional studies to discover the role of this gene in the postnatal kidney and urinary tract.

## Conclusion

This study represents the first report of *DCHS1* in association with UTI. We speculate that the site of biological action may be in the lower urinary tract and “bypassed” to a certain extent in individuals with VUR. Thus, *DCHS1* genetic variations associate with pediatric UTI risk, but the effect of these variations is masked by VUR. *DCHS1* can be added to the growing

body of disease-modifying genes such as *DEFA1A3*, in conjunction with patient characteristics such as VUR grade and bowel/bladder dysfunction to start creating UTI risk panels for patients at risk for UTI sequelae.<sup>13</sup> Further investigation is required to determine the role of *DCHS1* and innate immune functions of the kidney and urinary tract.

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### Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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