Polymorphisms in \( \alpha \)-Defensin–Encoding DEFA1A3 Associate with Urinary Tract Infection Risk in Children with Vescoureteral Reflux

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ABSTRACT

The contribution of genetic variation to urinary tract infection (UTI) risk in children with vesicoureteral reflux is largely unknown. The innate immune system, which includes antimicrobial peptides, such as the \( \alpha \)-defensins, encoded by DEFA1A3, is important in preventing UTIs but has not been investigated in the vesicoureteral reflux population. We used quantitative real-time PCR to determine DEFA1A3 DNA copy numbers in 298 individuals with confirmed UTIs and vesicoureteral reflux from the Randomized Intervention for Children with Vescoureteral Reflux (RIVUR) Study and 295 controls, and we correlated copy numbers with outcomes. Outcomes studied included reflux grade, UTIs during the study on placebo or antibiotics, bowel and bladder dysfunction, and renal scarring. Overall, 29% of patients and 16% of controls had less than or equal to five copies of DEFA1A3 (odds ratio, 2.09; 95% confidence interval, 1.40 to 3.11; \( P < 0.001 \)). For each additional copy of DEFA1A3, the odds of recurrent UTI in patients receiving antibiotic prophylaxis decreased by 47% when adjusting for vesicoureteral reflux grade and bowel and bladder dysfunction. In patients receiving placebo, DEFA1A3 copy number did not associate with risk of recurrent UTI. Notably, we found that DEFA1A3 is expressed in renal epithelium and not restricted to myeloid-derived cells, such as neutrophils. In conclusion, low DEFA1A3 copy number associated with recurrent UTIs in subjects in the RIVUR Study randomized to prophylactic antibiotics, providing evidence that copy number polymorphisms in an antimicrobial peptide associate with UTI risk.


Vesicoureteral reflux (VUR), which is present in 1%–2% of children, is a risk factor for the development of urinary tract infections (UTIs). The Randomized Intervention for Children with Vescoureteral Reflux (RIVUR) Trial recently randomized children with a history of UTI and VUR to daily antibiotic prophylaxis or placebo and followed them for 2 years to monitor for UTI recurrence risk. Additional details

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about the RIVUR Study design and outcomes have been previously reported as well as our methods. The RIVUR Study showed that 25.4% of children with VUR treated with placebo experience UTI recurrence over a 2-year period, whereas antibiotic prophylaxis reduces the UTI recurrence risk to 13%. These recurrent UTIs can lead to renal scarring, hypertension, and CKD. However, the broad application of antibiotic prophylaxis to all patients with VUR remains controversial, because only 30% of children with VUR will experience an initial UTI. Therefore, strategies are needed to identify a subset of patients with VUR who will experience UTIs and benefit from antibiotic prophylaxis.

The innate immune system is largely responsible for the body’s defense against UTIs. Innate immune effectors include antimicrobial peptides (AMPs) that rapidly destroy microbes and chemokines that result in inflammatory cell migration to the affected site and the inflammatory cells. AMPs, such as defensins, have a broad range of antimicrobial activity against a wide variety of microbes, including Gram-negative and -positive bacteria. Defensins are categorized into two families, the α-defensins and the β-defensins, depending on their disulfide-bridging pattern. B-Defensins are widely expressed by epithelial cells, whereas α-defensins are expressed by neutrophils or in the case of α-defensin-5 (DEFA5), the epithelia of the gastrointestinal tract, the genitourinary tract in girls, and the urinary tract.

Neutrophil-associated DEFA1 differs from an adjacent gene, DEFA3, by a single nucleotide. This gene locus has collectively been renamed DEFA1A3, and it encodes the protein Human Neutrophil Peptide 1–3 (HNPI–3). Historically, myeloid-derived cells were the only cells thought to produce HNPI–3. HNPI–3 possesses broad spectrum antimicrobial activity against a range of bacteria, including Escherichia coli, and urine levels of HNPI–3 increase eightfold with pyelonephritis versus controls. However, DEFA1A3/HNPI–3 has not been extensively evaluated in the kidney.

Genetic variations in humans range from single-nucleotide polymorphisms and point mutations occurring in genes, to larger changes to large microscopically visible chromosome defects. Single nucleotide polymorphisms (SNPs) are single nucleotide changes to large microscopically visible chromosome defects. Single nucleotide polymorphisms and point mutations occur more frequently, CNVs are more likely to be important components of genomic diversity and phenotypic variation by their ability to generate significant gene dosage effects.

CNVs that occur in >1% of the population have been termed copy number polymorphisms (CNPs). CNPs may represent excellent candidates for disease risk modifiers. For instance, DEFA1A3 CNPs have been associated with various inflammation and autoimmune diseases, including sepsis, Crohn disease, and Behcet disease. We hypothesize that deficiencies in the host innate immune defense, such as low DEFA1A3 DNA copy numbers, contribute to UTI risk in patients with VUR. The objectives of this study included (1) comparison of DEFA1A3 CNP in children from the RIVUR Trial who had a history of VUR and one or two UTIs with healthy controls and (2) determination of the biologic relevance of DEFA1A3 expression in the kidney.

RESULTS

Patient Cohorts Analyzed Were Matched for Sex and Ethnic/Racial Category

DNA was obtained on 298 white girl patients in the RIVUR Trial. Two hundred ninety-five white girl controls with no history of UTI or VUR were enrolled between 2010 and 2014. The mean age of the patients in the RIVUR Trial was 22.7 months (range =2–71) at the start of the 2-year study. The mean age of the control patients was 129.8 months (range =3.5–215). The patients in the RIVUR Trial were matched with controls the same age or older to have equal or more years to acquire a UTI. Complete clinical characteristics of our cohorts are summarized in Table 1.

Patients in the RIVUR Study Have Decreased DNA Copy Number of DEFA1A3 Compared with Controls

We determined DEFA1A3 gene copy number per diploid genome using quantitative real-time PCR and verified it in a subset of samples using pulsed-field gel electrophoresis. Patients in the RIVUR Trial and matched controls were copy typed. The DEFA1A3 copy number in the patients in the RIVUR Trial varied from three to 13, with a mean copy number of 6.3 ± 1.4. The DEFA1A3 copy number for controls ranged between four and 18, with a mean copy number 7 ± 2.0.

The distributions of copy numbers were significantly different between the two groups (P < 0.001) (Figure 1). Overall, a higher percentage of patients in the RIVUR Trial (n = 86; 29%) had five or fewer copies of DEFA1A3 compared with controls (n = 48; 16%; odds ratio [OR], 2.09; 95% confidence interval, 1.40 to 3.11; P < 0.001). Conversely, a higher percentage of controls (n = 93; 31%) had eight or more copies compared with patients in the RIVUR Trial (n = 55; 18%; OR, 0.50; 95% confidence interval, 0.34–0.73; P < 0.001). A similar statistically significant difference was seen in white boys in
the RIVUR Trial compared with control white boys (Supplemental Figure 1). Thus, a lower copy number of DEFA1A3 was found in patients in the RIVUR Trial (VUR and UTI).

**Certain Patients in the RIVUR Study with Breakthrough Infections Have a Significantly Lower Copy Number of DEFA1A3**

To determine if DEFA1A3 copy numbers were associated with subsequent infections in the RIVUR Trial population during the study, we analyzed the relation of DEFA1A3 copy number to UTI breakthrough events within the RIVUR Trial cohort. The distributions of DEFA1A3 copy number in those with breakthrough infections (n=70) during the 2-year clinical study compared with those who did not have breakthrough UTIs (n=228) were not statistically different (P=0.39).

The RIVUR Study was a placebo–controlled, randomized trial that showed that daily antibiotic prophylaxis significantly reduced UTI risk in patients.3 To explore the relations of CNV to recurrent UTI, we analyzed copy number in those with and without breakthrough UTIs in the antibiotic prophylaxis (n=154) and placebo (n=144) groups separately (Figure 2). For our study cohort, breakthrough infections occurred in 23 (15%) in the antibiotic group and 47 (33%) in the placebo group. Patients treated with antibiotic prophylaxis who experienced breakthrough UTIs (5.82 copies) averaged a 0.5-copy difference compared with those without breakthrough UTIs (6.34 copies; n=131). These results were statistically significant (P=0.04). Patients treated with placebo who experienced breakthrough infections (n=47) averaged 6.36 copies, similar to 6.39 copies in those who did not (n=97; P=0.90). This finding indicates that antibiotics in conjunction with a high (greater than or equal to eight) copy number of DEFA1A3 may decrease UTI risk in children with VUR (OR, 0; 95% confidence interval, 0-NaN; P=0.01) (Figure 2, B–D). Conversely, within the antibiotic group, low (less than or equal to five) DEFA1A3 copy number may increase UTI risk (OR, 2.51; 95% confidence interval, 1.02 to 6.22; P=0.04).

Because grade of VUR and bowel and bladder dysfunction (BBD) have been identified as risk factors for UTI in the RIVUR Trial cohort, we performed logistical regression analysis to account for these conditions (Table 2).4 Our analysis showed that DEFA1A3 copy number is a risk factor for UTI independent of BBD or high-grade/dilating VUR in the antibiotic prophylaxis group. For each additional copy of DEFA1A3, the odds of recurrent UTI decrease by 47% \(1 - (0.6307)^{100}\%\) when adjusting for VUR grade, BBD, treatment group, and interaction with copy number (Table 2). Additional analyses

**Figure 1.** Patients in the RIVUR Trial are more likely to have low DEFA1A3 copy number than control patients. (A) Bar graph representation of absolute DEFA1A3 copy number distribution between patients in the RIVUR Trial and control patients. (B) Bar graph representation of patients in the RIVUR Trial and control populations grouped according to low (less than or equal to five copies), midrange (six or seven copies), and high (greater than or equal to eight copies) number of DEFA1A3. (C) The ORs for patients in the RIVUR Trial cohort compared with controls and risk of having a UTI according to low, midrange, and high DEFA1A3 copy number. Patients in the RIVUR Trial have the additional risk factor of VUR, whereas controls do not.
Figure 2. DEFA1A3 copy number predicts antibiotic response. (A and B) When analysis for breakthrough UTI is restricted to patients on antibiotic prophylaxis, a lower absolute DNA copy number significantly correlates with UTI recurrence (P=0.04). (C) In patients who received prophylaxis, the breakthrough UTI ORs for greater than or equal to eight, six, or seven, and less than or equal to five copy number were 0.08, 0.98, and 2.51, respectively. (D and E) No difference was shown between DEFA1A3 copy number and recurrence in patients who received placebo (P=0.90). (F) The breakthrough UTI ORs were 0.70, 0.52, and 0.71 for greater than or equal to eight, six, or seven, and less than or equal to five copy number, respectively.
Table 2. Logistical regression coefficients for recurrent UTI risk among patients in the RIVUR Trial

<table>
<thead>
<tr>
<th>Variable</th>
<th>All Patients in the RIVUR Trial</th>
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<tbody>
<tr>
<td></td>
<td>Estimate</td>
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<tr>
<td>Intercept</td>
<td>2.029</td>
</tr>
<tr>
<td>DEFA1A3 CN</td>
<td>−0.631</td>
</tr>
<tr>
<td>BBD</td>
<td>0.373</td>
</tr>
<tr>
<td>VUR gradea</td>
<td>−1.57</td>
</tr>
<tr>
<td>Group (P)b</td>
<td>−3.087</td>
</tr>
<tr>
<td>Interaction of DEFA1A3 CN × group (P)</td>
<td>0.637</td>
</tr>
</tbody>
</table>

VUR divided into nondilating (grades 1 and 2) versus dilating. CN, copy number; P, placebo group.
aVUR graded by grade 1 and 2 versus grades 3 and 4.
bTreatment group (antibiotic prophylaxis group versus placebo).

The mRNA Level of DEFA1A3 in Kidney Is Correlated with the Gene Copy Number

Because significant differences in gene copy number could result in large ranges of tissue mRNA expression, we correlated tissue DNA copy number of DEFA1A3 to tissue DEFA1A3 mRNA. Analyzing 43 kidney samples, we observed a positive correlation between gene copy number and mRNA levels ($r^2=0.11; P=0.03$) (Figure 5).

DISCUSSION

In this study, we used a unique and well-characterized cohort from the previously published RIVUR Study to show that patients with VUR and UTIs have a higher frequency of low DEFA1A3 DNA copies compared with controls. Furthermore, we have shown that the protein product, HNP1–3, is also produced by renal tubular epithelium. Finally, we show that, despite the fact that DEFA1A3/HNP1–3 kidney expression was limited to the collecting duct, we can correlate host DNA copy number of DEFA1A3 to mRNA transcript levels in the kidney.

The role of the innate immune system and AMPs in kidney disease and infections of the kidney and urinary tract is an emerging concept. Our research group has shown the biologic relevance of AMPs ribonucleases 6 and 7 and human DEFA5 in the kidney and urinary tract in relation to UTIs. Prior studies have shown additional AMPs that may be important to the innate defense of the kidney and urinary tract. Now, HNP1–3 and its gene DEFA1A3 can be added to the growing portfolio of AMPs that are critical to the innate defense of kidney. Furthermore, the location of DEFA1A3 expression in the medullary collecting duct adds to the growing body of evidence that the collecting duct, specifically intercalated cells, is critical to defending the kidney from ascending infection. Additionally, this study represents the first report of genetic variations in an AMP gene being associated with UTI risk. DEFA1A3 has previously been implicated in inflammatory renal diseases, including lupus nephritis and IgA nephropathy. Thus, DEFA1A3 CNPs may be a disease-modifying factor in a range of kidney diseases. Previous groups have linked myeloid cell–derived HNP1–3 and genetic variations in DEFA1A3 under the presumption that this gene is not expressed by renal tissue. Our evidence that this gene is expressed by renal epithelia opens many new avenues for research in the fields of lupus nephritis and IgA nephropathy.

In healthy human kidney, DEFA1A3 mRNA expression ranged from 15 to 3979 (median =399; n=37) transcripts per 10 ng total RNA (Figure 3). Bladders had significantly lower levels, with a range of 11–91 (median =53; n=4) transcripts per 10 ng total RNA ($P<0.01$). To confirm DEFA1A3 parenchymal expression in the kidney, we performed in situ hybridization to localize expression in the kidney (Figure 4A, Supplemental Figure 4). Interestingly, DEFA1A3 transcripts localized to the epithelium of the nephron and were not present solely in resident neutrophils. To confirm translation of DEFA1A3 mRNA transcripts to the protein HNP1–3 and localize nephron segment, immunofluorescence was performed on healthy human kidney. HNP1–3 localized to the medullary collecting duct and was variably expressed by collecting duct intercalated and/or principal cells (Figure 4B), whereas no expression was present in negative control (Supplemental Figure 5).

DEFA1A3 Copy Number May Confer a Risk to Nonclassic UTIs

Each UTI during the RIVUR Study had urine cultured and bacteria speciated. We analyzed DEFA1A3 copy number in relation to the bacteria type. We grouped patients as E. coli (n=267) versus non–E. coli (n=31) UTI. The non–E. coli group had lower copy number compared with those with classic E. coli UTI ($P=0.05$) (Supplemental Figure 2).

DEFA1A3 Copy Number Was Not Associated with Acquired Renal Scarring in the RIVUR Trial Cohort

Within the analyzed patients in the RIVUR Trial, 22 of 240 (9%) acquired a new renal scar during the study period. DEFA1A3 copy number did not associate with these new scars (Supplemental Figure 3).

DEFA1A3 mRNA Transcripts and HNP1–3 Are Present in Collecting Duct Epithelium

In healthy human kidney, DEFA1A3 mRNA expression ranged from 15 to 3979 (median =399; n=37) transcripts per 10 ng total RNA (Figure 3). Bladders had significantly lower levels, with a range of 11–91 (median =53; n=4) transcripts per 10 ng total RNA ($P<0.01$). To confirm DEFA1A3 parenchymal expression in the kidney, we performed in situ hybridization to localize expression in the kidney (Figure 4A, Supplemental Figure 4). Interestingly, DEFA1A3 transcripts localized to the epithelium of the nephron and were not present solely in resident neutrophils. To confirm translation of DEFA1A3 mRNA transcripts to the protein HNP1–3 and localize nephron segment, immunofluorescence was performed on healthy human kidney. HNP1–3 localized to the medullary collecting duct and was variably expressed by collecting duct intercalated and/or principal cells (Figure 4B), whereas no expression was present in negative control (Supplemental Figure 5).
Thus, the link between genetic variations in this locus and UTIs is logical.

CNVs and CNPs have been statistically associated with several disease phenotypes; however, the functional relevance of these genetic variations remains largely unknown. Here, we show that higher DEFA1A3 copy number results in increased mRNA expression, likely from increased gene dosage. The fact that prior analyses of DEFA1A3 copy number and expression levels in leukocytes did not show a correlation may be attributed to the observation that DEFA1A3 is largely transcribed and translated in bone marrow as opposed to circulating leukocytes.16,18,19 Therefore, decreased copy number in the patients in the RIVUR Trial theoretically results in decreased kidney expression of HNP1–3. Our findings present the kidney as an advantageous location for future studies evaluating the correlation between gene copy number, protein expression, and disease risk.

Our group and others have shown that urine levels of HNP1–3 markedly increase during UTI and pyelonephritis.11,20,40 We speculate that low HNP1–3 expression related to decreased DEFA1A3 copy number contributes to UTI risk on the basis of lower DEFA1A3 copy number in patients in the RIVUR Trial and the correlation between DEFA1A3 copy number and mRNA expression. To definitively show a causal relationship, future studies will need to measure urine HNP1–3 levels during UTI and DEFA1A3 copy number along with controlling for the amount of pyuria and the severity of UTI in addition to renal epithelial versus tubular DEFA1A3 infection. Some functions of HNP1–3 have previously been shown to be dose dependent, such as activity against Mycobacterium tuberculosis, tissue remodeling properties, and inhibition of adenovirus.43–45 Possibly, mechanisms other than copy number–protein correlation are responsible for low DEFA1A3–associated UTI risk.

Others have accurately copy-typed DEFA1A3 using PCR-based methodologies and single-nucleotide polymorphism association with high/medium/low copy numbers.16,46 We

![Figure 3](image-url) **Figure 3.** DEFA1A3 is variably expressed in the kidney. DEFA1A3 is expressed in the kidney (n=37) in variable levels (mRNA copies per 10 ng total RNA), with a median of 399.0 and a range from 15.0 to 3973.0. The bladder (n=4) DEFA1A3 expression was minimal, with a median of 52.5 and a range from 11.0 to 91.0 (significantly less than the kidney; P<0.01; n=37 kidneys and n=4 bladders).

![Figure 4](image-url) **Figure 4.** DEFA1A3 is expressed in the renal collecting duct. (A) In situ hybridization for localization of DEFA1A3 in human kidney sections. The hybridization of a DEFA1A3 antisense probe (purple staining) can be seen in isolated renal tubular epithelial cells (arrows). (B) HNP1–3 kidney expression localizes to the medullary collecting duct. HNP1–3 localization in the human kidney is presented in (B) ×40 and (C) ×100 images. Collecting ducts are labeled with aquaporin-2 (red), HNP1–3 is green, and nuclei are blue. HNP1–3 immunolabeling (arrows) localized to the collecting tubules represented by aquaporin-2 staining (red, arrowheads) and isolated round polymorphonuclear cells consistent with neutrophils (asterisks). Rare co-localization of HNP1–3 and aquaporin-2 is noted (bolts). Scale bars, 50 μm.
used multiple methodologies to accurately quantitate the absolute copy number of \textit{DEFA1A3} in this study. The finding that DNA copy number is associated with UTIs and VUR expands genetic variations associated with VUR pathology from renal development genes to innate immune genes. Indeed, many children with VUR never develop infections and seem to have no clinical consequences from the retrograde flow of urine in the upper urinary tract. Our finding of decreased \textit{DEFA1A3} copy number in children with VUR and UTIs raises the possibility that genetic variations in innate immunity are a risk factor for UTI in the subset of patients who experience recurrent infections.

In the RIVUR Study, 25.4% of children treated with placebo had a breakthrough UTI during the 2-year study period. This rate of UTI was almost twice the rate of children treated with antibiotics prophylaxis.\textsuperscript{29} The patients in the RIVUR Trial randomized to placebo and analyzed in this study did not seem to have different breakthrough UTIs on the basis of \textit{DEFA1A3} copy number. Presumably, the anatomic defect of VUR or host characteristics overwhelmed the innate defenses, and therefore, no differences could be detected. Intriguingly, \textit{DEFA1A3} copy number is significantly lower in children with VUR on antibiotic prophylaxis who develop breakthrough UTIs. Antibiotic prophylaxis seems largely sufficient to prevent recurrent UTIs in children with high \textit{DEFA1A3} copy number. However, when low \textit{DEFA1A3} copy number is present, the efficacy of antibiotic prophylaxis decreases. Thus, adding antibiotic support to individuals with high and low \textit{DEFA1A3} copy numbers reveals the added burden caused by reduced \textit{\alpha}-defensin expression. Therefore, \textit{DEFA1A3} copy number determination represents a potential strategy to identify patients with VUR who will respond well to antibiotic prophylaxis. Future prospective studies with appropriately powered randomized cohorts are needed to prove this potentially causal relationship.

Currently, many recommendations exist to limit unnecessary or invasive testing to diagnose VUR or renal scarring. The AAP recommendations and the NICE guidelines help guide the clinician in determining which patients should be imaged with what imaging modality.\textsuperscript{47,48} Clear evidence–based recommendations are not available on how to treat the patient with known VUR, and this topic is a hot area of debate among clinical circles. Our study may provide the foundation for future studies to incorporate genetic data into clinical decision-making algorithms for patients with VUR at risk for recurrent UTIs.

We acknowledge that this study has some limitations. Because the largest cohort from the RIVUR Trial was white girls, our results may not apply to other cohorts and nonrefluxing patients. The \textit{DEFA1A3} copy number data from white boys also must be cautiously interpreted, because this comparison involved a small sample size. Future studies will focus on other cohorts with various UTI risk phenotypes and sex, race, and ethnicity.

We have correlated \textit{DEFA1A3} copy number with kidney mRNA expression to complement a prior report of a positive correlation with HNP1–3 expression in neutrophils.\textsuperscript{49} The correlation of kidney mRNA expression to DNA copy number is exciting and usually very elusive in the field of DNA CNVs. Ideally, we would like to correlate urine protein levels with \textit{DEFA1A3} copy number. Infected urine samples from the RIVUR Study were not available. HNP1–3 is not present in the urine during sterility.\textsuperscript{40} Ultimately, we would like to be able to determine if the source of urinary HNP1–3 during infection is intercalated cells or neutrophils and whether levels correlate with \textit{DEFA1A3} copy number. Because UTI is a heterogeneous event with a wide spectrum in degree of pyuria, upper tract versus lower tract involvement, uropathogenic cellular death and sloughing, and urinary concentration and osmolality as well as virulent factors with each uropathogen that may affect neutrophil degranulation or intercalated cell secretion of HNP1–3, designing an appropriate experiment would require conditional knockout mouse models to account for source and then, transgenic mice to recapitulate CNVs. Additionally, we aim to determine whether \textit{DEFA1A3} copy number accounts for differences in neutrophil bacterial killing between individuals. Finally, quantitative real–time PCR has limitations at discerning small copy number differences at high copy numbers, although previous studies have shown that PCR-based methodologies are accurate for determining copy numbers in multiallelic loci.\textsuperscript{50,51}

**Figure 5.** \textit{DEFA1A3} mRNA expression correlates with copy number. Regression analysis shows that increased \textit{DEFA1A3} gene copy number results in increased mRNA expression (black line). The 95% confidence intervals are represented by gray lines (n=43 kidneys).

\begin{itemize}
\item **r^2=0.110, \* p=0.03**
\end{itemize}
Review Board (IRB) Protocols IRB07–00383 and IRB10–00319 and The Ohio State University Wexner Medical Center IRB Protocol OSURB2013H0255. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki.

Genetic Samples

Patient Population. Samples were used from the National Institutes of Health–funded RIVUR Study.2,3 Informed consent for genetic samples was obtained as a component of enrollment to the parent RIVUR Study.2,3 The RIVUR Study consisted of 607 children ages 2–71 months of age presenting with confirmed grades 1–4 VUR and history of first or second UTI. The patients in the RIVUR Trial were randomized to antimicrobial prophylaxis or placebo and followed for 2 years. UTI guidelines in patients in the RIVUR Trial were adapted from the American Academy of Pediatric guidelines and defined as a positive culture with a single organism of $\geq 50,000$ CFU/ml on a catheter specimen or $>100,000$ CFU/ml on a clean catch with evidence for pyuria on the urinalysis. DNA from 455 of these children was obtained by the RIVUR Study. The patients in the RIVUR Trial were subgrouped by parameters followed in the RIVUR Study, including randomization to placebo versus antibiotic prophylaxis, breakthrough UTIs defined as UTIs during the study period and not including the study eligibility entry UTI, BBD as defined by dysfunctional voiding questionnaire score, and VUR grade along with acquired renal scarring defined by new DMSA–positive scars during the study period.

Control Population. Control patients were enrolled from patients presenting to the NCH Emergency Department. Patients were screened by a study staff interview for history of UTIs or known congenital anomalies of the kidney or urinary tract, including VUR, and eligible for inclusion if the aforementioned conditions were not present. Written informed consent was obtained. Because different races and ethnicities can have different copy number profiles, this analysis was limited to whites.52 Additionally, sex is a confounder in UTI risk, and therefore, the analysis was limited to girls separate from boys.

Human Biospecimens.

DNA. Control DNA was obtained from patients presenting to the NCH Emergency Department with no history of UTI. Saliva samples were obtained using Oragene DNA Collection Kits (DNA Genotek, Kanata, ON, Canada). Saliva samples were transferred to 15-ml conical tubes and incubated at 50°C for a minimum of 2 hours. DNA was extracted using prepIT L2P Reagent (PT-L2P-45; Genotek) according to the manufacturer’s manual purification protocol for whole samples. After purification and precipitation with ethanol, DNA was rehydrated with 100 $\mu$l Tris-EDTA solution. DNA concentration (nanograms per microliter) and DNA purity (a260-to-a280 and a260-to-a230 ratios) were determined using the Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

Tissue. We obtained noninfected bladder tissue ($n=4$) from children undergoing ureteral reimplantation for reasons other than recurrent UTI, such as UPV obstruction. Kidney tissue ($n=43$) was provided by the Cooperative Human Tissue Network, which is funded by the National Cancer Institute.53 No tissue was obtained from patients with recurrent UTI. Tissue samples were snap frozen or preserved as neutral formalin–fixed, paraffin–embedded sections. Kidney tissues were removed from patients with suspected tumor, and we used adjacent tissue free of disease or inflammation.

DEFA1A3 Copy Typing.

Pulse–Field Gel Electrophoresis. To develop absolute DEFA1A3 copy number to use for the PCR, PacI-PFGE and Southern blot analysis of DEFA1A3 in nine different human subjects were used. Mononuclear suspension cells were harvested by centrifugation at 1000×g for 10 minutes at 4°C and rinsed with PBS. Approximately 2–5×10^6 cells were resuspended in 100 $\mu$l molten low–gelling temperature agarose to a final concentration of 0.5% (agarose), and the mixture was set in a plug mold. Agarose plugs with intact genomic DNA were equilibrated with restriction enzyme buffer, digested with PacI enzyme (15 U per reaction) for 2 hours, and resolved with pulsed–field gel electrophoresis for DNA fragments with sizes between 50 and 400 kb. The agarose gel with resolved genomic DNA fragments was subjected to modified Southern blot procedures as described previously.54 Genomic DNA transferred to nylon membrane was hybridized with a DEFA1A3–specific genomic probe labeled by $\alpha$25P–dCTP and subjected to x-ray film autoradiography at −80°C for 3–14 days. Because each segment of duplication for the DEFA1A3 gene is 19.1 kb in size, the absolute copy number could be determined by the size of band on the λ-ladder and the number of haplotype bands (Figure 6).

High-Throughput Copy Typing. We used multiplex real–time PCR using Taqman Dye Chemistry (Applied Biosystems, Foster City, CA) and the comparative cycle threshold difference (ΔΔCT) method to copy type each individual’s total DEFA1A3 copy number. The ZNF80 gene served as a two copies per diploid genome reference gene. Individuals with known copy number served as calibrators to determine copy number for unknown samples. Calibrators with eight total copies were determined by pulsed–field gel electrophoresis. The DEFA1A3 probe was FAM labeled, and the reference ZNF80 probe was VIC labeled. Briefly, each multiplex reaction consisted of 0.9 $\mu$m each forward and reverse primers, 100 nM target (FAM-labeled DEFA1A3) and reference (VIC-labeled ZNF80) probes, 30 ng patient genomic DNA, and 2× Taqman Universal PCR Master Mix. The final volume was adjusted to 20 $\mu$l with molecular-grade water. Each sample was performed in duplicate. Real-time PCR was performed using the ABI 7500 Real-Time PCR System, with cycles of 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute.

Relative quantitation with the ΔΔCT method was used to calculate the copy number of the DEFA1A3 gene. This method measured the cycle threshold difference (ΔCT) between DEFA1A3 as a target and ZNF80 as a reference and then, compared the ΔCT values of samples with the calibrator sample known to have eight copies of the target
Copy numbers were rounded up or down to the closest integer.

sequence. Known samples with DEF1A3 copy numbers of six, seven, and 11 were included on each plate as quality controls:

\[
\Delta C_T = C_T(\text{target}) - C_T(\text{reference}),
\]

\[
\Delta \Delta C_T = \Delta C_T(\text{sample}) - \Delta C_T(\text{calibrator}),
\]

and total DEF1A3 copy number(sample) = copy number(calibrator) \times 2^{\Delta \Delta C_T}.

Copy numbers were rounded up or down to the closest integer.

**Nucleic Acid and Protein Isolation from Human Kidney Samples**

Kidney tissue samples were divided into 100-mg pieces. Tissue samples were homogenized in a bullet blender (BBY-24M; Next Direct, Averill Park, NY) using two or three 3.2-mm stainless steel beads and five or six 2.9-mm-diameter zirconium oxide beads (Next Direct) under standard conditions. The extracted DNA was resuspended in buffer 10 mM Tris-HCl and 1 mM EDTA (pH. 8.0).

RNA was extracted using the Trizol method. Adjacent pieces of the same kidney specimens used for DNA and RNA extraction were homogenized in RIPA buffer with protease inhibitors to achieve tissue lysate for protein analysis.

**Tissue DEF1A3 mRNA Expression**

mRNA expression was analyzed as previously described. Briefly, RNA extracted from kidney, bladder, and ureter was reverse transcribed into cDNA using the Verso cDNA Kit (Thermo Scientific). Quantitative real-time PCR was performed using cDNA as the template with a specific primer pair (forward primer: 5'-CCCTCGCCCATGCTTGGTGC-3' and reverse primer: 5'-CTTGAGGCTGATGCTTGGAGGC-3'). The DEF1A cDNA clone (cDNA clone MGC: 120826 IMAGE: 7939636; American Type Culture Collection, Manassas, VA) was used as a standard to quantify the mRNA copy numbers for each sample. The PCR conditions were as follows: initial denaturation at 95°C for 10 minutes followed by 40 cycles, with each cycle consisting of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The reactions were performed using the 7500 Real-Time PCR System (Applied Biosystems). Primers and probes are listed in Table 3.

**DEF1A3 In Situ Hybridization**

RNA was interrogated in 4-µm paraffin–embedded human kidney sections using the Quantigene ViewRNA ISH Tissue Assay according to the manufacturer’s protocol (Affymetrix, Santa Clara, CA). Prehybridization conditions were optimized with 5 minutes of incubation in pretreatment solution (90°C) and 5 minutes of treatment with protease QF (40°C). Sections were hybridized for 3 hours (40°C) with a viewRNA Probe Set designed against the DEF1A3 mRNA. The probes were then amplified (PreAmplifier and Amplifier molecules), conjugated to alkaline phosphatase (Label Probe 1-AP), and bound with Fast Red Substrate. Finally, sections were counterstained with hematoxylin and imaged with the EVOS FL Auto Cell Imaging System (Life Technologies, Grand Island, NY).

**Immunofluorescence**

After deparaffinization, rehydration and permeabilization with 0.1% Triton X-100, antigen with Tris-EDTA (pH 9.0), and blocking superblock solution (Scy Tek, Logan, UT) were performed. Sections were incubated with anti-human HNP1-3 (1:300 dilution; Hycult Biotech, Plymouth Meeting, PA) overnight at 4°C. After washing with PBS, 10% superblock sections were incubated with anti–mouse IgG-AF488 (1:200 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA) and anti–rabbit IgG-AF594 (1:200 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour. Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (1:10,000 dilution; Invitrogen, Carlsbad, CA) and imaged with the EVOS FL Auto Cell Imaging System.

**Table 3. Genotyping Primers**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEF1A forward</td>
<td>5’-CCCTGCCCAGGCTCAAGGA-3’</td>
</tr>
<tr>
<td>DEF1A reverse</td>
<td>5’-CATAGCGACGTTCCTTGCAAT-3’</td>
</tr>
<tr>
<td>Probe</td>
<td>FAM-GGTCTTGCAGGATACC-MGB</td>
</tr>
<tr>
<td>ZNF80 forward</td>
<td>5’-CTCTGTTCATCAGGTACG-3’</td>
</tr>
<tr>
<td>ZNF80 reverse</td>
<td>5’-CTTTGCACCTGTCAGGCTTT-3’</td>
</tr>
<tr>
<td>Probe</td>
<td>VIC-GAAGACCGTCCTACCATCGCTT-3’</td>
</tr>
</tbody>
</table>

FAM, 6-carboxyfluorescein; MGB, minor groove binder; VIC, 4,7,2-trichloro-7’-phenyl-6-carboxyfluorescein.
West Grove, PA) for 30 minutes at room temperature. For Aquaporin staining, sections were labeled for 30 minutes at room temperature with a 1:400 dilution of anti-AQP2-Rhodamine antibody (anti-AQP2-ATTO-550; Alomone Laboratory, Jerusalem, Israel) and counterstained with DAPI (H-1500; Vector Laboratories, Burlingame, CA) visualized on a Keyence Microscope (Keyence, Osaka, Japan) with appropriate filters and brightness along with contrast adjusted with Keyence imaging software. Secondary antibody alone was used as a negative control.

**Replicates and Outliers**

Genotyping experiments were performed in triplicate; otherwise, all other experiments were performed in duplicate. No outlying data were excluded.

**Statistical Analyses**

Gene copy number analysis was done using t tests to assess the distribution difference (mean values) of copy number between independent two–sample groups (e.g., patients in the RIVUR Trial versus controls, entry versus recurrence groups, E. coli versus non-E. coli, and breakthrough UTI risk between the antibiotic and the placebo groups). To perform comparisons between ratios of groups (e.g., patients in the RIVUR Trial versus controls and breakthrough UTI risk between the antibiotic and the placebo groups) with low, medium, or high DEFA1A3 copy number, the chi-squared test was performed, provided that ≥80% of the cells have an expected frequency of greater than or equal to five and no cell has an expected frequency <1.0; otherwise, the Fisher exact test was used. To perform statistical analysis for HNP1–3/DEFA1A3, expression differences were evaluated with the Mann–Whitney test. Linear regression analysis was performed to reveal the linear relationship between CNV and mRNA, and confidence intervals are presented. To assess the risk for recurrent UTI related to CNVs, the group receiving antibiotics, and their interaction, we performed logistical regression analysis with an adjustment for VUR grade and BBD using the function glm in R. Statistical analyses were done using stats in the open source statistical software R (www.r-project.org), Vassarstats (http://vassarstats.net), and Prism software (GraphPad Software, La Jolla, CA). All statistical tests were two sided, with a significant level of 95% (i.e., P value <0.05).

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**DISCLOSURES**

None.

**REFERENCES**


31. DEFA1A3 and UTI Risk


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