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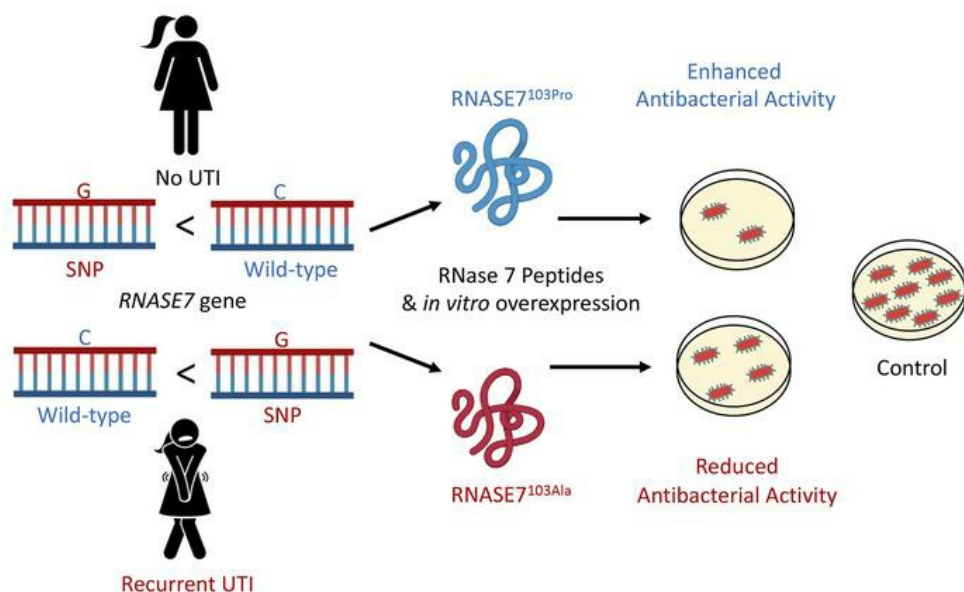
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Ribonuclease 7 polymorphism rs1263872 reduces antimicrobial activity and associates with pediatric urinary tract infections

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Ribonuclease 7 (RNase 7) is an antimicrobial peptide that prevents urinary tract infections (UTI); however, it is yet unknown how *RNASE7* genetic variations affect its antimicrobial activity and its mitigation of UTI risk. This study determined whether the *RNASE7* SNP rs1263872 is more prevalent in children with UTI and defined how rs1263872 affects RNase 7's antimicrobial activity against uropathogenic *E. coli* (UPEC). We performed genotyping for rs1263872 in 2 national UTI cohorts, including children enrolled in the Randomized Intervention for Children with Vesicoureteral Reflux trial or the Careful Urinary Tract Infection Evaluation study. Genotypes from these cohorts were compared with those of female controls with no UTI. To assess whether rs1263872 affects RNase 7's antimicrobial activity, we generated RNase 7 peptides and genetically modified urothelial cultures encoding wild-type RNase 7 and its variant. Compared with controls, girls in both UTI cohorts had an increased prevalence of the *RNASE7* variant. Compared with the missense variant, wild-type RNase 7 peptide showed greater bactericidal activity against UPEC. Wild-type RNase 7 overexpression in human urothelial cultures reduced UPEC invasive infection compared with mutant overexpression. These results show that children with UTI have an increased prevalence of *RNASE7* rs1263872, which may increase UTI susceptibility by suppressing RNase 7's antibacterial activity.

Introduction

Urinary tract infections (UTI) are one of the most common bacterial infections. In children, 8% of girls will develop a UTI by 7 years of age and up to 50% of affected children will suffer from at least 1 UTI recurrence. In acute cases, UTI can lead to kidney injury, bacteremia, and urosepsis. Long-term UTI complications include hypertension, proteinuria, renal fibrosis, and chronic kidney disease. Although all children are susceptible to UTI, evidence suggests that different variables influence UTI risk. Depending on age, vesicoureteral reflux (VUR), bowel and bladder dysfunction, and sexual activity augment UTI susceptibility (1, 2). However, these conditions do not completely explain why some people develop UTI, more severe UTI, or recurrent UTI. Identifying additional UTI risk factors may improve the health of children and reduce healthcare costs.

Recently, sizable advancements have been made in identifying host responses that protect the kidneys and bladder from uropathogens. Evidence suggests the innate immune system provides the front-line defense against UTI. Among these innate defenses, antimicrobial peptides (AMPs) play important roles in shielding the urothelium from uropathogenic *E. coli* (UPEC) — the most common pathogen causing UTI (3). AMPs are small cationic peptides produced and secreted by cells involved in host defense. In the kidney and urinary tract, AMPs are produced by the bladder urothelium and kidney tubules. Some AMPs are produced at high basal concentrations to prevent bacterial attachment while others are induced to facilitate UPEC clearance. When AMP production is disrupted, UTI risk increases. In humans, cathelicidins, defensins, and ribonucleases are the major AMP families (3, 4).

Previously, we identified ribonuclease 7 (RNase 7) as an essential and abundant human AMP that shields the urinary tract from invasive infection (5–7). RNase 7 has antimicrobial activity against Gram-positive and Gram-negative uropathogens, including UPEC and multidrug resistant (MDR) UPEC (5, 7). RNase 7's catalytic activity is not required for its bactericidal activity. Instead, RNase 7's antimicrobial functions are dependent on its binding affinity for LPSs on the bacterial cell wall as well as its ability to permeate and disrupt bacterial membranes. These mechanisms of bacterial membrane disruption differ from those of antibiotics, which kill or prevent bacterial replication by inhibiting cell wall synthesis, DNA replication, RNA transcription, or protein synthesis (8–10).
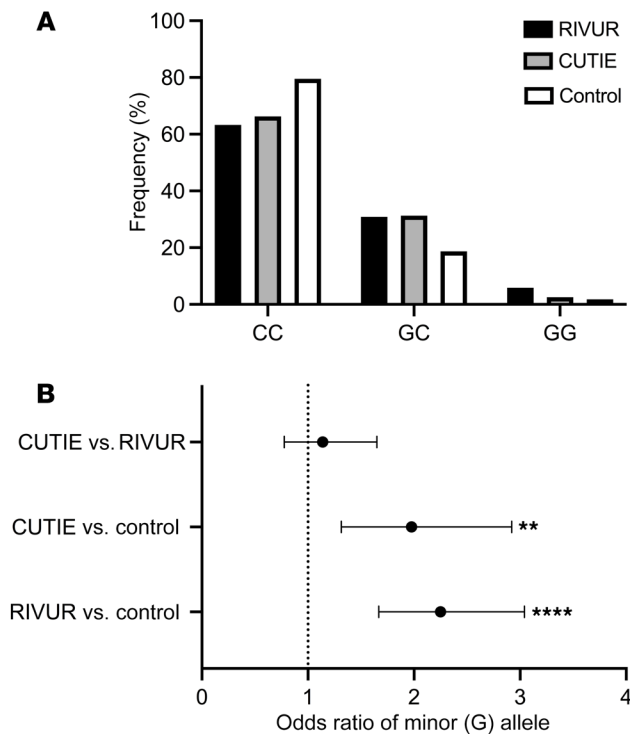
Figure 1. Distribution and odds ratio of minor *RNASE7* allele in clinical cohorts. (**A**) Relative percentage of each genotype across study cohorts. Female participants enrolled in the RIVUR (black bars, *n* = 424) and CUTIE (gray bars, *n* = 160) studies have a higher prevalence of GC and GG genotypes compared with controls (white bars, *n* = 482). 63.3% and 66.3% of participants in the RIVUR and CUTIE studies had the CC genotype, respectively. In contrast, 79.5% of controls had the CC genotype. (**B**) Odds ratios comparing the presence of the minor allele (influence on UTI risk) in different cohorts. Comparing the presence of the minor allele in the RIVUR cohort with that controls resulted in an odds ratio of 2.25 (95% CI 1.67–3.04, *P* < 0.0001). Comparing the presence of the minor allele in the CUTIE cohort and that in controls resulted in an odds ratio of 1.98 (95% CI 1.32–2.92, *P* = 0.0011). No difference was seen between the RIVUR and CUTIE cohorts (odds ratio 1.14, 95% CI 0.78–1.65), indicating that VUR status does not affect UTI risk in relation to genotype. All comparisons were made using Fisher's exact test. *******P* < 0.01, ****P < 0.0001.

While genetic variations in AMP genes have been associated with infection propensity, no published data to our knowledge interrogate how *RNASE7* genetic variations effect UTI risk. Thus, the objectives of this study were to (a) evaluate the frequency of the *RNASE7* SNP rs1263872 in clinical cohorts comprising female participants with UTI and controls without UTI and (b) to define the effect of SNP rs1263872 on the antimicrobial activity of the encoded RNase 7 peptide against UPEC. SNP rs1263872 has a C-to-G nucleotide substitution and confers a proline-to-alanine amino acid substitution at amino acid position 103 (P103A) of the RNase 7 peptide. This substitution predicts a change in RNase 7's secondary structure and possibly its antimicrobial activity (11).

Results and Discussion

We queried the dbSNP (https://www.ncbi.nlm.nih.gov/snp/) and identified 5 nonsynonymous variants in the coding region of the *RNASE7* gene. At the time of our search, rs1263872 was the most prevalent SNP with a minor allele frequency of 0.07–0.19 (11). To our knowledge, there are no published data that link this SNP to clinical outcomes. Next, we performed genotyping for rs1263872 on controls without UTI and children with UTI enrolled in the Randomized Intervention for Children with Vesicoureteral Reflux (RIVUR) trial or the Careful Urinary Tract Infection Evaluation (CUTIE) study, as outlined in the Supplemental Methods (12, 13). Allele frequencies of rs1263872 in the control cohort were found to be in Hardy-Weinberg equilibrium and did not differ from those reported in dbSNP (11). Genotype frequencies are shown (Figure 1). Compared with controls, we identified an increased prevalence of the minor G allele in both UTI cohorts. Thus, we postulated that this missense polymorphism leads to an amino acid substitution that negatively affects RNase 7's antimicrobial activity. In the RIVUR cohort we did not detect a statistical difference in the

prevalence of the minor G allele between the low-grade, nondilating VUR and higher-grade, dilating VUR groups. Additionally, we did not identify a correlation with the minor G allele and kidney scarring following UTI (data not shown).

Next, we generated RNase 7 peptides to reflect *RNASE7* SNP rs1263872 wild-type (RNASE7[103Pro]) and its missense variant (RNASE7[103Ala]). To test these peptides' antimicrobial activity against *E. coli*, bactericidal activity assays were performed by coincubating each RNase 7 peptide with *E. coli* commensal strain MG-1655 or UPEC clinical strains from phylogenetic group B2 — the most predominant *E. coli* clade associated with UTI (14–16). We determined the O serogroup in all tested UPEC strains and assessed the expression of 10 virulence factors contributing to UTI pathogenicity (17). Published sequencing data identify added genomic differences between MG1655 and UPEC strains CFT073, 536, and UTI89 (18–20). In addition, the functional activity of type 1 pili, which promote UPEC adherence to urothelial cells, was detected by hemagglutination in the presence and absence of mannose (21). Genotyping these clinical strains supports data showing that UPEC is a heterogeneous pathotype (Table 1). When these *E. coli* strains were incubated with RNase 7 peptides, our results show that the minor allele RNASE7[103Ala] had diminished bactericidal activity compared with wild-type RNASE7[103Pro] (Figure 2A). We did not observe a correlation between UPEC genotype and RNase 7 susceptibility, as RNase 7 killed each strain similarly. Future studies are needed to identify UPEC virulence factors that may confer a fitness advantage.

Because rs1263872 is predicted to cause a change in RNase 7's secondary structure, we determined if this amino acid substitution (P103A) affects the ability of RNase 7 to bind the bacterial cell wall. We assessed the ability of the RNASE7[103Ala] and RNASE7[103Pro] to bind LPS using the Bodipy TR Cadaverine probe

Table 1. Characteristics of *E. coli* strains used in this study

Strain	CFT073	J96	536	MDR58	MDR12	MDR51	UTI89	UTI89 Δ*fimH*	MG1655
O serotype	O6	O4	O6	O25	Other	Other	O18	O18	O16
Phylogenetic group	B2	B2	B2	B2	B2	B2	B2	B2	A
Strain type	Pyelonephritis	Pyelonephritis	Pyelonephritis	Pyelonephritis	Cystitis	Cystitis	Cystitis	Cystitis	Commensal
Strain source	Blood	Urine	Urine	Urine	Urine	Urine	Urine	Urine	Stool
Iron acquisition systems									
iutA	+	–	–	+	+	+	+	+	–
fyuA	+	+	+	+	+	+	+	+	–
Pili									
fimH	+	+	+	+	+	+	+	–	+
papAH	+	+	+	+	–	–	+	+	–
papEF	+	+	+	+	–	–	+	+	–
papC	+	+	+	+	–	–	+	+	–
sfaS	–	+	+	+	–	–	+	+	–
Toxins									
cnf1	–	+	–	+	–	–	+	–	–
hlyA	+	+	+	+	–	+	+	–	–
Other									
kpsMTII	+	–	+	–	–	–	+	+	–
kpsMTIII	–	+	–	–	–	–	–	–	–
HA dilution	8	16	4	4	–	4	64	–	8
Mannose-sensitive HA	+	+	+	+	N/A	+	+	N/A	+

For virulence factors, + indicates that gene expression was detected and – indicates that gene expression was not detected. For mannose-sensitive HA + identifies mannose-sensitive HA. Hemagglutination (HA) dilution indicates the minimum dilution in which HA of guinea pig erythrocytes was detected.

(BC). BC binds the lipid A portion of LPS and measures the competitive displacement of BC by LPS-binding molecules such as RNase 7. When compared with RNASE7[103Pro] peptide, RNASE7[103Ala] showed reduced affinity binding toward LPS (Figure 2B). This finding may explain the reduced antibacterial capacity of RNASE7[103Ala]. Prior reports suggest that LPS binding is required for RNase 7's antibacterial activity (8).

To confirm this phenotype in vitro, human UROtsa cells were stably infected with retroviral constructs expressing these *RNASE7* alleles or empty vector. UROtsa cells have minimal endogenous RNase 7 expression (7). Western blot confirmed cellular RNase 7 overexpression and ELISA quantified secretion into the media (Figure 2, C and D). To evaluate extracellular UPEC bactericidal activity, we collected 3-day-old culture media from confluent RNase 7–expressing cells and control cells. Isolated media was inoculated with UPEC pyelonephritis strain 536, cystitis strains MDR12 and UTI89, and the avirulent mutant UTI89Δ*fimH*. After a 90-minute incubation, survival of all tested UPEC strains decreased in media isolated from wild-type RNASE7[103Pro]-expressing cells compared with that in mutant RNASE7[103Ala]-expressing cells (Figure 2E). To assess if cellular RNase 7 shields the urothelium from bacterial challenge, confluent UROtsa cells were challenged with these same UPEC strains. The percentage of UPEC adhering to and invading wild-type RNASE7[103Pro]-overexpressing cells was significantly reduced compared with mutant RNASE7[103Ala] (Figure 2, F and G). UPEC MDR12, which demonstrated little functional type 1 pili activity by hemagglutination, and the attenuated UTI89Δ*fimH* showed reduced urothelial binding and minimal urothelial invasion. However, UPEC attachment ratios were comparable to those

of UTI89 — suggesting that RNase 7 does not interfere with type 1 pilus-meditated bacterial adhesion (Figure 2H). These results suggest that extracellular and cellular bacterial killing is the major upstream antimicrobial mechanism of RNase 7. Additionally, they further demonstrate that wild-type RNASE7[103Pro] has enhanced antimicrobial activity against multiple UPEC strains compared with its variant, RNASE7[103Ala] — suggesting that the *RNASE7* rs1263872 minor allele can effect UPEC susceptibility.

The role of host defense peptides, such as RNase 7, in UTI prevention and UPEC clearance is a relatively new concept. Our research group and others have shown that bacteriostatic iron-regulatory peptides, such as hepcidin and lipocalin 2, or bactericidal AMPs, such as cathelicidin, defensins, and ribonucleases, prevent UTI (3, 4). To our knowledge, this is the first report describing a SNP in an AMP gene that may affect UTI susceptibility. Previously, we demonstrated that α-defensin copy number variations in the *DEFA1A3* gene may affect UTI risk. In a subset of children in the RIVUR study, we found low *DEFA1A3* copy numbers associated with recurrent UTI — indicating that AMP copy number polymorphisms may increase UTI severity (22).

Data in this study suggest that a SNP altering RNase 7's primary structure can increase UPEC susceptibility. The data generated here, in conjunction with our previously published findings (6, 7), suggest that RNase 7 has a key role in preventing UTI. In addition to its antimicrobial activity against UPEC and MDR UPEC, our data show that overexpressing RNase 7 in vitro reduces the ability of UPEC to attach to or invade urothelial cells. When we subjected transgenic, humanized RNase 7–expressing mice to experimental UTI, RNase 7's in vivo antibacterial activity
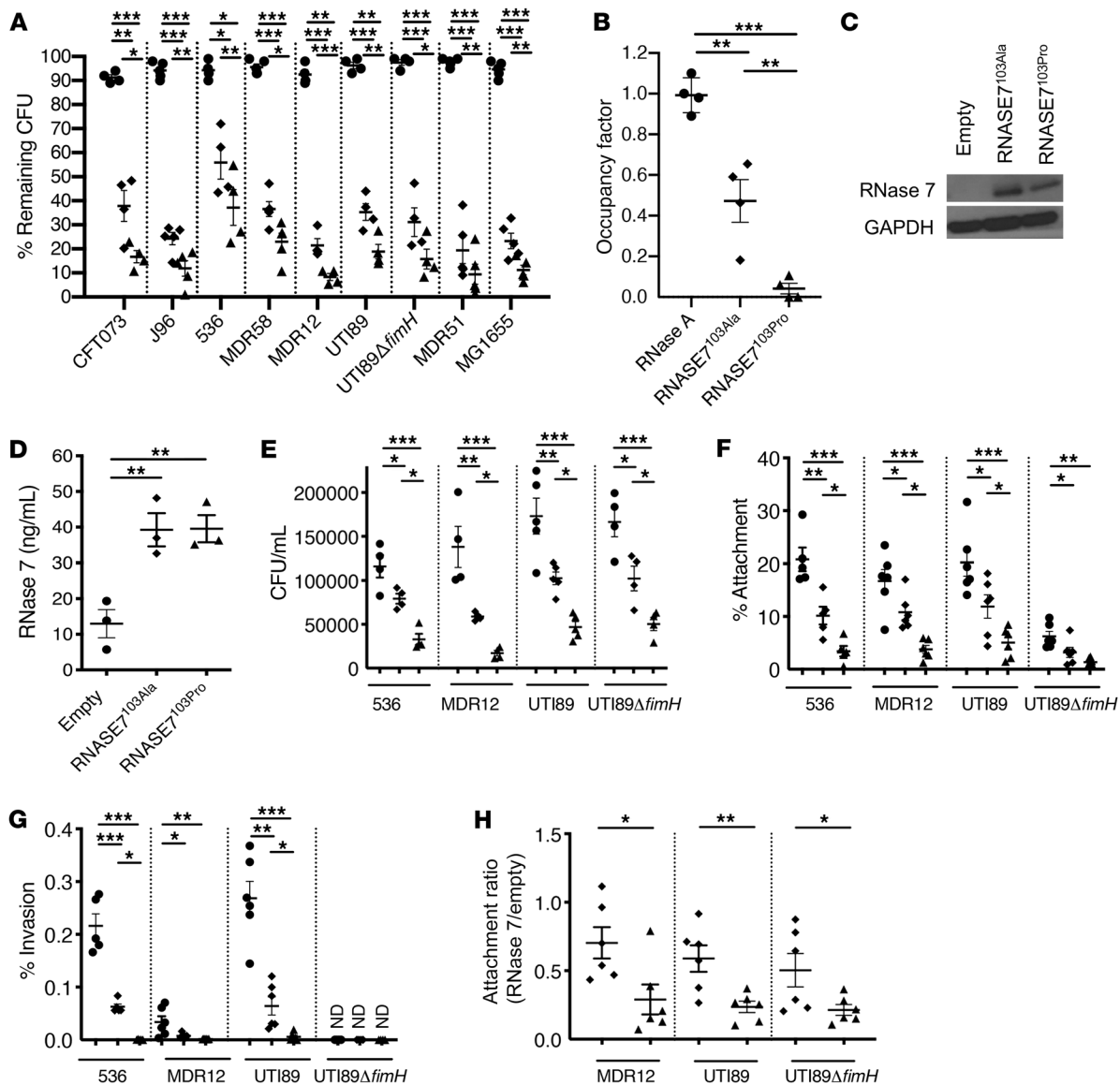
Figure 2. Compared with wild-type RNase 7, *RNASE7*[103Ala] has reduced antimicrobial activity. (**A**) *E. coli* strains were incubated with 2.5 μM RNASE7[103Ala] (diamonds) or wild-type RNASE7[103Pro] (triangles). An irrelevant peptide (circles) served as control. After incubation, UPEC strains were plated on LB agar and colonies were enumerated. Results are from 4 to 5 experiments performed in duplicate (*n* = 4–5). Data shown represent percentages of remaining colony-forming units (mean ± SEM). (**B**) Displacement of LPS-bound Bodipy TR Cadaverine with 0.5 μM RNase A (circles, negative control), RNASE7[103Ala] (diamonds), and wild-type RNASE7[103Pro] (triangles). Results show the mean and SEM from 4 experiments performed in duplicate (*n* = 4). (**C** and **D**) UROtsa cells were infected with retroviral constructs expressing wild-type *RNASE7*[103Pro], *RNASE7*[103Ala], or empty vector. Mean ± SEM. (**C**) Representative Western blot, probed for RNase 7 and GAPDH. (**D**) ELISA quantified RNase 7 in culture media. Results are from 3 experiments performed in triplicate (*n* = 3). Mean ± SEM. (**E**) Extracellular bactericidal activity assays were performed using culture media from empty vector (circles), *RNASE7*[103Ala] (diamonds), and *RNASE7*[103Pro] (triangles) retrovirus-infected cells, as described in the Supplemental Methods. Graphs show the mean UPEC survival and SEM. Results are from 4 to 5 experiments performed in triplicate (*n* = 4–5)(mean ± SEM). (**F** and **G**) UPEC attachment (**F**) and invasion (**G**) assays were performed as outlined in the Supplemental Methods. Shown are the percentage of UPEC adhering to the cellular surface and invading empty vector– (circles), *RNASE7*[103Ala]- (diamonds), and *RNASE7*[103Pro]-transduced (triangles) UROtsa cells. Results are from 5 to 6 experiments performed in quadruplicate (*n* = 5–6) (mean ± SEM). ND, not detected. (**H**) To account for the attenuated attachment of MDR12 and UTI89Δ*fimH*, the ratio of UPEC attachment in *RNASE7*[103Ala]- (diamonds) and *RNASE7*[103Pro]-overexpressing (triangles) cells compared with empty vector–expressing cells is shown. (**A**, **B**, and **D**–**G**) Pairwise comparisons were made by 1-way ANOVA with Tukey's modification or (**H**) Student's *t* test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

promoted UPEC clearance (7). Conversely, silencing urothelial RNase 7 expression in vitro increased invasive UPEC infection and blocking urinary RNase 7 antibacterial activity increased UPEC growth in human urine (6). In addition, our published data demonstrate that female participants with recurrent UTI have lower urinary RNase 7 concentrations compared with controls, and urinary RNase 7 concentrations negatively correlate with the number of UTI (7). Together, these data provide evidence that altering RNase 7's structure, expression, or antimicrobial activity affects UTI susceptibility.

We acknowledge that this study has limitations. The RIVUR and CUTIE studies primarily comprised non-Hispanic, White female participants of European ancestry (12, 13). Thus, our results must be cautiously interpreted in children of different sex, race, or ethnicity. While the use of recombinant RNase 7 peptides and in vitro tissue culture experiments suggest the RNase 7 proline-to-alanine substitution negatively affects RNase 7's bactericidal activity, these results may not translate in vivo. To fully define the use of RNase 7 as a UTI prognostic, a large, prospective analysis in children with different UTI risk phenotypes and racial/sex profiles is needed to determine if children with *RNASE7* genetic variations have an increased propensity to develop recurrent UTI, more severe UTI, or atypical infections. These prospective studies may identify at-risk children with abnormal RNase 7 profiles, who benefit from close UTI surveillance, antibiotic prophylaxis, or new approaches to boost endogenous UTI defenses (7).

To conclude, a growing body of evidence suggests that RNase 7 has an important role in UTI prevention. Findings in this study show that the *RNASE7* SNP rs1263872, which encodes an RNase 7 peptide with reduced antibacterial activity, is more common in females participants with UTI. These data provide a foundation that may ultimately help clinicians make more informed decisions regarding UTI treatment or prevention in children at risk for UTI complications.

Methods

See Supplemental Methods for details on study cohorts and study methods.

Study approval. Research in human participants was performed in accordance with the principles of the World Medical Association's Declaration of Helsinki and approved by the University of Tennessee Health Science Center IRB (protocol 14-03325-XP) and Nationwide Children's IRB (protocol 07-00383).

Author contributions

DSH and JDS supervised the project and contributed to the design and interpretation of all experiments. KP and SC performed the human genotyping experiments and analysis. AS completed the UPEC genotyping. TE performed the PINCO and hemagglutination experiments. JDS and CMV generated the RNase 7 recombinant peptides and performed the UPEC bactericidal assays, respectively. KP, TE, ALS, DSH, and JDS wrote the manuscript with input from all coauthors.

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