16. Hasler U, Mordasini D, Bens M *et al*. Long-term regulation of aquaporin-2 expression in vasopressin-responsive renal collecting duct principal cells. *J Biol Chem* 2002; 277: 10379–10386
17. Nakamura S, Yamamura Y, Itoh S *et al*. Characterization of a novel nonpeptide vasopressin V(2)-agonist, OPC-51803, in cells transfected human vasopressin receptor subtypes. *Br J Pharmacol* 2000; 129: 1700–1706
18. Serradeil-Le Gal C. An overview of SR121463, a selective non-peptide vasopressin V(2) receptor antagonist. *Cardiovasc Drug Rev* 2001; 19: 201–214
19. Deen PMT, Van Balkom BWM, Savelkoul PJ *et al*. Aquaporin-2: COOH terminus is necessary but not sufficient for routing to the apical membrane. *Am J Physiol Renal Physiol* 2002; 282: F330–F340
20. Deen PMT, van Aubel RA, van Lieburg AF *et al*. Urinary content of aquaporin 1 and 2 in nephrogenic diabetes insipidus. *J Am Soc Nephrol* 1996; 7: 836–841
21. Deen PMT, Croes H, van Aubel RA *et al*. Water channels encoded by mutant aquaporin-2 genes in nephrogenic diabetes insipidus are impaired in their cellular routing. *J Clin Invest* 1995; 95: 2291–2296
22. Li Y, Shaw S, Kamsteeg EJ *et al*. Development of lithium-induced nephrogenic diabetes insipidus is dissociated from adenylyl cyclase activity. *J Am Soc Nephrol* 2006; 17: 1063–1072
23. Kanno K, Sasaki S, Hirata Y *et al*. Urinary excretion of aquaporin-2 in patients with diabetes insipidus. *N Engl J Med* 1995; 332: 1540–1545
24. Yun J, Schoneberg T, Liu J *et al*. Generation and phenotype of mice harboring a nonsense mutation in the V2 vasopressin receptor gene. *J Clin Invest* 2000; 106: 1361–1371
25. Promeneur D, Kwon TH, Frokiaer J *et al*. Vasopressin V(2)-receptor-dependent regulation of AQP2 expression in Brattleboro rats. *Am J Physiol Renal Physiol* 2000; 279: F370–F382

Received for publication: 22.3.09; Accepted in revised form: 17.7.09

Nephrol Dial Transplant (2010) 25: 54–60
doi: 10.1093/ndt/gfp453
Advance Access publication 10 September 2009

Analysis of *TSHZ2* and *TSHZ3* genes in congenital pelvi-ureteric junction obstruction

Dagan Jenkins[1], Xavier Caubit[2], Aleksandar Dimovski[3], Nadica Matevska[3], Claire M. Lye[4], Feryal Cabuk[5], Zoran Gucev[6], Velibor Tasic[6], Laurent Fasano[2] and Adrian S. Woolf[7]

[1]Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DS, UK, [2]Institut de Biologie du Développement de Marseille-Luminy (IBDML), UMR6216, CNRS, Université de la Méditerranée, F-13288 Marseille cedex 09, France, [3]Department of Molecular Biology and Genetics, Institute of Pharmaceutical Chemistry, Faculty of Pharmacy, Skopje, Macedonia, [4]Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge, UK, [5]Department of Medical Biology and Genetics, Kırıkkale University, Medical School, Kırıkkale, Turkey, [6]Department of Pediatric Nephrology, University Children's Hospital, Skopje, Macedonia and [7]Nephro-Urology Unit, UCL Institute of Child Health, 30 Guilford Street, London, UK

Correspondence and offprint requests to: Dagan Jenkins; E-mail: dagan.jenkins@imm.ox.ac.uk

Abstract

Background. Congenital pelvi-ureteric junction obstruction (PUJO) affects 0.3% of human births. It may result from aberrant smooth muscle development in the renal pelvis, resulting in hydronephrosis. Mice that are null mutant for the *Teashirt3* (*Tshz3*) gene exhibit congenital PUJO with defective smooth muscle differentiation and absent peristalsis in the proximal ureter.

Methods. Given the phenotype of *Tshz3* mutant mice, we considered that *Teashirt* genes, which code for a family of transcription factors, might represent candidate genes for human PUJO. To evaluate this possibility, we used *in situ* hydridization to analyse the three mammalian *Tshz* genes in mouse embryonic ureters and determined whether *TSHZ3* was expressed in the human embryonic ureter. *TSHZ2* and *TSHZ3* were sequenced in index cases with non-syndromic PUJO.

Results. *Tshz2* and *Tshz3* genes were detected in mouse ureters and TSHZ3 was expressed in the human embryonic renal pelvis. Direct sequencing of *TSHZ2* and *TSHZ3* did not identify any mutations in an initial cohort of 48 PUJO index cases, excluding these genes as a major cause of this condition. A polymorphic missense change (E469G) in *TSHZ3* was identified at a residue highly conserved throughout evolution in all Teashirt proteins, although subsequently no significant difference between the E469G allele frequency in Albanian and Macedonian PUJO index cases (3.2%) versus 633 control individuals (1.7%) was found ($P = 0.18$).

Conclusions. Mutations in *TSHZ2* and *TSHZ3* are not a major cause of PUJO, at least in Albanian and Macedonian populations. Expression of these genes in the human fetal ureter emphasizes the importance of analysing these genes in other groups of patients with renal tract malformations.

Keywords: pelvi-ureteric junction obstruction; Teashirt genes

Introduction

Congenital pelvi-ureteric junction obstruction (PUJO) occurs in 0.3% of human births and is usually diagnosed by postnatal investigations prompted by detecting fetal hydonephrosis on ultrasound screening [1–3]. PUJO can be associated with impaired kidney excretory function in which case surgery to refashion the PUJ may be undertaken [4,5]. Congenital unilateral PUJO occasionally occurs in association with a contralateral, more severe anomaly such as multicystic dysplastic kidney [6], so PUJO may represent the mild end of a malformation spectrum with aberrant morphogenesis of the ureter and renal pelvis. PUJO can occur in more than one member of the same family [7–10], so it may have a genetic basis, at least in certain cases. In some kindreds, there appears to be a dominant inheritance pattern, and Izquierdo [10] provided data to support linkage to chromosome 6p. In other families, however, PUJO is not linked to this locus [9,11], suggesting genetic heterogeneity.

PUJO is a heterogeneous condition at the anatomical level, sometimes being associated with intrinsic defects of the ureteric wall, discussed below, while in other cases a 'crossing vessel' causes external compression [7]. Antonakopoulos *et al.* [12] reported abnormal smooth muscle (SM) arrangements at the junction of the proximal ureter and renal pelvis in resected PUJs; the normal interwoven pattern was absent and instead SM bundles were arranged in 'an outer circular and an inner longitudinal layer'. dell'Agnola *et al.* [13] reported that both time between fetal diagnosis and PUJ resection and the size of the hydronephrosis were 'related to the frequency of muscle hypertrophy and fibrosis of both pelvis and ureter just above and below the junction'. Murakumo *et al.* [14] examined PUJO specimens with 'intrinsic obstruction' and noted that 'muscle fascicles were sparse and thin'. Another study [15] reported that resected PUJs were 'inflamed and markedly thickened due to a combination of muscle hypertrophy and fibrosis around muscle fibres'. While some of these aberrations are probably disruptions caused by excessive distension and/or superimposed infection, the observations also give rise to the hypothesis that at least some cases of congenital PUJO are caused by primary defects of SM differentiation and morphogenesis.

There are three mammalian *Teashirt* (*Tshz*) genes and they code for a family of transcription factors, with homologues first described in flies where they modulate anterior–posterior embryonic patterning and tissue morphogenesis [16–18]. In mice, *Tshz3* is expressed in mesenchymal cells that form the wall of the embryonic ureter, and *Tshz3* function is required for upregulation of myocardin [19], a molecule involved in the transcriptional machinery that directs the expression of SM contractile proteins [20]. Mice with null mutation of *Tshz3* do not form SM cells in the proximal ureter [19]. On a CD1 background, homozygous mutant mice invariably have bilateral fetal hydonephrosis, whereas a subset of animals with just one mutant allele have unilateral hydronephrosis [19]. Although these ureters are not anatomically blocked, urine flow is impaired because of functional obstruction caused by absent peristalsis in the proximal ureter [19]. Aspects of the mouse phenotype, with SM pathology and congenital hydronephrosis, are reminiscent of human PUJO.

Therefore, the possibility that *TSHZ* genes may be implicated in human PUJO represents a hypothesis worthy of serious study. To this end, in the current paper, we first ascertained whether the other two *Teashirt* gene family members (i.e. *Tshz1* and *Thsz2*) are also expressed during formation of the mouse ureter. We then went on to determine whether humans with congenital PUJO might have mutations of either *TSHZ2* or *TSHZ3*, the two members of the gene family expressed in fetal ureters.

Table 1. PCR primers used for PCR amplification of TSHZ2 and TSHZ3

Primer	Sequence (5′ to 3′)
HTSHZ3-F	AGTGACATCCGCAATCAACA
HTSHZ3-R	TTTTGACGTGTGGCTCTCTG
TSHZ2 e1F	GTGGGACTGGAGCGAAGTAG
TSHZ2 e1R	AAAGCTTAAGTGGGTGGTGC
TSHZ2 e2aF	GGCATGGGAAGCACTTAGTC
TSHZ2 e2aR	GACCGAGAAGGCAAGTTCTG
TSHZ2 e2bF	AAGACGCTCTGTCCAAAAGC
TSHZ2 e2bR	CCCACACTCCATGCACTTTA
TSHZ2 e2cF	GCCTGCAAGTCCCAGATCTTA
TSHZ2 e2cR	TGTCTTCTGACTCTTTCTTGACTTG
TSHZ2 e2dF	CACTCAAGTCAAGAAAGAGTCAGAA
TSHZ2 e2dR	ATCCTCAAAGCGCCTGACAT
TSHZ2 e2eF	GCTGGAAATGGATGTCAGG
TSHZ2 e2eR	AAAGCTCCTCACCAGGCTAA
TSHZ3 e1F	CCTCCCTCCCTGTCCTCAG
TSHZ3 e1R	GGAAGAGGAGGAGGAGAGCA
TSHZ3 e2aF	CCTGTCTCTCTTTCCCACCT
TSHZ3 e2aR	ACTCCACCAGGGTGTCGTAG
TSHZ3 e2bF	AGCAAGTTCCGCTGTAAGGAA
TSHZ3 e2bR	GGCTTCCCCTTTTTCATAGC
TSHZ3 e2cF	CCACTTCATCAAGGTCACCA
TSHZ3 e2cR	TCTCCTCCACTTTGGCAACT
TSHZ3 e2dF	GGAGCTGGTGAAAAAGGTCA
TSHZ3 e2dR	ACATGAATGATACGACGGCA
TSHZ3 e2eF	CCTCATCCACGGTGACAAC
TSHZ3 e2eR	TGCACTGATAGGAAGTCCCC
TSHZ3 e2fF	ATCCAAACTGTCCACCGAAC
TSHZ3 e2fR	CCTTCCACAGTTTCCCTCAA
E469GdigF	CCTACCATCACAACCCTGCT
E469GdigR	TTTCTCCTTGTCGACTTCCTTCT**G**G**G**CC[a]

60°C annealing temperature was used for all *TSHZ3* primers and for E469Gdig; a 66°C annealing temperature was used for all *TSHZ2* primers.
[a]Mismatches incorporated to engineer a diagnostic restriction site are underlined and in bold.

Patients and methods

In situ hybridization (ISH)

ISH using digoxigenin-labelled or radioactive probes was performed on sections as described [18]. For ISH of human embryos, a 997 bp TSHZ3 DNA fragment was generated by PCR with primers *HTSHZ3-F* and *HTSHZ3-R* (Table 1). The PCR product was cloned into pGEM-T Easy vector (Promega), and sense and antisense probes were generated with S^{35}UTP. Slides were exposed for 7 days at 4°C under desiccant. The human fetal renal tract to be analysed by ISH was obtained

Table 2. Summary of PUJO patients used in this study

All PUJO patients			
		Male[a]	Female
Left[b]	44	34	10
Right	20	12	8[c]
Bilateral	3	3	0
Total[d]	67	49	18

[a]Ratio of left versus right unilateral PUJO in males significantly deviates from 1:1, $\chi^2 = 10.52$ ($P = 0.0012$).
[b]Ratio of male versus female PUJO in left-sided patients significantly deviates from 1:1, $\chi^2 = 13.09$ ($P = 0.0003$).
[c]One patient was homozygous for the E469G allele.
[d]Ratio of males to females in all patients significantly deviates from 1:1, $\chi^2 = 14.34$ ($P = 0.0002$).

from the MRC-Wellcome Trust Human Developmental Biology Resource (http://www.hdbr.org/) as described [21,22].

Patients

Index cases with congenital PUJO (one each from 49 families) were ascertained by two of the authors (V. T. and Z. G.) in their clinics in Macedonia. All these individuals had presented hydronephrosis visualized on antenatal ultrasound screening. Postnatally, the specific diagnosis was confirmed using appropriate imaging, including ultrasonography to demonstrate persistent hydronephrosis and the exclusion of vesicoureteric reflux as a cause for renal tract dilatation. One case had neurofibromatosis type 1 and she was excluded from further analysis because this condition can itself be associated with renal tract anomalies [23]. Another case had distal renal tubular acidosis type 1 as well as PUJO but was retained in the current analysis. Therefore, no index case included in our primary genetic analyses had features of a recognizable, multi-organ syndrome and they were thus typical of PUJO cases presenting to Paediatric Nephrology and Urology clinics. Note that relatives of index cases had not been systematically screened to detect renal tract anomalies; however, in a few families, siblings were known to be affected (e.g. see Results section). Of these 48 index cases, 21 were of Macedonian origin, 22 were of Albanian origin, 2 were of Turkish origin, 2 were of Gypsy descent and 1 was of Bosnian origin. Subsequently, the *THSZ3* gene variant E469G was analysed in an additional 19 non-syndromic PUJO index cases (11 of Macedonian, 7 of Albanian and 1 of Gypsy descent) when DNA became available. As in other clinical studies of PUJO (e.g. 24), our cohort of 67 cases was mostly male (χ^2 test, $P = 0.0003$), and PUJO was predominantly unilateral (Table 2). In these males, there was a significant skewing towards left-sided PUJO in unilateral cases (χ^2 test, $P = 0.0012$).

Sequencing of human genomic DNA

Consent was obtained for collection of blood to be used for DNA extraction from leucocytes. *TSHZ2* and *TSHZ3* (Ensembl accession numbers OTTHUMG00000033058 and OTTHUMG00000071116, respectively) exons were amplified in standard PCR reactions from human leucocyte DNA with primers listed in Table 1. Following treatment (37°C for 30 min followed by 85°C for 15 min) with Exonuclease I (10 U; NEB) and shrimp alkaline phosphatase (2 U; Roche), PCR products were sequenced using the Big Dye Terminator kit (Applied Biosystems). To rapidly detect the *THSZ3* E469G variant, we used a diagnostic restriction digest (*Apa*I (+)) with primers E469GdigF and E469GdigR (Table 1).

Results

Gene expression in developing ureters

In embryonic mice, SM precursor cells in the wall of the proximal ureter upregulate expression of contractile proteins from embryonic day (E) 15 [19], equivalent to the end of the first third of human gestation [25]. Using ISH (Figure 1A–I), we found that expression of both *Tshz2* and *Tshz3* preceded this differentiation event because transcripts were present in mesenchymal cells in proximal ureters at E14; these patterns were also observed one day later. By contrast, no specific ureteric ISH signal for *Tshz1* could be detected in the proximal ureter. We detected expression of *TSHZ3* in peri-urothelial cells of the proximal ureter and renal pelvis in a human embryo at 9 weeks of gestation (Figure 1J and K).

Table 3. *TSHZ2* and *TSHZ3* variants identified in the initial 48 PUJO index cases

dbSNP accession	DNA	Protein	Carrier frequency in PUJO
		TSHZ2 variants	
rs739869	339G>C	R113S	7/48 (including 3 homozygotes)
rs739870	852C>T	F284Y	1/48
New SNP	1668G>C	P556P	1/48
		TSHZ3 variants	
New SNP	486C>T	S162S	1/48
New SNP	1406A>G	E469G	5/48 (including 1 homozygote)
New SNP	1692G>C	L564L	2/48
New SNP	1797G>A	T599T	1/48
rs3745784	2283T>C	A761A	13/48

These 48 individuals consisted of 21 patients of Macedonian, 23 of Albanian, 1 of Bosnian, 2 of Turkish and 1 of 'Gypsy' origin.
A number of *TSHZ3* double heterozygotes were identified—one patient was doubly heterozygous for c.1406A>G and c.2283T>C; one patient was homozygous for c.1406 A>G and heterozygous for c.2283 T>C; one patient was doubly heterozygous for c.1406 A>G and c.1692 G>C.

TSHZ genomic analyses

We sequenced the complete coding regions of *TSHZ2* and *TSHZ3* in the 48 PUJO index cases initially available to us. As well as three known single nucleotide polymorphisms (SNPs), we identified five variants, one in *TSHZ2* and four in *TSHZ3* (Table 3), previously unreported in the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/). Four variants were silent (i.e. would not be predicted to alter the encoded protein), suggesting that they were unlikely to be pathogenic. The other was a 1406A>G transition in *TSHZ3* coding for an E469G missense change in which glutamic acid, a negatively charged and hydrophilic molecule, is changed to glycine, a nonpolar and hydrophobic residue. This substitution was found to be heterozygous in four index cases and homozygous in other one (Tables 3 and 4; Figure 2). This variant alters an amino acid conserved as a charged residue in all three Teashirt proteins residing in a highly evolutionarily conserved stretch of amino acids (Figure 2B).

We next used an *Apa*I(+) diagnostic restriction digest to confirm the genotypes in the original 48 patients and to genotype an additional 19 PUJO index cases collected since our study initiated. We also sought the variant in control DNA samples from individuals of Albanian, Macedonian and Turkish descendants and additionally in UK Caucasian controls. Although the E469G allele frequency in

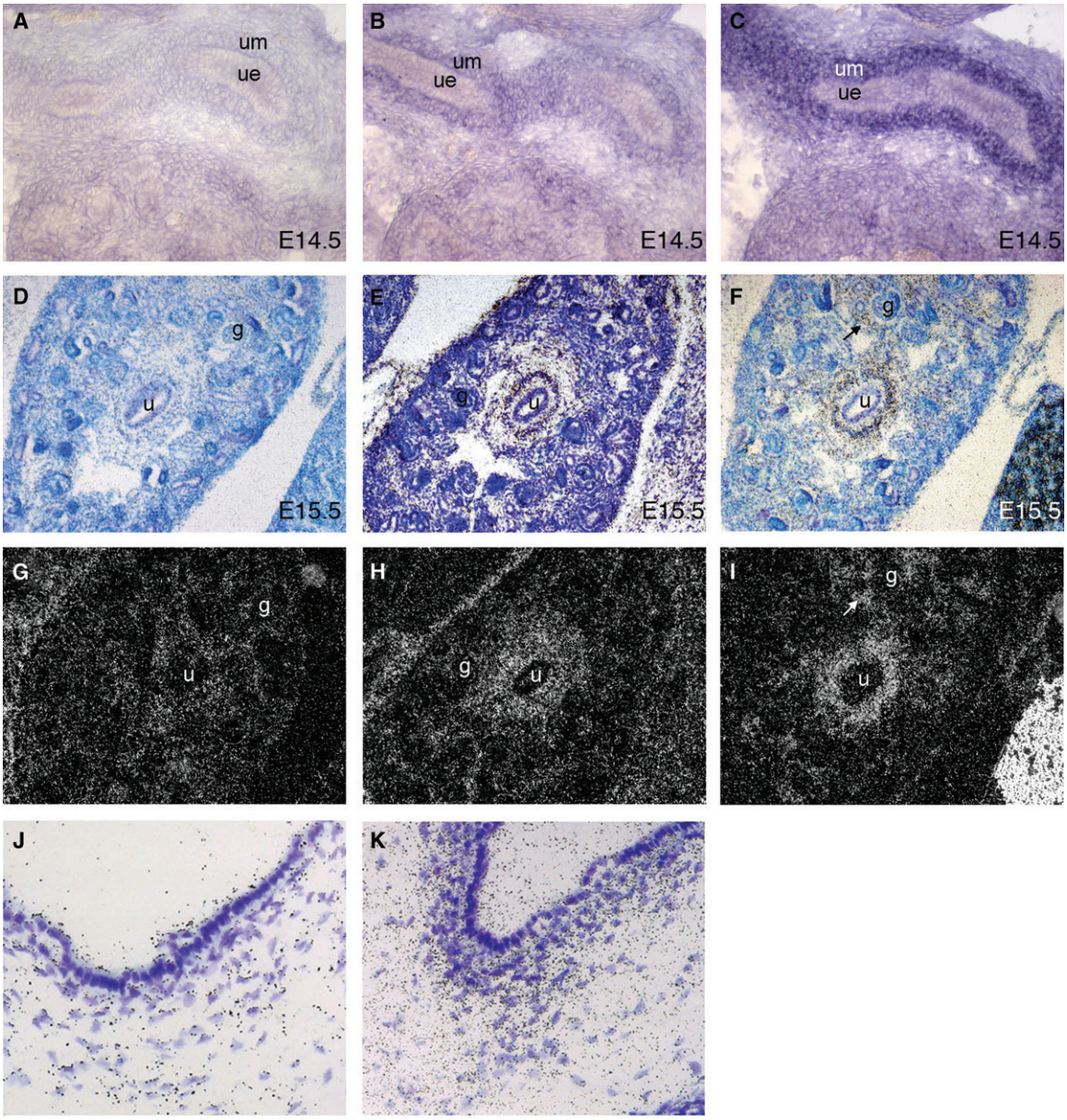

Fig. 1. *In situ* hybridization of Tshz1–3 in mouse, and TSHZ3 in human ureters. (**A–C**) Longitudinal sections of E14 wild-type proximal ureters with ISH for *Tshz1* (**A**), *Tshz2* (**B**) and *Tshz3* (**C**). Note the positive signal (purple) for *Tshz2* and *Tshz3*. (**D–I**) Transverse sections of E15 wild-type kidneys with ISH for *Tshz1* (**D** and **G**), *Tshz2* (**E** and **H**) and *Tshz3* (**F** and **I**). (**D–F**) and (**G–I**) are respective light-field (positive signal represented by black dots) and dark-field views (positive signal appears as white dots) of the same sections, with the proximal ureter/nascent renal pelvis shown in the centre of the kidney. g, glomerulus; u, ureter; ue, ureteric epithelium; um, ureteric mesenchyme. (**J** and **K**) Respective ISH of normal human proximal ureter/renal pelvis at 9-week gestation with sense control (**J**) showing only background signal, but the antisense *TSHZ3* probe showing (**K**) signal (black dots) in cells which surround the urothelium (intense blue layer).

Albanian PUJO patients (3 heterozygous individuals) was almost double that observed in 37 Albanian control individuals (5.0% versus 2.7%), the difference was not significant ($P = 0.40$). There was no difference in the allele frequency between the one heterozygous Macedonian PUJO index case and 275 Macedonian control individuals (1.6% versus 2.0%; $P = 0.73$). Neither the Bosnian nor the two Gypsy PUJO index cases carried the variant allele. One of the two Turkish PUJO index cases, a female, was homozygous for the E469G allele. Considering the frequency of this allele in a Turkish control population (5.45%), the observation of a homozygote does not conform to Hardy–Weinberg equilibrium. This, together with the fact that the frequency of the E469G allele in 37 Turkish control individuals was elevated in comparison to all other control groups combined (5.54% versus 1.7%; $P = 0.045$), led us to exclude the Turkish patients from further statistical comparisons to avoid population stratification effects. We found that the frequency of the E469G allele did not differ significantly between Albanian, Macedonian and the 321 UK Caucasian control individuals (Table 4) and so, to maximize our statistical power, we performed a combined analysis comparing

Table 4. Statistical analysis of the E469G allele in PUJO

Group (number of individuals)	E469G alleles detected	E469G allele frequency	Fisher exact test of E469G allele frequency
Albanian PUJO (30)	3/60	5.0%	$P = 0.40^{b,d}$ $P = 0.09^{b,f}$
Macedonian PUJO (32)	1/64	1.6%	$P = 0.74^{b,e}$
Turkish PUJO (2)	2/4[a]	50.0%	$P = 0.028^{b,h}$
Gypsy PUJO (2)	0/4	0.0%	–
Bosnian PUJO (1)	0/2	0.0%	–
Albanian plus Macedonian PUJO (62)	4/124	3.2%	$P = 0.18^{b,f}$
Albanian controls (37)	2/74	2.7%	$P = 0.28^{c,g}$
Macedonian controls (275)	11/550	2.0%	$P = 0.36^{c,g}$
UK Caucasian controls (321)	8/642	1.3%	–
Albanian plus Macedonian plus UK Caucasian controls (633)	21/1266	1.7%	$P = 0.045^{c,h}$
Turkish controls (37)	4/74	5.45%	–

[a]One patient was homozygous; [b]One-tail test; [c]Two-tail test; [d]versus Albanian controls; [e]versus Macedonian controls; [f]versus Albanian plus Macedonian plus UK Caucasian controls; [g]versus UK Caucasian controls; [h]versus Turkish controls.

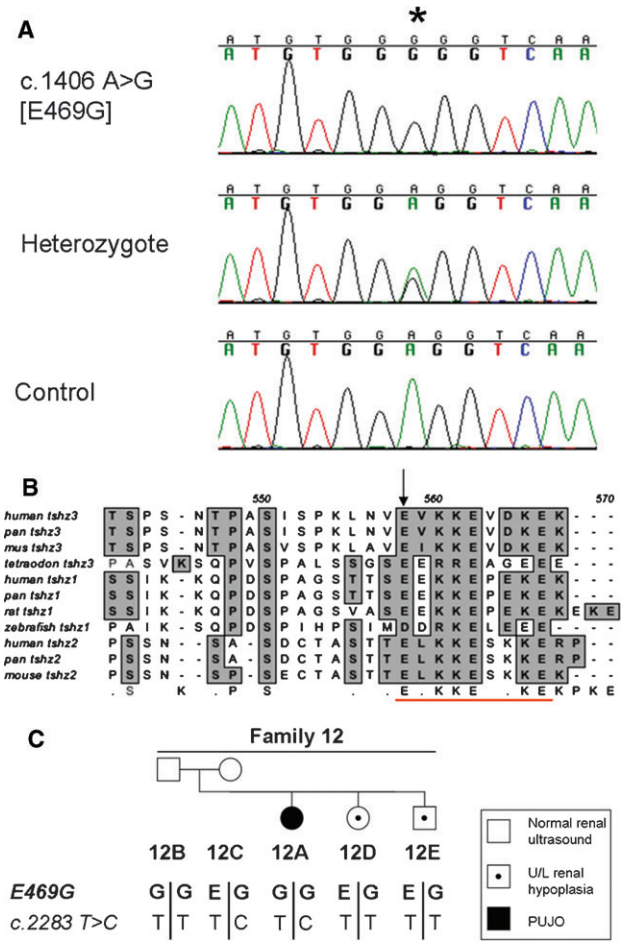

Fig. 2. The E469G polymorphism in *TSHZ3* in human PUJO. (**A**) Sequence chromatograms showing the c.1406 A > G variant encoding the E469G missense change in a homozygote (index case Family 12, top panel) and a heterozygote (middle panel). (**B**) Alignment of Teashirt proteins from a variety of species showing conservation of residue 469 (numbered for TSHZ3, indicated by the arrow) as a charged amino acid throughout evolution. Note that this residue forms part of stretch of charged residues (underlined in red) conserved in all Teashirt proteins that may represent an important structural motif. (**C**) Analysis of the haplotypes on which the E469G variant resides in a Turkish family, as determined by segregation of SNPs. Genotyping of the 2283T>C SNP (rs3745784) allowed the maternal and paternal E469G-harbouring haplotypes to be distinguished, showing that they are not shared identical-by-descent.

the allele frequency in 62 Albanian and Macedonian PUJO index cases versus all 633 Albanian, Macedonian and UK Caucasian controls. There was no evidence for an elevated frequency of the E469G allele in the disease group ($P = 0.18$), suggesting that the E469G variant is not a risk factor for PUJO in Albanian and Macedonian populations.

Figure 2C depicts the family of Turkish descent, in which the index PUJO case was homozygous for the E469G variant. Interestingly, it had been found that her two heterozygous siblings each had unilateral small kidney (i.e. presumed renal hypoplasia), with an overtly normal contralateral renal tract. Given the E469G allele frequency in Turkish controls, the difference in the proportion of homozygotes between Turkish cases (1/2) and controls (0/37) is significant ($P = 0.05$; Fisher exact test). If we assume, in the most conservative scenario, that the homozygous father is the product of a first-cousin marriage, the correct segregation of the E469G allele in the three siblings (assuming a semi-dominant disease model) would only be expected with a frequency of 2.8 per 1000 by chance. However, the fact that the unaffected father was also homozygous makes it unclear whether this variant is related to disease.

Discussion

This study shows that *Tshz3* transcripts are expressed in the proximal part of the embryonic ureter, both directly before (E14) and at the onset (E15) of muscularization [19,26]. This finding was to be expected, given the fact that the protein encoded by this gene is known to be present in these locations during mouse embryogenesis [19]. The fact that we also detect *TSHZ3* transcripts in the human embryonic ureter can be taken as evidence that gene function in ureter SM formation might be conserved between mouse and man. We also report the novel observations that *Tshz2* but not *Tshz1* is expressed in the differentiating ureter, as assessed by ISH. By contrast, the same methodology has detected *Tshz1* expression in other parts of the embryo [27], thus excluding a negative finding due to technical error.

Whether an individual is born with a urinary tract malformation is most likely determined by a complex interplay between genes and environmental factors rather than because of mutation in a single 'PUJO gene'. Indeed, only a subset of *Tshz3*$^{+/-}$ mutant mice on an outbred background have congenital hydronephrosis [19]. Moreover, the fact that congenital human PUJO, as in the current series, is predominantly unilateral similarly suggests a complex causation. Previously, variants of other genes expressed in the urinary tract have been implicated in the pathogenesis of urinary tract anomalies. These genes include *AT2*, *RET*, *ROBO2* and *UPK2* and *3* [21,22,28–32], variously implicated in duplicated renal tract, vesicoureteric reflux and

other phenotypes. Furthermore, reports of unaffected mutation carriers in affected families show that these variants are incompletely penetrant, and this is frequently manifested as unilateral disease.

Collectively, the expression data presented in this paper were consistent with *TSHZ2* (chromosome 20q13.2) and *TSHZ3* (19q13.11) as candidate genes for congenital PUJO. To our knowledge, before the current study, neither gene has been analysed in humans with congenital disease. Overall, our results suggest that mutations in these two genes do not play a major role in the pathogenesis of human PUJO. We failed to find any variants of *TSHZ2* that would be of functional significance. We did, however, identify a non-synonymous allele of *TSHZ3* at a residue that shows extensive evolutionary conservation across Teashirt proteins. Indeed, the E469 variant forms part of a motif, VEVKKEV, that may regulate sumoylation of *TSHZ3*; the amino acids YKXE represent a core sumoylation motif, and K472 is predicted to undergo sumoylation using the web-based tool, SUMOsp 2.0 (http://bioinformatics.lcd-ustc.org/gps2). Sumoylation, a protein degradation pathway, is known to regulate transcription by modifying the DNA-binding activity of transcription factors and their subcellular localization and interactions between transcription factors and their co-regulators [33]. This suggested that the E469G substitution would affect the function of the encoded protein.

To investigate whether this variant contributes to disease, we extensively analysed its frequency in several populations, but found no evidence for the association of the E469G variant with human PUJO. It remains possible, however, that this variant could contribute to disease in other groups of patients with urinary tract malformations, and this warrants future investigation. Our characterization of the genetic variation at the *TSHZ2* and *TSHZ3* loci, and especially our identification of particular haplotypes segregating with the E469G *TSHZ3* variant (Figure 2C), offers valuable information for future association studies.

Our current analysis was limited to direct sequencing of *TSHZ2* and *TSHZ3* coding regions, and so this study does not exclude heterozygous deletions or mutations in regulatory elements. It will therefore be necessary to analyse patients with PUJO for copy number variations using methods such as array-based comparative genomic hybridization or multiplex ligation-dependent probe amplification. Interestingly, translocations and a chromosomal deletion disrupting the 19q12 region in which *TSHZ3* resides have also been reported in patients with multi-organ malformations, with renal tracts affected by hydonephrosis and multicystic dysplastic kidney [34,35], implicating the disruption of long-range elements in regulating the expression of *TSHZ3*. Therefore, it may be informative to seek mutations in conserved non-coding elements of *TSHZ3* in patients because mutations in *cis*-regulatory elements have been shown to cause human disease [36] and the *Tshz* family is known to have a large number of such duplicated elements [37]. We suggest that it may also be worthwhile to screen patients with urinary tract malformations other than PUJO (such as multicystic dysplastic kidney) for *TSHZ* mutations. As well as hydronephrosis, *Tshz3* null-mutant mice exhibit respiratory problems at birth [19] that may reflect a function of the *Tshz3* gene in the brain stem and/or lungs where it is expressed (unpublished data). It is therefore possible that humans with major *TSHZ3* mutations may not survive the neonatal period, thus explaining why mutations are not found in non-syndromic survivors with PUJO.

Conflict of interest statement. None declared.

References

1. Gunn TR, Mora JD, Pease P. Antenatal diagnosis of urinary tract abnormalities by ultrasonography after 28 weeks' gestation: incidence and outcome. *Am J Obstet Gynecol* 1995; 172: 479–486
2. Ismaili K, Hall M, Donner C et al.; Brussels Free University Perinatal Nephrology Study Group. Results of systematic screening for minor degrees of fetal renal pelvis dilatation in an unselected population. *Am J Obstet Gynecol* 2003; 188: 242–246
3. Ek S, Lidefeldt KJ, Varricio L. Fetal hydronephrosis; prevalence, natural history and postnatal consequences in an unselected population. *Acta Obstet Gynecol Scand* 2007; 86: 1463–1466
4. Josephson S, Dhillon HK, Ransley PG. Post-natal management of antenatally detected, bilateral hydronephrosis. *Urol Int* 1993; 51: 79–84
5. Decramer S, Wittke S, Mischak H *et al*. Predicting the clinical outcome of congenital unilateral ureteropelvic junction obstruction in newborn by urinary proteome analysis. *Nat Med* 2006; 12: 398–400
6. Shaheen IS, Watson AR, Broderick N *et al*. Multicystic dysplastic kidney and pelviureteric junction obstruction. *Pediatr Surg Int* 2005; 21: 282–284
7. Aaron G, Robbins MA. Hydronephrosis due to aberrant vessels: remarkable familial incidence with report of cases. *J Urol* 1948; 60: 702–705
8. Jewell JH, Buchert WI. Unilateral hereditary hydronephrosis: a report of four cases in three consecutive generations. *J Urol* 1962; 88: 129–136
9. Mackintosh P, Almarhoos G, Heath DA. HLA linkage with familial vesicoureteral reflux and familial pelvi-ureteric junction obstruction. *Tissue Antigens* 1989; 34: 185–189
10. Izquierdo L, Porteous M, Paramo PG *et al*. Evidence for genetic heterogeneity in hereditary hydronephrosis caused by pelvi-ureteric junction obstruction, with one locus assigned to chromosome 6p. *Hum Genet* 1992; 89: 557–560
11. McHale D, Porteous MEM, Wentzel J *et al*. Further evidence of genetic heterogeneity in hereditary hydronephrosis. *Clin Genet* 1996; 50: 491–493
12. Antonakopoulos GN, Fuggle WJ, Newman J et al.. Idiopathic hydronephrosis. Light microscopic features and pathogenesis. *Arch Pathol Lab Med* 1985; 109: 1097–1101
13. dell'Agnola CA, Carmassi LM, Merlo D *et al*. Duration and severity of congenital hydronephrosis as a cause of smooth muscle deterioration in pyelo-ureteral junction obstruction. *Z Kinderchir* 1990; 45: 286–290
14. Murakumo M, Nonomura K, Yamashita T *et al*. Structural changes of collagen components and diminution of nerves in congenital ureteropelvic junction obstruction. *J Urol* 1997; 157: 1963–1938
15. Zhang PL, Peters CA, Rosen S. Ureteropelvic junction obstruction: morphological and clinical studies. *Pediatr Nephrol* 2000; 14: 820–826
16. Fasano L, Röder L, Coré N *et al*. The gene teashirt is required for the development of Drosophila embryonic trunk segments and encodes a protein with widely spaced zinc finger motifs. *Cell* 1991; 64: 63–79
17. Manfroid I, Caubit X, Kerridge S *et al*. Three putative murine Teashirt orthologues specify trunk structures in Drosophila in the same way as the Drosophila teashirt gene. *Development* 2004; 131: 1065–1073
18. Caubit X, Tiveron MC, Cremer H *et al*. Expression patterns of the three Teashirt-related genes define specific boundaries in the developing and postnatal mouse forebrain. *J Comp Neurol* 2005; 486: 76–88

19. Caubit X, Lye CM, Martin E *et al*. Teashirt 3 is necessary for ureteral smooth muscle differentiation downstream of SHH and BMP4. *Development* 2008; 135: 3301–3310
20. Pipes GC, Creemers EE, Olson EN. The myocardin family of transcriptional coactivators: versatile regulators of cell growth, migration, and myogenesis. *Genes Dev* 2006; 20: 1545–1556
21. Jenkins D, Bitner-Glindzicz M, Malcolm S *et al*. *De novo Uroplakin IIIa* heterozygous mutations cause human renal adysplasia leading to severe kidney failure. *J Am Soc Nephrol* 2005; 16: 2141–2149
22. Jenkins D, Bitner-Glindzicz M, Malcolm S *et al*. Mutation analyses of Uroplakin II in children with renal tract malformations. *Nephrol Dial Transplant* 2006; 21: 3415–3421
23. Morelli G, Mancini P, Lencioni R *et al*. Transperineal biopsy leads to the diagnosis of genitourinary neurofibromatosis in association with a congenital malformation of the urinary tract. *Eur Urol* 1996; 29: 491–493
24. Karnak I, Woo LL, Shah SN *et al*. Prenatally detected ureteropelvic junction obstruction: clinical features and associated urologic abnormalities. *Pediatr Surg Int* 2008; 24: 395–402
25. Matsuno T, Tokunaka S, Koyanagi T. Muscular development in the urinary tract. *J Urol* 1984; 132: 148–152
26. Yu J, Carroll TJ, McMahon AP. Sonic hedgehog regulates proliferation and differentiation of mesenchymal cells in the mouse metanephric kidney. *Development* 2002; 129: 5301–5312
27. Coré N, Caubit X, Metchat A *et al*. Tshz1 is required for axial skeleton, soft palate and middle ear development in mice. *Dev Biol* 2007; 308: 407–420
28. Nishimura H, Yerkes E, Hohenfellner K *et al*. Role of the angiotensin type 2 receptor gene in congenital anomalies of the kidney and urinary tract, CAKUT, of mice and men. *Mol Cell* 1999; 3: 1–10
29. Yang Y, Houle AM, Letendre J *et al*. RET Gly691Ser mutation is associated with primary vesicoureteral reflux in the French-Canadian population from Quebec. *Hum Mutat* 2008; 29: 695–702
30. Lu W, van Eerde AM, Fan X *et al*. Disruption of ROBO2 is associated with urinary tract anomalies and confers risk of vesicoureteral reflux. *Am J Hum Genet* 2007; 80: 616–632
31. Feather SA, Malcolm S, Woolf AS *et al*. Primary, non-syndromic vesicoureteric reflux and its nephropathy is genetically heterogeneous with a locus on chromosome 1. *Am J Hum Genet* 2000; 66: 1420–1425
32. Belk RA, Thomas DF, Mueller RF *et al*. A family study and the natural history of prenatally detected unilateral multicystic dysplastic kidney. *J Urol* 2002; 167: 666–669
33. Watts FZ. SUMO modification of proteins other than transcription factors. *Semin Cell Dev Biol* 2004; 15: 211–220
34. Groenen PM, Garcia E, Debeer P *et al*. Structure, sequence, and chromosome 19 localization of human USF2 and its rearrangement in a patient with multicystic renal dysplasia. *Genomics* 1996; 38: 141–148
35. Kulharya AS, Michaelis RC, Norris KS *et al*. Constitutional del(19)(q12q13.1) in a three-year-old girl with severe phenotypic abnormalities affecting multiple organ systems. *Am J Med Genet* 1998; 77: 391–394
36. Kleinjan DA, van Heyningen V. Long-range control of gene expression: emerging mechanisms and disruption in disease. *Am J Hum Genet* 2005; 76: 8–32
37. McEwen GK, Woolfe A, Goode D *et al*. Ancient duplicated conserved noncoding elements in vertebrates: a genomic and functional analysis. *Genome Res* 2006; 16: 451–465

Received for publication: 22.12.08; Accepted in revised form: 11.8.09

Nephrol Dial Transplant (2010) 25: 60–68
doi: 10.1093/ndt/gfp451
Advance Access publication 10 September 2009

Klotho reduces apoptosis in experimental ischaemic acute kidney injury via HSP-70

Hidekazu Sugiura, Takumi Yoshida, Michihiro Mitobe, Satsuki Yoshida, Shunji Shiohira, Kosaku Nitta and Ken Tsuchiya

Department of Medicine IV, Tokyo Women's Medical University, Tokyo, Japan

Correspondence and offprint requests to: Takumi Yoshida; E-mail: tyoshida@kc.twmu.ac.jp

Abstract

Background. High Klotho expression has been detected in the kidney, and since the results of a recent study suggested that Klotho induction mitigates ischaemic damage in the kidney, in the present study we explored the mechanism by which Klotho expression reduces renal ischaemia-reperfusion injury (IRI).

Methods. Male mice were subjected to bilateral renal ischaemia for 30 min and reperfusion for 24 h, or to a sham operation. Both the IRI group and the sham group were intravenously injected with an adenovirus harbouring the mouse Klotho gene (ad-kl) before renal IRI. In addition, mIMCD3 cells induced to overexpress Klotho by transferring the Klotho gene with ad-kl were analysed by DNA microarray and real-time PCR. Renal expression of Klotho and several genes selected by DNA microarray were assessed by real-time PCR or Western blotting, and TUNEL staining was performed to assess apoptosis.

Results. Prior administration of ad-kl to the mice resulted in robust induction of Klotho mRNA in the kidney and liver. Ad-kl transfer improved the plasma creatinine values and mitigated the histological damage and apoptosis induced by IRI. Expression of several genes was altered in mIMCD3 cells as a result of the change in Klotho expression, and