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Research Article
Molecular Screening of *PAX2* Gene Polymorphism in Primary Vesicoureteral Reflux Patients in Taif Governorate, KSA

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Abstract

Background and Objective: Primary Nonsyndromic Vesicoureteral Reflux (PVUR) is a widespread genetic malformation and considered a prevalent Congenital Abnormality of the Kidney and Urinary Tract (CAKUT). Mutations in the *PAX2* gene have been associated with abnormalities in the kidney extending from CAKUT to oncogenic processes. The present study analyzes the *PAX2* polymorphisms and their association with primary VUR in Saudi children patients from the Taif governorate. **Materials and Methods:** Fifteen children with primary VUR were identified and screened for gene mutations in the *PAX2* gene by direct sequencing method of purified Polymerase Chain Reaction (PCR) products of all exons to elucidate the correlation between *PAX2* gene and VUR. **Results:** Seven new variants have been defined. Three polymorphic missense variants in homozygous genotype form were found in intron 8 and detected in eight patients, One missense mutation was found in exon 10 in the site of transactivation domain and detected in ten patients and *in-silico* analysis predicted it as a pathogenic one, Three mutations were found in exon 11 and detected in all patients as a compound homozygous. **Conclusion:** *PAX2* is important for normal kidney development and mutations in the gene possibly lead to disturbance in the protein structure and could be non-functional thus mutations in *PAX2* may be one of the causes of PVUR in Saudi Arabia. Further investigation is necessary to understand the aetiology of disease and maybe other genes implicated in VUR.

Key words: Vesicoureteral reflux, molecular screening, *PAX2*, *in-silico* analysis, mutations, genetic malformation, oncogenic process

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Congenital Abnormalities of the Kidney and Urinary Tract (CAKUT) are famous for a high inter and intra-familial variability in phenotypic outcome[1]. They are involved in some defects such as kidney agenesis, hydronephrosis, hydroureter and vesicoureteral reflux[2]. These events are often correlated with chronic renal disease in children. Vesicoureteral reflux (VUR) refers to a case in which the abnormal retrograde flow of urine from the bladder into the ureter and toward the kidney secondary to the abnormal functional vesicoureteric junction. This junction usually acts as a one-way valve, permitting urine flow from the ureter into the bladder and closing during urination, preventing backflow[3]. The effective function of the valve is based on the harmonious action of the length of the submucosal ureter, the width of the ureteric opening, the muscles of the trigone and ureter and coordinated ureteric peristalsis[4]. VUR is revealed generally during emptying, when intravesical pressure elevates or at any time in the voiding cycle, especially when the bladder function is abnormal[5].

The intensity of reflux is expressed as grades, which is dependent on the system decided by the International Reflux Study in Children[6]. Two types of VUR in children: the primary type, in which the baby is born with an inaccurate valve situated where the ureter and the bladder join, while the secondary type illustrates the presence of a blockage somewhere in the urinary system, caused probably by a bladder infection that makes the ureter swell, leading to urine reflux to the kidneys[7,8]. This study focused on Primary Vesicoureteral Reflux (PVUR).

Primary Vesicoureteral Reflux (VUR, OMIM, 193000) is a nonsyndromic congenital anomaly that affects about 1% of young children and considers one of the most prevalent congenital anomalies affecting humans[9]. PVUR is thought to be linked with the abnormally lateral insertion of the ureters into the bladder leading to an enlarged ureteral opening and a shortening of the segment of the ureter, which normally crosses indirectly through the bladder wall. Individuals with VUR are susceptible to Urinary Tract Infection (UTI), indeed, about 30% of children who suffered from UTI have VUR[9]. VUR has a strong hereditary history, with about a 30-50 folds increase in incidence in first-degree relatives of VUR probands[10].

Some genes are involved in the urinary tract, kidney development and Ureteric Budding (UB). Most mutations in these genes specified encode transcription factors and signaling molecules that arrange these processes and are believed to be possible candidate genes for VUR susceptibility[11-14]. The country of parents in which they were born and their Heritage could define the ethnicity of their children because some reports suggested the effect of that on the diagnosis of children with VUR[15]. *PAX2* is one gene of the ''paired-box'' family of transcription factors that plays an important role during embryonic development in the formation of tissues and organs and mutations in this gene have been associated with the development of CAKUT[16]. Two intact copies of *PAX2* are required for normal renal development and its codes for a transcription factor necessary for differentiation of the epithelial components of the fetal kidney and ureter[17]. Expression of the *PAX2* gene is critical for the development of the optic nerve, inner ear, CNS and urogenital tract[17,18]. The mechanisms by which the *PAX2* gene takes part in the urinary tract and renal morphogenesis are incompletely defined.

The *PAX2* gene maps to human chromosome 10[19] and consists of 12 exons spread over about 86 kb of genomic DNA and consists of some domains[18,20]. Exons 1-4 (amino acids 16-142) include the paired box domain which has recognized DNA-binding properties. Exon 5 contains another highly conserved octapeptide domain (amino acids 185-192). The octapeptide domain seems to have some roles in the suppression of transactivation of target genes[21]. Exon 7 (amino acids 256-278) encodes partial homeodomain that functions in DNA binding in collaboration with the paired box[22,23].

The transactivation domain is encoded by exons 7-12 (amino acids 279-373) and located at the carboxy-terminal portion of the *PAX2* protein and is expected to be accountable for the ability of *PAX2* to regulate the transcription of target genes, this domain rich in serine, threonine and proline and seems to function as a repressor and an activator for transcription of gene[24].

Some studies showed that *PAX2* polymorphisms are associated with renal-coloboma syndrome, Multicystic Dysplastic Kidney (MCDK), Renal Hypodysplasia (RHD) and Vesicoureteral Reflux (VUR)[23,25-27]. This association is probably explained by the fact that *PAX2* codifies a necessary transcription factor to the regulation of the polarization and induction of epithelial structures of the kidney and ureter[28].

The rate of consanguinity in the Saudi population is high (52-56% of marriages) and leads to a high percentage of genetically intermediated renal diseases[29]. It was demonstrated, the high prevalence of genetically transmitted renal diseases, particularly those with an autosomal recessive pattern[30]. The prevalence of renal diseases is not exactly known because only a few studies of literature on this subject were done based on clinical features in Saudi children and this insufficient to evaluate the scale of VUR in some areas in Saudi Arabia[31-33].

Mutational screening of *PAX2* may let to understand the molecular basis of this disease in a better method and offer physicians a future method for the diagnosis and management of VUR. In Saudi Arabia, there is a lack of molecular screening studies of VUR, therefore, the major objective of the present study is to detect the mutations in the *PAX2* gene in primary VUR children patients in Taif province that will allow better diagnosis and evaluates a possible role of *PAX2* mutations in the VUR patients.

MATERIALS AND METHODS

Patients: Prospective study of 15 children with primary VUR was performed and the pediatric nephrologists and radiologists of the outpatient services of hospitals assessed the patients. This study was carried out at molecular genetics Laboratory, Deanship of Scientific Research, Taif University, KSA from January, 2017 and December 2018 and was approved by the bioethics committee of Al-Hada Armed Forces Hospital (PTRC#15-05-227) and informed consent for children was obtained from their parents. Patients with VUR were chosen according to the special inclusion criteria: the absence of any other malformation diagnosis except for primary VUR, children age between 0-12 years. exclude all patients with secondary VUR (e.g., structure bladder outflow obstruction, neurogenic bladder and posterior urethral valves) or presence of syndromic features or other abnormalities in the urinary tract (e.g., complex multiorgan syndromes, ureterocele and obstructive hydronephrosis) and adult patients. For every patient, site of VUR, the grade of VUR, association with UTI, hypertension, CKD, Renal U/S and family history for VUR and UTI were detected.

All Primary VUR patients either unilateral or bilateral were diagnosed by Voiding Cystourethrography (VCUG) and grade I-V according to the vesicoureteric reflux international system of radiographic grading[6]. Samples of primary VUR affected patients of any grade for this study were collected from the Armed Forces and Children's Hospitals in the Taif region, KSA. Extraction of Genomic DNA: Total DNA was extracted immediately from peripheral blood by Genomic DNA Purification Kit (Thermo Fisher, Waltham, MA, USA) as described by the protocol of the manufacture. The purity of the extracted genomic DNA was evaluated through agarose gel electrophoresis.

Amplification of DNA via PCR: To determine the mutations in *PAX2* and their roles in human VUR, specific primers[34] in Table 1 were used for amplification of extracted genomic DNA fragments spanning all coding sequences and exon-intron borders of *PAX2* gene by polymerase chain reaction (PCR). PCR reaction mixture of the final volume of 50 µL included: 50 ng of genomic DNA, 0.2 µM of forward and reverse primer and 25 µL of GoTaq® green master mix, with the remaining volume comprising Nuclease-Free Water (Promega, USA). The quality and purity of PCR products were confirmed by agarose gel electrophoresis.

Direct DNA Sequencing: Sequencing of the purified PCR specific fragments using the same primers were employed in the PCR amplification process on both forward as previously described[35] and reverse directions and it was repeated to confirm reproducible results. The PCR purified products were sequenced on both strands using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit on a 3130 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA) and the raw sequencing results were collected using the Data collection and analyzed using sequencing analysis software version 3.1 (Life Technologies, Grand Island, NY, USA). The DNA sequences were analyzed using the SeqScape software version 2.7 (Applied Biosystems, USA) for base-calling and mutation

Table 1: Primers and PCR conditions used to amplify genomic DNA segments of the PAX2 gene

Primers	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temperature (°C)/time (sec)	Product size (bp)
PA_1	GTTCACTCATCCTCCCTCCCCCACC	GGAGCCGGGCGCGGGTACTC	65/30	179
PA_2	CTGTGTGTGGGGTGTTGTGTT	AAGGCGTCTCTCCCGGGACAGCTGC	60/30	246
PA_3	CCGGCTTTCCCGGCGCAGGTA	GAGGAAGCTGGAGTCCAGCC	60/30	262
PA_4	CGGAATAGGAGTGGCATTTGA	CTCTAGGTGGGATCTGGTTT	54/30	181
PA_5	TGATGCCATTTCCTCCTTCC	GCCACACCTCTTCCCTCCT	54/30	175
PA_6	CAGTGTTTGTCTGTCTCTTATTTGCT	ATGTTCCCTCTGGCCCTCA	54/30	121
PA_7	CGCCCCGAGTGTCCATGTGTT	TACTTCTGCAAGCAGAAAGCTCCCT	60/30	234
PA_8	CCTTTCTCTGTGCGTGCATCAATAGA	GGCACCCTCCACTGACAGCAG	60/30	227
PA_9	CCCTTCCCCTTTGTGTTTTT	AGGCAGCTGCAGCATTGT	50/30	151
PA_10	CCCCTCCCTGCAAACCAC	CGCTGTGAGGGCCATGAC	54/30	150
PA_11	GCAGGCGTCACATCCCCACTC	CCGGCCACCAGGTGGCGT	60/30	148
PA_12	ATGTGGAGGCCGAAGCTG	TCTGACCCAGCCCATTCTTCT	54/30	163

detection. The results of mutations and polymorphic variants detected in this study were named through the nomenclature of the Human Genome Variation Society.

***In-silico* analysis:** To appreciate the possible effect of the pathogenic mutations detected in the present study, the PolyPhen2 web-site program (http://genetics.bwh.harvard.edu/pph2/) was used, this program employs the sequence homology and information of the 3D structure of the protein to expect the probable effect of an amino acid substitution on the structure and function of a human protein. The results are assorted as benign, possibly damaging, probably damaging or unknown[36].

RESULTS

Clinical data: Fifteen randomly chosen patients of PVUR were used in the study for the screening of mutations in *PAX2*, there is no information that renal diseases were found across their relatives. Ten males (67%) and five females (33%) enrolled in this study. Their ages ranged from 15 days-12 years. The mean age at diagnosis was 39.04±months. All patients are suffered from severe PVUR (five patients have grade IV and the other ten with grade V). Nine patients were detected as having additional UTI (60%). VCUG studies showed that the VUR was bilateral in 9/15 (60%) of patients, unilateral right-sided VUR in 2/15 (13.3%) and unilateral left-sided VUR in 4/15 (26.7%). Two patients 2/15 (13.3%) have Chronic Kidney Disease (CKD). Six patients 6/15 (40%) have renal scars as appeared by DMSA renal scan for the patients.

Screening for mutations in *PAX2*: Direct DNA sequencing of purified PCR products of all 12 exons of *PAX2* in all primary VUR patients revealed the presence of seven novel variants. These variants have not been previously described in the *PAX2* database and detected intron 8 and exons 10, 11. Three polymorphic missense variants in homozygous genotype form c.4398 C>T, c.4402 A>T and c.4408 A>T were found in intron eight, these variants were detected in eight patients (8/15), five boys and three girls as a homozygous form (Fig. 1). One missense mutation in c4756C>T and p.P373L in the sequence of exon 10 in the site of transactivation domain. This mutation was detected in ten patients (10/15), seven boys and three girls as a heterozygous genotype (Fig. 2). This mutation according to the PolyPhen-2 web-site program is predicted to be probably damaging and considered as a pathogenic one and consequently affect the function of the *PAX2* protein. Three mutations were found in exon 11. The first one was

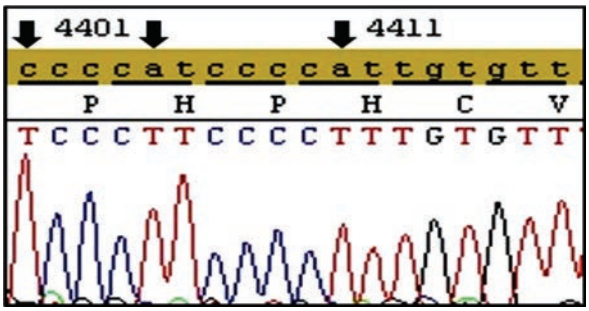

Fig. 1: Three polymorphic variants detected in intron 8 of the *PAX2* gene in primary VUR patients
Polymorphic variants in homozygous genotype form c.4398 C>T, c.4402 A>T and c.4408 A>T. Black arrows indicate the site of variants

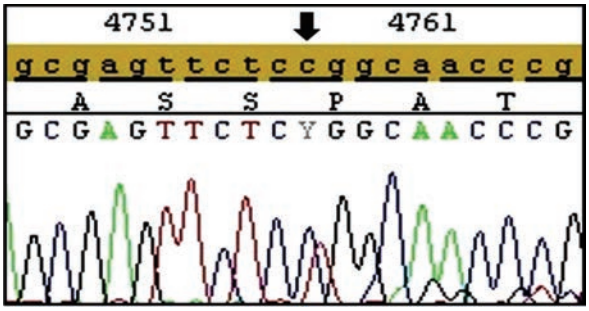

Fig. 2: Mutation detected in exon-10 of the *PAX2* gene in primary VUR patients
A missense mutation in heterozygous genotype form c.4756 C>T, p.P373L. Black arrows indicate the site of mutation

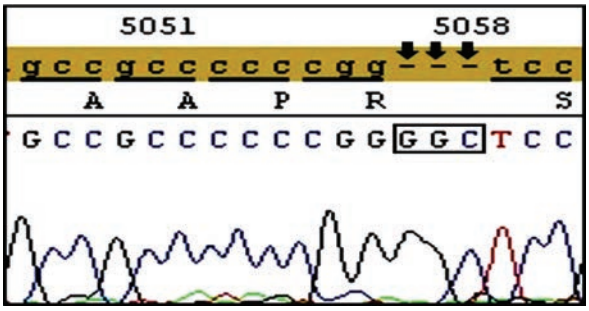

Fig. 3: Mutation detected in exon-11 of the *PAX2* gene in primary VUR patients
Insertion mutation c.5057_5058 InsGGC, InsGly. Black arrows indicate the site of mutation

found as the insertion of three nucleotides between the position c.5057_5058InsGGC, InsGly (Fig. 3). The second and third mutations in exon 11 were detected respectively, in positions c5073 C>G and c5074 G>C, p.R409A as a homozygous genotype (Fig. 4). It was interesting that all

Table 2: Summary of PAX2 variants detected in VUR patients in this study

Location	DNA sequence changes	AA changes	Number of patients	Protein domain
Intron-8	c.4398 C>T	Intronic	8	
	c.4402 A>T	Intronic	8	
	c.4408 A>T	Intronic	8	
Exon-10	c.4756 C>T	p.P373L	10	Transactivation
Exon-11	c.5057_5058 InsGG	Ins, Gly	All	Transactivation
	c.5073 C>G	p.R409A	All	
	c.5074 G>C	p.R409A	All	

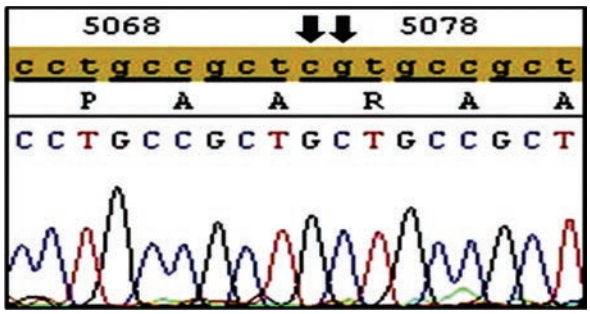

Fig. 4: Mutations detected in exon-11 of the *PAX2* gene in primary VUR patients
A missense mutation in homozygous genotype form c.5073 C>G and c.5074 G>C p.R409A. Black arrows indicate the site of mutation

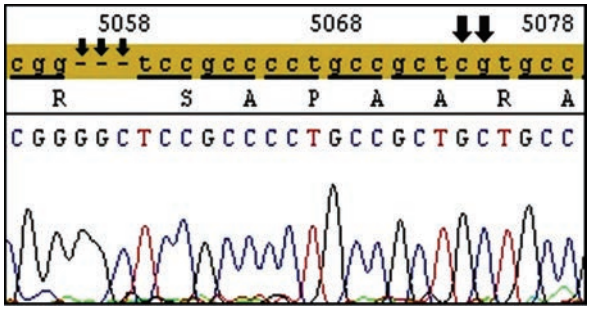

Fig. 5: Compound mutations detected in exon-11 of the *PAX2* gene in all patients of primary VUR used in this study
Insertion mutation c.5057_5058 InsGGC and the other two missense mutations in homozygous genotype forms c.5073 C>G and c.5074 G>C p.R409A. Black arrows indicate the site of mutation

patients were carried all three mutations in this exon as a compound homozygous (Fig. 5). It was observed in some cases the presence of more than one mutation in the same patient. Thus, seven new polymorphic variants detected in VUR patients in this study, three in intron-8 and one in exon-10 and three in exon-11 (Table 2).

DISCUSSION

In this study, 7 new variants have been detected in the *PAX2* gene in Saudi children with PVUR. The prevalence of VUR is about 1% and considers as one of the most detected congenital abnormalities[37]. Genetic factors consider the most important factors in the aetiology of primary VUR. Currently, VUR has been diagnosed through radionuclide micturating cystography or by voiding cystourethrography in young children, which are harmful and costly, thus, the detection of genes implicated in VUR could lead to the progression in diagnosis by genetic screening for infants[9].

The family of *PAX* genes are developmental genes encoding nuclear transcription factors, It plays an important role during the development of the urinary tract, the previous studies show that *PAX2* plays an important role in the multiple cell lines development and proliferation, organs development and repair processes[18,38], branching of the ureteric bud[39], *PAX2* influence the branching process by organizing the interaction between the UB and the metanephric blastema[39,40] and supposed to act as a transcription factor and affect the regulating some genes required for normal development of kidney and eye[41]. Irregular expression of *PAX2*, both increased or decreased, during the life span and this may be harmful to renal function and structure[42]. Association *of PAX2* knockout mice models with the syndrome of renal coloboma proposes that *PAX2* would be a suitable nominee gene as causative factors in the pathogenesis of VUR[43].

Mutations in the *PAX2* have been demonstrated to cause some renal diseases[44-46]. The previous study was showed that the correlation between *PAX2* polymorphisms and vesicoureteral reflux in Brazilian patients' samples of congenital anomalies of the kidney and urinary tract[27].

The protein level of *PAX2* and the expression of messenger RNA were remarkably increased in VUR patients, proposing a correlation with VUR[47]. The Association of *PAX2* polymorphisms with VUR can be elucidated by the fact that *PAX2* codifies a fundamental transcription factor to the organization of the induction and polarization of epithelial.

This study demonstrates for the first time, polymorphism analysis of the *PAX2* gene in primary VUR patients of Saudi children in the Taif governorate, Saudi Arabia.

Seven variant polymorphisms have been detected for the first time in the *PAX2* gene, three polymorphisms are intronic and four are detected in the transactivation domain.

The remarkable effect of *PAX2* in the development of the human renal system was previously reported[47-49]. The fundamental role of the expression of the transcription factor is encoded by the DNA binding domain of *PAX2* for renal epithelium development. A single *PAX2* mutant allele in humans displays vesicoureteral reflux, renal hypoplasia and optic nerve colobomas[17]. The expressions of two copies of the *PAX2* gene, in both the early mesenchymal derived epithelium and in the branching ureteric bud, were required for normal renal development in humans[17]. Overexpression of *PAX2* in both humans and mice is associated with overgrowth of the epithelium with tumor or cyst formation, while its deficiency leads to defective growth of the fetal kidney and ureter[17]. A decrease in the number of nephrons resulted from the deletion of a single *PAX2* allele in mice, frequently correlated with megaureter suggesting vesicoureteral re ux[50].

Heterozygous mutations of *PAX2* are correlated with the reduction in the branching of the ureteric bud and increase in apoptosis, because of a lowering in *PAX2* gene dosage during a crucial time in kidney development previously[51,52].

Segments of intronic DNA probably retain in the mRNA due to the intronic polymorphisms, altered pre-mRNA splicing or whole exons being spliced out of the mRNA. These variations could result in a non-functional protein production[53]. It was demonstrated that an intronic variant can reduce the *PAX2* expression level during the development of fetal kidney[54]. Thus, the three intronic variants in intron eight in this study may affect the level of *PAX2* expression or production of the non-functional protein of *PAX2* that may lead to VUR.

Heterozygous mutation in exon 10 results in the partial substitution of essential, non-polar and neutral amino acid proline with non-essential, non-polar and neutral amino acid leucine and this may cause a change in the resulted protein and its functions.

It is known that every single codon would code for a new amino acid, the glycine insertion occurred in exon 11 resulted in a frameshift, resulting in completely different protein coded for during translation.

Polar, hydrophilic and strong basic wild type amino acid residue, arginine was mutated to the non-polar, hydrophobic and neutral amino acid residue, alanine, this changes in amino acids in exon 11 in the transactivation domain of *PAX2* can be very important and affect the structure and the function of a protein.

The precise effect of these four mutations in exons 10 and 11 detected in the site of the transactivation domain is not known but they possibly lead to disruption in the structure of this part of the *PAX2* protein and produce a nonfunctional protein that leads to loss of its normal function. This agrees with the previously demonstrated that, the carboxy-terminal portion of the *PAX2* protein, encoded by exons 7-12, is a threonine and serine-rich domain and expected to be significant for the *PAX2* protein function as transcriptional activation of target genes[45,52]. Thus, mutations detected in the transactivation domain may affect this process.

It was demonstrated that mutations in *PAX2* can lead to some disease spectrum of CAKUT, including renal cysts, renal hypodysplasia, VUR and multicystic dysplastic kidneys[23,27]. Moreover, several possible nominee genes implicated in the pathogenesis of VUR and related urinary tract malformation were demonstrated in previous genetic studies of syndromes with associated VUR[12] and elucidate that the genetic pattern of VUR is heterogeneous. Despite the *PAX2* plays a remarkable role during the development of the kidney, the accurate effect of *PAX2* mutations on pathogenesis is not known. The present study has revealed that the importance of the *PAX2* gene for normal kidney development and mutations in this gene possibly lead to disturbance in the protein structure and could be non-functional thus, the mutations in *PAX2* may be one of the causes of PVUR in Saudi Arabia and can use as a genetic diagnostic tool for PVUR. The number of patients is small and consider as a clear limitation. Further, an investigation is necessary to understand the aetiology of disease and maybe other genes implicated in VUR.

CONCLUSION

In conclusion, to the extent of our information, this study demonstrates for the first time that PAX2 mutations in PVUR children patients in Saudi Arabia thought to be involved in the pathogenesis of VUR in our samples and support the role of the *PAX2* gene as an important candidate gene that affects Vesicoureteral Reflux (VUR) and suggests that VUR is genetically heterogeneous.

SIGNIFICANCE STATEMENT

This study discovers and elucidates for the first time the existence of the *PAX2* gene mutations in PVUR Saudi children patients. These results are helpful to understand the role of such genes in the development and pathogenesis of PVUR among Saudi children and can benefit as a diagnostic tool for the disease.

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