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Evaluation of exon-1 HOX-B7 mutations in Turkish reflux nephropathy patients

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ABSTRACT. We examined a possible influence of HOX gene mutations, which encode factors controlling anterior-posterior development, on vesicoureteral reflux (VUR). For this purpose, we evaluated the HOX-B7 gene as a promoter of the RET gene. From May 2014-June 2015, 33 pediatric patients diagnosed as having VUR at a university urology clinic were enrolled in the study. Patients with other urological malformations were excluded. The mean age was 69 ± 37 months. We found single genetic polymorphisms in 21 patients on HOX-B7 (exons 1 and 2), and 17 patients had polymorphisms only on exon 1. This variant is in a non-encoding area on the gene locus. Nevertheless, the results showed that these variants could be involved in VUR pathogenesis. Exon 1 mutation was found in six patients who had moderate VUR (grade III) and in seven patients who had severe VUR (grades IV and V). Eight patients with exon 1 variants had renal scarring due to reflux, and seven had homozygote mutations. When we examined the correlation between the duration of VUR and renal scarring, the duration of renal scar development in patients with exon 1 variant clustered at 15 months, but there was no significant difference between groups (P = 0.15), and there was no association between exon 1 variations and renal scarring (P = 0.86). The results showed that these variants could be an important gene for VUR pathogenesis. We concluded that a HOX-B7 variant of exon 1 was associated with

moderate to severe VUR. Our study support the conclusion that HOX-B7 is an important gene for VUR development, but there was no significant association with renal damage.

Key words: Pediatric; Urine reflux; Genes; Mutations; Ureter

INTRODUCTION

Vesicoureteral reflux (VUR) is the abnormal retrograde urinary flow from the bladder to the kidney. VUR affects approximately 1 to 2% of the general population and is commonly associated with recurrent urinary tract infections, renal malformations and reflux nephropathy in children (Risdon et al., 1993; Williams et al., 2008; Briggs et al., 2009). Furthermore, VUR also can be associated with healthy renal development during the prenatal period (Cendron, 2008). Voiding cystourethrogram (VCUG) is the most important diagnostic tool for the diagnosis and grading of VUR (Darge, 2002; Williams et al., 2008; Briggs et al., 2009).

The incidence of VUR is 30-50% in first-degree relatives, 50% concordance among dizygotic twins, and 100% concordance among monozygotic twins (Briggs et al., 2009). This incidence is due to genetic factors that have been implicated in the pathogenesis of VUR. Mackie and Stephens (1975) described the ureteric bud (UB) theory, in which a caudal location from the orthotropic side would cause a refluxing ureter and a malformed kidney. UB development is regulated by various gene loci, including PAX, Agtr2, UPK, SLIT/ROBO; overexpression or downregulation of these genes gives rise to malformed kidneys and VUR by affecting UB growth (Nishimura et al., 1999; Oshima et al., 2001; Kong et al., 2004; Murawski et al., 2007; Liaw et al., 2018).

Homeobox (HOX) genes encode transcription factors that control anterior-posterior development during the embryological period. HOX genes are characterized by a highly conserved trihelix homeodomain that binds specific DNA sequences. A total of 39 HOX genes have been described from paralogous clusters (Rubin et al., 2007). Over 13 HOX genes are expressed in the ureteric bud and its branches. HOX-B7 expresses the ureteric bud during early embryogenesis (Patterson and Potter, 2004). The first descriptive study about HOX-B7 showed that overexpression of exon 1 and exon 2 were associated with heart malformation and kidney anomalies (Argao et al., 1995). Another early study described RET signaling, which is regulated by HOX-B7. RET is a proto-oncogene that first demonstrated a role in the development of UB, and a study showed that a HOX-B7/RET transgene caused new or ectopic branches, thereby inhibiting kidney development (Sirinivas et al., 1999). Additionally, a French-Canadian study showed that RET mutation was found in 70% of VUR patients. (Yang et al., 2008)

Furthermore, similar to other HOX genes, HOX-B7 was also associated with cancer pathogenesis and studies have shown that HOX-B7 is involved in the regulation of proliferation, angiogenesis of cancers and hematopoiesis (Calvo et al., 2000; Miller et al., 2003; Samuel and Naora, 2005; Segara et al., 2005; Wu et al., 2006). HOX genes are also transcriptional regulatory genes that are activated during embryologic development; when they are dysregulated, neoplastic diseases can occur (Miller et al., 2003; Samuel and Naora, 2005; Segara et al., 2005).

Though the abovementioned studies described UVJ development, in the current study, we aimed to identify HOX-B7 gene mutations in children with primary VUR.

MATERIAL AND METHODS

From May 2014 to June 2015, 33 Turkish pediatric VUR patients were evaluated for primary VUR and graded as I to V, according to International Reflux Study Guidelines (Lebowitz et al., 1985). This study was approved by the University of Cukurova Ethics Committee, approval number #H8/4,2014. The parents or legal guardians of the patients were informed that the clinical and laboratory data would be used for scientific purposes, and their written consent was obtained. We obtained blood samples and leukocyte DNA samples for the study. We recruited Turkish pediatric VUR patients. All past medical histories and radiological examinations were collected. All past medical histories, including ultrasound images, voiding cystourethrography, renal scintigraphy, and dimercaptosuccinic acid (DMSA) data were collected. Renal dysplasia was defined as a difference of size compared to the contralateral kidney on ultrasonography and reduced uptake of DMSA (Cendron, 2008).

Sequencing and Sequence Analyses

All patients were screened using established screening protocols, which included sequencing the coding region, splice junctions and flanking intronic sequences amplified by polymerase chain reaction (PCR) in duplicate samples of genomic DNA from each individual. The DNA was extracted using the GF-1 blood DNA extraction kit (catalog number GF-BD-050), according to the manufacturer's instructions. Approximately 100 ng of genomic DNA was amplified in a 50 mL reaction volume containing 5 µL Taq buffer (10X containing KCl), 200 mM dNTPs, 0.75 mM MgCl2, 20 pmol of each primer and 0.5 units of Taq DNA polymerase. The two sets of primers were as follows:

F1: 5' (M13F)-TCACGTGGTCCGGAGAGGA 3'
R1: 5' (M13R)-CGGTGCGCGGCGTTA 3'
F2: 5' (M13F)-GCCGGCGTTTTCTTTACTCAC 3'
R2 5' (M13R)-CTGAACCCCTTCCCAGCTTTA 3'

These primers were used for amplification. Samples were denatured at 94°C for 3 min, followed by 35 cycles of denaturation (94°C, 30 s), annealing (58°C, 30 s for F1–R1, 55°C, 30 s for F2-R2), extension (72°C, 30 s) and a final extension of 7 min at 72°C in a thermal cycler. PCR products were cleaned using ExoSAP-IT (Applied Biosystems) according to the manufacturer's instructions. Finally, sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's instructions. Samples were analyzed in an ABI Prism 3100 (Applied Biosystems). Sequence alignments were performed using Sequencer (version 4.0.5). Allele frequencies were compared to published frequencies in NCBI (www.ncbi.nlm.nih.gov).

Prediction of Mutation analyses

We used Mutation Taster (www.mutationtaster.org) to identify HOX-B7 gene mutations (Schwarz et al., 2014). We researched the most frequently misleading locus of HOX-B7 (exon 1-2). Any known variants were searched in ExAC or the 1000G database.

Statistical analyses

SPSS, version 23.0 and R studio were used to perform statistical analyses. The Chi-square test was used to compare the categorical measurements between the groups. The Kolmogorov Smirnov and the Mann Whitney U test were used for comparisons. A p-value of less than 0.05 indicated statistical significance.

RESULTS

There were 33 children, 13 males, and 20 females, in the study. The mean age was 69.1 (9-144) months at the time the blood sample was taken. Twenty patients had bilateral VUR; seven had left VUR, and six had right VUR. Nineteen patients had evidence of renal scarring on DMSA, and 13 patients had renal atrophy with reflux nephropathy. Recurrent urinary tract infections were described in 25 patients. Almost all patients with renal atrophy had severe VUR (grades IV-V), and all of them had taken antibiotic prophylaxis after the diagnosis of VUR. Table 1 shows the demographic data of patients. Figure 1 shows a possible mechanism of variants on the development of reflux.

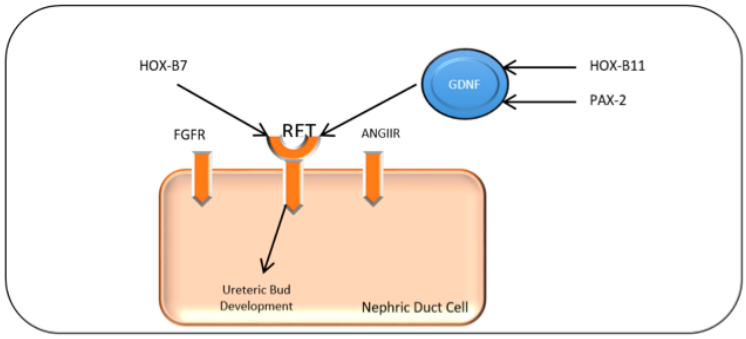

Figure 1. Mechanism of HOX-B7 affecting development of vesicoureteral reflux.

Table 1. Demographic data of the Turkish reflux nephropathy patients.

	Number of cases
Age (months)	69.1
Sex	
Female	23
Male	10
Grade of VUR	
1	4
2	2
3	10
4	7
5	10
Side	
Left	7
Right	6
Bilateral	20
Recurrent UTI	25
Renal scarring	19
Renal atrophy	13

UTI: Urinary tract infection, VUR: vesicoureteral reflux

Seventeen patients had one genetic mutation at the 61st nucleotide position (A/A) on exon 1, which is the longest allele and is frequently mutated. Four patients had heterozygous variants (A/G), and 13 patients had homozygous variants (G/G) (Figure 1a-b). This gene area does not encode a protein. However, the result of the gene database search was disease-causing.

Seven patients with exon 1 variants had severe VUR (grades IV and V), and six had moderate VUR (grade III) (Table 2). One patient had a single duplex system on the right side. Eight patients with exon 1 variants had renal scarring, and seven of them had homozygous variants. There was no significant correlation between exon 1 variant and renal scarring (P = 0.56). When we assessed the relationship between the duration of VUR and renal scarring, patients with exon 1 variants had a bit earlier renal damage, and the time of renal scar development in patients with exon variants was observed to be clustered at 15 months (Figure 2). But there was no significant difference between patients with and without this exon 1 variant (P = 0.15).

Table 2. Demographic data of vesicoureteral reflux patients with Exon 1 and 2 variants.

	Exon 1		**Exon 2**	
	Homozygote	**Heterozygote**	**Homozygote**	**Heterozygote**
Age (months)[a]	61 ± 32 (9-110)	53 ± 13 (40-66)	78 ± 18 (60-96)	54 ± 18 (36-72)
No. Cases[b]	13	4	2	2
Female	10	3	1	2
Male	3	1	1	-
Side[b]				
Left	4	2	-	-
Right	3	1	1	-
Bilateral	6	1	1	2
Grade Of VUR				
1	-	2	-	-
2	-	1	-	-
3	6	1	-	-
4	4	-	2	-
5	3	-	-	2
Renal Scar[b]	7	1	-	-
Duplex System[b]	1	-	-	-

[a]Data expressed as mean±SD. [b]Data expressed as n. VUR: vesicoureteral reflux

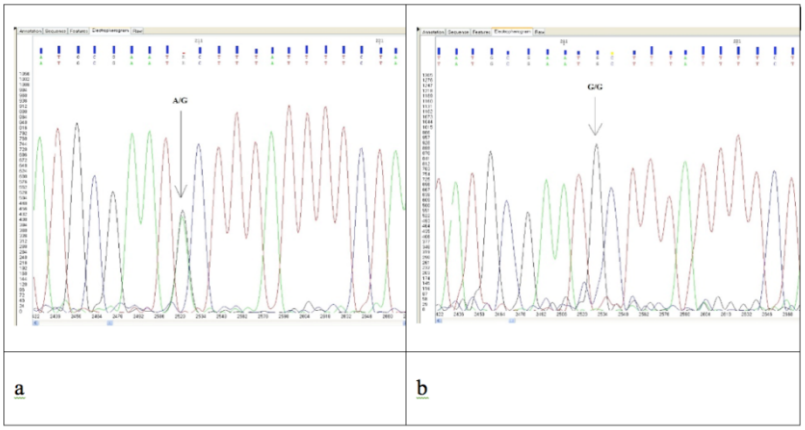

Figure 2. Examples of allelic variants found in the exon 1 region of the HOX-B7 gene in our patient group. The green peaks adenine, the black peaks guanine. a) heterozygote and b) homozygous A> G mutation in the exon 1 region of the HOX-B7 gene in our patients.

An exon 2 variation at the 2721st nucleotide position, a C/C allele, was determined in four patients. One heterozygous variant was identified, the allele of exon 2 (C/T). One heterozygous (C/A) and two homozygous variants (A/A) were determined on the 2737 allele of exon 2 (Figure 3a-b). All patients with exon 2 variants also had homozygote exon 1 variants (G/G). These gene areas do not encode a protein, and the result of the exon 2 mutation, according to the gene database, was a polymorphism.

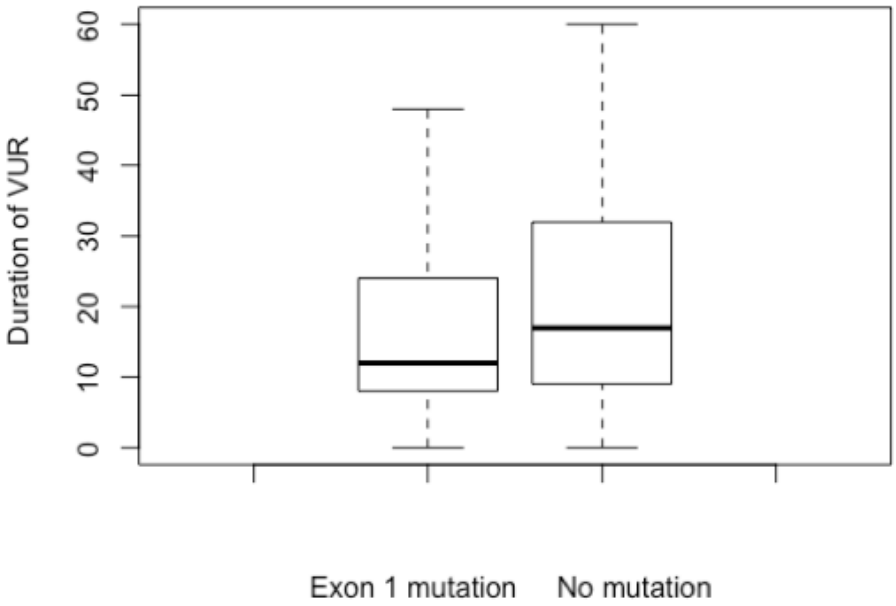

Figure 3. Duration of vesicoureteral reflux (VUR) to renal scarring.

DISCUSSION

VUR is one of the most significant diseases in pediatric patients because it causes renal parenchymal fibrosis, hypertension and decreased renal function; a large portion of VUR patients progress to renal scarring and end-stage renal disease (Mehls et al., 1996; Lashdes-Vasama et al., 2006). Furthermore, as with many other disorders, VUR has various natural processes and clinical outcomes; some of the cases resolve spontaneously, and persistent cases present an increased risk for complications (Mehls et al., 1996; Smellie et al., 1998; Lashdes-Vasama et al., 2006, Carvas et al., 2010). Consequently, in the management of VUR, it is essential for clinicians to identify children with risk factors to prevent potential renal damage (Murawski et al., 2008). By analyzing studies, 14 genes and more than 10 potentially interesting loci have were identified as being associated with VUR. Therefore, VUR is genetically heterogeneous. One hypothesis is that a few critical genes regulate ureteric bud formation and urinary tract development.

The first investigated gene was in a mouse model of VUR, the angiotensin type II receptor (Agtr2), which is a component of the renin-angiotensin system that is implicated in renal development. Mice with Agtr2 gene block have hypoplastic kidneys, VUR, and duplicated urinary tracts (Nishimura et al., 1999; Oshima et al., 2001). Another early study

involved the Pax gene and UPK genes, which demonstrated that mice with gene mutations had various changes in the ureter and had VUR, reflux nephropathy and malformed kidneys (Vermillion and Heale, 1973; Kong et al., 2004; Murawski et al., 2007). Uroplakin (UPK) protein-encoding gene-null mice have hydronephrosis, reduced renal function; UPKII and UPKIII gene null mice have reflux and nephropathy (Vermillion and Heale, 1973).

Homeobox (HOX) genes act as master regulators in the development of anterior-posterior patterning, cell proliferation, and organ morphogenesis (Roberts et al., 1998; Kuure et al., 2000; Patterson et al., 2001). In our study, we determined that a HOX-B7 variant of exon 1 was associated with moderate and severe VUR. Low-grade reflux was not found in patients with homozygote exon 1 variations. This gene area was not protein-encoding. Nevertheless, the database showed that this single base exchange could be a disease-causing locus. Similar to our study, an early study demonstrated that mice with overexpression of exon 1 and exon 2 of HOX-B7 had renal malformations, including a doubled renal system and mispositioned pelvis (Argao et al., 1995). Another study showed that HOX genes, including HOX-B7, HOX-A11, and HOX-D11, are necessary for kidney development, and 13 HOX genes are expressed in the ureteric bud and its branches during development (Patterson and Potter, 2004). HOX-B7 is expressed in the urogenital system in the mesonephros stage, as well as in the ureter and collecting system (Kuure et al., 2000; Peterson and Potter, 2004). Early studies showed that HOX-B7 involved a RET mutation. Similar to our research, Srinivas et al. (1999) showed that the HOX-B7 promoter was exposed to express RET throughout the ureteric bud branches; and RET mutations caused renal dysplasia and VUR in mice. Another RET gene study showed that VUR pathogenesis was associated with the glial-derived neurotrophic factor (GDNF). Manipulation of the level of GDNF showed that this defect caused insufficient RET signaling. GDNF caused various inhibitions of ureteric bud growth and branching (Argao et al., 1995; Jeanpierrre et al., 2011). Additionally, this study was the first study showing that HOX-B7 also encodes a human mammary epithelial cell (MCF10A) protein; overexpression of HOX-B7 in mice caused anomalies in number such as renal duplications (Majumdar et al., 2003; Peterson and Potter, 2004). In our study, we found only one patient with anomalies in the collecting system number who had a duplex collecting system in the right kidney. Novel studies also showed a relation and expression was correlated between HOX-B7 and cancers (Liao et al., 2011; Komatsu et al., 2016).

A total of 24% of patients with exon 1 variants had renal scarring. There was no association and the cumulative effect of HOX-B7 gene variations on renal damage. Kuroda et al. (2007) showed that transforming growth factor beta-1 (TGF-β1) is a cytokine that regulates healthy cellular growth and differentiation on embryogenesis; it has been reported to play essential roles in renal diseases, including renal scarring. There may be a potential correlation between the TGF-b1 polymorphisms 509CC and 869TT and VUR/renal scarring.

Variants in both genes (exon 1 and exon 2) were not associated with recurrent urinary tract infections. The reason for this outcome is that there was a small number of patients, and the results may be more meaningful with a higher number of patients.

A limitation of the study was the small number of patients, and another limitation was the absence of a control group. Nevertheless, we believe that our research may play a role in the comprehension of VUR pathogenesis; HOX-B7 is an important locus affecting various diseases.

CONCLUSIONS

Finding the genetic basis of VUR can provide us with a better understanding and identification of the ways to manage this disease best. Our study evaluated the role of the HOX-B7 gene development of this disease. Exon 1 of the HOX-B7 mutation could play a crucial role in the development of VUR and this gene was mainly found related to higher susceptibility of the carrier to have mild and severe reflux; but we did not found any association with exon variants and renal function loss in the Turkish population.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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