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ASSOCIATION OF SINGLE NUCLEOTIDE POLYMORPHISM IN IL12B WITH CONGENITAL ANOMALIES OF THE KIDNEY AND URINARY TRACT AMONG BULGARIAN CHILDREN

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ABSTRACT

Congenital anomalies of the kidney and urinary tract (CAKUTs) comprise a wide range of renal system structural and functional malformations. Some cytokines were associated with clinical consequences of CAKUT. However, the role of IL-12-related cytokines and functional polymorphisms in *IL12B* gene was not explored as a factor influencing immune mechanisms of CAKUT development.

The aim of the study was to determine the distribution of +16974A/C polymorphism in *IL12B* (rs3212227) among Bulgarian children with CAKUT and to investigate its significance for clinical manifestation of the disease.

Genotyping of +16974A/C polymorphism in *IL12B* was performed by restriction fragment length polymorphism assay among 119 CAKUT cases and 284 controls.

The CC-genotype and C-allele were overrepresented among patients with renal hypo-dysplasia compared to controls (CC-genotype: 25% *vs.* 7%, p=0.005 and C-allele: 37.5% *vs.* 22%, p=0.023, respectively). The carrying of C-allele in the homozygous genotype was associated with 4.711-fold increased risk of the development of renal hypo-dysplasia among Bulgarian CAKUT patients. Respectively, the frequency of AA-genotype was lower in cases compared to controls (50% vs. 63%).

We conclude that the carrying of CC-genotype of +16974A/C polymorphism of *IL12B* could be accepted as a risk factor for the development of renal hypo/dysplasia among Bulgarian CAKUT patients.

Key words: cytokine, genotype, rs3212227, renal hypoplasia and dysplasia

INTRODUCTION

Congenital anomalies of the kidney and urinary tract (CAKUTs) comprise a wide range of renal system structural and functional malformations-renal hypoplasia and dysplasia, unilateral renal agenesia, hydronephrosis , megaureter, posterior urethral valves (PUV), vesicoureteral reflux (VUR) and double or horseshoe kidney. Mutations in renal developmental genes (HNF1β, Pax2, UMOD, Eya1 ect.) are detected in minority (1.9-20%) of patients with nonsyndromic CAKUT from different population (1). Thus, the contribution of genetic mutations and polymorphisms to the cause of nonsyndromic CAKUT in the majority of children remains unresolved.

On the other hand, a number of clinical and experimental studies have shown the relation between renal diseases and cytokines production and leave no doubt about the role of inflammation in renal diseases. Many authors associated cytokines with relevant clinical consequences of CAKUT such as acute pyelonephritis, urinary tract obstruction, and renal scarring (2). Sheu et al. (3-4) evaluated serum and urinary levels of interleukin-1β (IL-1β), IL-6, and CXCL8/IL-8 in children with acute pyelonephritis and have reported that the significantly reduced levels of IL-1β in children with renal scarring. The role of others cytokine as TGF-β and TNF-α have been evaluated in

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experimental hydronephrosis (5-6). TGF-β is a multifunctional cytokine involved in tubulointerstitial fibrosis and in the processes of renal wound healing. Respectively, higher urinary level of TGF-β was associated with higher risk of developing obstructive hydronephrosis (7-8).

Functional genetic polymorphisms that altered gene expression of cytokines are potential factors that could modulate the development and progression of CAKUT. Some of these polymorphisms were also associated to reflux nephropathy and progressive renal damage. Associations between gene polymorphisms of TNF-α, TGF-β and VEGF with vesicoureteral reflux were found, although the results are manifold. Several studies have investigated the potential role of TGF-β1 polymorphisms at different genetic loci in the development of VUR and/or renal scarring. In recent studies was shown that homozygous genotype- TT of -509 polymorphism in TGF-β gene is a risk factor for primary VUR (9) and renal scaring (10).

However, the role of IL-12-related cytokines and functional polymorphisms in *IL12B* gene was not explored as a factor influencing the pathogenesis of CAKUT. One of the functional polymorphism in the *IL12B* is a single-nucleotide polymorphism (SNP) in 3'-untranslated region (UTR) with number rs3212227. A considerable body of evidences has been accumulated over past years, which demonstrated the functional role of this polymorphism and its effect on IL-12p40 production (11-14).

In regards to the above, the aim of the present study was to determine the distribution of +16974A/C polymorphism in *IL12B* (rs3212227) among Bulgarian children with CAKUT and to search association between genotypes and clinical manifestations of the disease.

MATERIALS AND METHODS

Genomic DNA was extracted from venous blood from 119 CAKUT cases, 80 unaffected children and 204 unaffected adults by a standard method using proteinase K digestion followed by standard salting-out technique and stored at -80°C until use. DNA concentration and quality was determined spectrophotometric and all used samples have A260/A280 ratio above 1.7.

Genotyping of +16974A/C polymorphism in *IL12B* was performed by restriction fragment length polymorphism assay (RFLP-PCR) after amplification of 1,046 bp fragment as was described previously (13). The PCR products were treated with TaqI restriction enzyme and the 16974*C allele yielded two fragments, 906 and 140 bp, respectively. PCR products and digested products were visualized on a 2% agarose gel stained with ethidium bromide (0.5 mg/ml).

Statistical analysis was carried out using SPSS software, version 21 (IBM, Chicago, IL). Allelic and genotype frequencies were compared between groups by chi-square ($\chi 2$) test or Fisher exact test (as appropriate). The strength of the associations between genotypes of +16974A/C SNP in *IL12B* and CAKUT was presented as odds ratios (OR) with corresponding 95% confidence intervals (CIs) using logistic regression method. The homozygous genotype-AA for the common allele in Caucasians was used as the reference category. The limit of significance for all analyses was defined as a p-value of 0.05.

RESULTS

In the cohort of 119 CAKUT patients from Bulgaria, 66 (0.56) were homozygous for the wild +16974*A allele, 37 (0.31) patients were heterozygous, and the rest of 16 (0.13) were homozygous for the variant +16974*C allele **(Table 1).** The distribution and frequencies of genotypes of +16974A/C SNP in 3'UTR of *IL12B* were calculated among two control groups: one composed of 80 unaffected children with range of age between 3 months and 18 years and second control group of unaffected adults with range of age between 20-81 years. Genotype distribution among unaffected children was the following: AA-genotype 51 (0.64), AC-genotype 23 (0.29), and CC-genotype 6 (0.07) and among unaffected adults: AA-genotype 128 (0.63), AC-genotype 63 (0.31), and CC-genotype 13 (0.06). Obviously, the genotype distribution among unaffected children and adults is quite similar ($\chi 2$=0.205; df=2; p=0.903). Based on this result, we merged unaffected children and adults into a total group of 284 unaffected controls. Compared the genotype distribution among CAKUT patients and total group of unaffected controls we observed an elevation of CC-genotype and reduction of AA-genotype frequencies in patients group ($\chi 2$=5.219; df=2;

p=0.074). The homozygous CC-genotype was overrepresented among CAKUT patients compared to controls (13% vs. 7% with OR=2.284; 95% CI: 1.045-4.983; p=0.022). Respectively, the frequency of AA-genotype was lower in cases compared to control (56% vs. 63%), while the frequency of AC-genotype was equal (31% vs. 30%). According to allelic frequency, we observed higher frequency of variant-C allele among CAKUT patients than in controls (29% vs. 22% with OR=1.462; 95% CI: 1.022-2.091; p=0.03)

To evaluate whether the +16974A/C SNP in 3'UTR of *IL12B* affects a broad phenotypic spectrum of CAKUT, patients were subdivided according to the kind of anomalies into four subgroups: i) Congenital kidney malformations (hypoplasia and dysplasia); ii) obstructive uropathies (hydronephrosis, megaureter), iii) vesicoureteral reflux and iv) double or horseshoe kidney.

Table 1. *Genotype and allelic frequencies of +16974 A/C SNP in IL112B among CAKUT patients and healthy controls*

Cases/controls	**Genotype frequency n (%)**			**Allelic frequency n (%)**	
	AA	**AC**	**CC**	**A -allele**	**C -allele**
CAKUT patients (n=119)	66 (56)	37 (31)	16 (13)	169 (71)	69 (29)
Unaffected children (n=80)	51 (64)	23 (29)	6 (7)	125 (78)	35 (22)
Unaffected adults (n=204)	128 (63)	63 (31)	13 (6)	319 (78)	89 (22)
Total group of unaffected controls (n=284)	179 (63)	86 (30)	19 (7)	444 (78)	124 (22)

In attempt to achieve more homogenous subgroup of CAKUT patients, we omitted those patients (n=7) with more than one anomaly and those patients (n=5) with unilateral renal agenesia. Thus, the patient group appeared to consist of 107 persons. The comparison of genotype and allelic frequencies between patients' subgroups and unaffected controls is presented in **Table 2**.

Analyzing the difference in the frequency of +16974A/C SNP in 3'UTR of *IL12B,* we found that patients with renal hypo-dysplasia showed a significant different genotype distribution of investigated polymorphism ($\chi 2$=8.618; df=2; p=0.013). The CC-genotype and C-allele were overrepresented among patients with renal hypo-dysplasia. The carrying of C-allele in homozygous genotype was associated with 4.711 fold increased risk of development of renal hypo-dysplasia among Bulgarian CAKUT patients. However, we should note a slight elevation of CC-genotype among VUR patients compared to controls (12.5% vs. 7%; with OR=2.048; 95% CI: 0.603-6.584; p=0.184) and C-allele (27.5% vs. 22% with OR=1.358; 95% CI: 0.772-2.375; p=0.256) without reaching the statistical significance. The rest subgoups of CAKUT patients (patients with obstructive uropathies and double or horseshoe kidney) showed a similar distribution of investigated polymorphism to the controls.

DISCUSSION

To the best of our knowledge, this is a first study exploring the significance of +16974A/C polymorphism in *IL12B* for the development of congenital anomalies of the kidney and urinary tract. Here we presented genotype-CC of investigated polymorphism as a risk factor for the development of renal hypo-dysplasia among Bulgarian children with congenital anomalies of the kidney and urinary tract.

Table 2. *Genotype and allelic frequencies of +16974A/C SNP in IL112B among subgroups of CAKUT patients according to the kind of anomalies compared to control group*

Cases	Genotype frequency n (%)			Allelic frequency n (%)	
	AA	**AC**	**CC**	**A -allele**	**C -allele**
renal hypo/dysplasia (n=20)	10 (50)	5 (25)	5 (25)	25 (62.5)	15 (37.5)
Odds Ratio (95% CI); p-value	1.00	1.041 (0.299-3.445); 0.944	**4.711 (1.245-17.240) 0.005**	1.00	**2.148 (1.042-4.396) 0.023**
obstructive uropathies (n=27)	17 (63)	7 (26)	3 (11)	41 (76)	13 (24)
Odds Ratio (95% CI) p-value	1.00	0.857 (0.309-2.298) 0.741	1.663 (0.351-6.834) *0.434*	1.00	1.135 (0.559-2.273) 0.704
vesicoureteral reflux (n=40)	23 (57.5)	12 (30)	5 (12.5)	58 (72.5)	22 (27.5)
Odds Ratio (95% CI) p-value	1.00	1.086 (0.483-2.413) 0.828	2.048 (0.603-6.584) 0.184	1.00	1.358 (0.772-2.375) 0.256
double or horseshoe kidney. (n=20)	11 (55)	8 (40)	1 (5)	30 (75)	10 (25)
Odds Ratio (95% CI) p-value	1.00	1.514 (0.533-4.242) 0.388	0.856 (0.039-7.080) *1.0*	1.00	1.194 (0.530-2.631) 0.640

The presented Odds Ratio with 95% confidence interval (95% CI) were estimated comparing the group of patients with unaffected controls with reference AA-genotype; The probability computed by Fisher exact test is presents in italic*;* $P < 0.05$ are bolded

Although mutations in many different single renal developmental genes are associated with broad phenotypic spectrum of CAKUT (15) variability in genotype–phenotype correlation in renal system anomalies points to the essential roles of other genetic factors. Recently, the role of broad spectrum of cytokines, growth factors, chemokines in CAKUT pathogenesis have been explored. However, it should be noted the majority of these clinical studies are focused on ureteropelvic junction obstruction and vesicoureteral reflux. Cytokines as TGF-β, TNF-α, IL-6, IL-8, IL-1β may contribute to the initiation and progression of renal fibrosis, tubulointerstitial injury in obstructed kidney, glomerular and tubulointerstitial scarring after a urinary tract infections (3, 4, 6, 10).

Respectively, functional polymorphisms in cytokines genes than altered gene expression could be additional genetic factors influenced the CAKUT pathogenesis. Associations between gene polymorphisms of TNF- α, TGF- β and VEGF with VUR were found (16-17). Hussein et al. (18) showed that T-allele of SNP in promoter region of TGF-β gene at position -509 is associated with an increased risk for the development of renal scarring. Kuroda et al (19) demonstrated that the genotype-CC of the same polymorphism -509C/T TGF-β was significantly increased in the familial vesicoureteral reflux group compared to controls and supposed that this genotype may have increased susceptibility to vesicoureteral reflux.

TGF-β and IL-6 together direct T cell to Th17 phenotype that recently was pointed as a key cell subset in renal immunophatology (20). IL-23 is an important cytokine for stabilizing Th17 cells. Recent studies implicate IL-17, IL-23, and Th17 responses in experimental glomerulonephritis IL-23 is a heterodimeric cytokine composed of IL-12p40 subunit and specific IL-23p19. IL-TGF-β and IL-6 together direct T cell to Th17 phenotype that recently was pointed as a key cell subset in renal immunophatology (20). IL-23 is an important cytokine for stabilizing Th17 cells. Recent studies implicate IL-17, IL-23, and Th17

responses in experimental glomerulonephritis (21). IL-23 is a heterodimeric cytokine composed of IL-12p40 subunit and specific IL-23p19. IL-12p40 is also presents as a heterodimeric Th1 cytokine IL-12p70 (IL-12p40/p35), homodimeric (IL-12p80) and monomeric form (IL-12p40). However, the genetic polymorphism +16974A/C in *IL12B* gene was not explored previously as a factor influence the CAKUT phenotype. In our current study, we demonstrated a significant elevation of homozygous genotype-CC and C-allele among CAKUT patients with renal hypoplasia and dysplasia. Previously, we and other authors have demonstrated that this genotype is associated with lower level of IL-12p40 subunit and this association is stimuli dependent (11, 13). We hypothesizes that lower-IL-12p40 genotype (+16974*CC) could contribution to development of renal hypoplasia and/or dysplasia via lower IL-23 quantity leading to less Th17 cells or via lower IL-12p70 quantity leading to diminished Th1 response. However, further investigations are necessary to determine the exact IL-12-related cytokine, contained IL-12p40 subunit, involved in the renal hypoplasia and/or dysplasia. In addition, this association could help in understanding the immune mechanisms of CAKUT development and progression of renal injury

CONCLUSION

We could assume that +16974A/C polymorphism of *IL12B* has a role in the pathogenesis of renal hypo-dysplasia. The carrying of CC-genotype could be accepted as an additional risk factor for the development renal hypo-dysplasia among Bulgarian CAKUT patients.

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