**ORIGINAL ARTICLE**

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# Implication of genetic variations in congenital obstructive nephropathy

**Abstract** The renin–angiotensin system (RAS) has long been implicated in kidney development, and it has been reported that disruption of angiotensin type 2 receptor (AGTR2) results in a wide range of congenital anomalies of the kidney and urinary tract. We investigated the allele frequencies of the AGTR2 and other RAS genes in Korean patients with ureteropelvic junction obstruction, multicystic dysplastic kidney (MCDK), and unilateral renal agenesis (RA). Fifty-three Korean children were enrolled: 37 boys and 16 girls, 27 with hydronephrosis, 23 with MCDK, and 3 with RA. Among 100 healthy Koreans, the frequencies of A and G alleles at the A–G transition site of intron 1 of the AGTR2 gene were 70% (140/200) and 30% (60/200), respectively. In the patient group, the A allele frequency was 57% (60/106) and the G allele frequency was 43% (46/106), significantly higher than in the general population ($P$=0.024). There was no significant difference of allele frequency between boys and girls. Angiotensin-converting enzyme insertion/deletion, angiotensinogen M235T, and the angiotensin 2 type 1 receptor A1166C genotype distribution showed no difference from those of the control subjects. These findings indicate that the AGTR2 gene may play a major role in the development of congenital obstructive nephropathy.

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## Introduction

Congenital anomalies of the kidney and urinary tract (CAKUT) are common in humans, and the incidence is increasing with recent advances in prenatal ultrasonographic examinations. They account for more than 50% of abdominal masses found in neonates and involve some 0.5% of all pregnancies [1, 2], and, in spite of recent advances in early diagnosis and surgical intervention, they constitute a major cause of end-stage renal failure [3].

The etiologies of most of these anomalies have not been defined, but it has long been noted that they have a familial pattern with variable penetrance and are often concurrently present [4], and a common genetic background for these anomalies has been speculated [5]. Nephrogenesis is controlled by many genes that regulate growth by affecting cell survival, proliferation, differentiation, and morphogenesis [6]. Recently, several genes that are implicated in nephrogenesis have been identified, whose derangement results in renal maldevelopment [6, 7, 8, 9, 10], and some of the implicated genes are described as causing syndromes involving multi-organ dysgenesis [8, 9, 10].

The renin–angiotensin system (RAS) has long been implicated in kidney development [11], and experimental studies have shown that derangement of the renin–angiotensin system during fetal life results in renal maldevelopment [12]. The angiotensin type 2 receptor (AGTR2) is the recently identified isoform, and, in mice, its gene *Agtr2* is actively transcribed at the onset of, and throughout, the embryonic development of the kidney and urinary tract system, and is mostly inactivated by the time of birth [13]. Nishimura et al. reported that *Agtr2* mutant mice have the phenotypes of all key features characterizing common human CAKUT, and, in a concurrent human study, showed that *AGTR2* genotype in Caucasians exhibited a significant association with CAKUT [14].

**Table 1** The primers and probes prepared for investigation of sequence of angiotensinogen, AGTR1, and AGTR2

| Moiety | Primers and probes | |
|---|---|---|
| Angiotensinogen M235T | Sense primer | 5'-TCCTGAGAAGATTGACAGGTTCAT-3' |
| | Antisense primer | 5'-CCAGGGTGCTGTCCACACT-3' |
| | Thr (C) allele probe | FAM-5'-CTCCCTGACGGGGAG-3'-MGB |
| | Met (T) allele probe | VIC-5'-CTCCCTGATGGGGAG-3'-MGB |
| AGTR1 A1166C | Sense primer | 5'-GAGAACATTCCTCTGCAGCACTT-3' |
| | Antisense primer | 5'-CGGTTCAGTCCACATAATGCAT-3' |
| | A allele probe | FAM-5'-AATGAGCATTAGCTACTT-3'-MGB |
| | C allele probe | VIC-5'-ATGAGCCTTAGCTACT-3'-MGB |
| AGTR2 A-1332G | Sense primer | 5'-GGATGAACTTCTGTTTTTCCTGTGT-3' |
| | Antisense primer | 5'-GCAGTTATCATAAATCAGCTTGCTTAGT-3' |
| | A allele probe | FAM-5'-ACTCCTAAATTATTTAGCTGC-3'-MGB |
| | G allele probe | VIC-5'-CTCCTGAATTATTTAGCTGC-3'-MGB |

However, a recent study in 66 Japanese boys disclosed no evidence for *AGTR2* gene derangement [15].

In this study, we examined the associations of AGTR2 and other RAS gene polymorphisms in Korean CAKUT patients, in particular in ureteropelvic junction obstruction (UPJO), multicystic dysplastic kidney (MCDK) and unilateral renal agenesis patients.

## Methods

### Patients

Children who visited our hospital from March 1995 to June 2004 and were diagnosed with MCDK, renal agenesis (RA), or unilateral hydronephrosis (HN) of Society for Fetal Urology (SFU) grades 3 or 4 [16] were enrolled in this study. The study was approved by the institutional review boards of the Asan Medical Center. MCDK and RA were diagnosed by ultrasonography (USG) and [99m]technetium dimercaptosuccinic acid (DMSA) scans. HN was diagnosed by USG and [99m]technetium mercaptoacetyltriglycerine (MAG3) scan. All children underwent voiding cystourethrography (VCUG). Patients with associated anomalies in the ipsilateral/contralateral kidney, including vesicoureteral reflux (VUR), ureterovesical junction obstruction (UVJO) and mega-ureter (MU), were excluded. Fifty-three children were enrolled: 37 boys and 16 girls. HN was found in 27, MCDK in 23, and RA in 3.

As a control group, 100 healthy Korean subjects from a routine screening program were included. All had undergone blood pressure measurement, abdominal USG and urinalysis, and renal function was estimated by measurement of serum creatinine. Control subjects were enrolled in regular sequence, excluding those with any abnormal findings. The age distribution of the control group was 26–73 years (median age 53 years), 55 men and 45 women. Following the manufacturer's instructions, we extracted genomic DNA from peripheral blood of the healthy controls and patients, using the QIAamp DNA blood mini-kit (Qiagen, Hilden, Germany).

The subjects were analyzed for polymorphisms in four renin–angiotensin system genes: the angiotensin converting enzyme (ACE) insertion/deletion (I/D), angiotensinogen (Agt) M235T, angiotensin 2 type 1 receptor (AGTR1) A1166C, angiotensin type 2 receptor (AGTR2) A-1332G.

### Genotyping of ACE gene I/D alleles by real-time PCR and melting curve analysis

Primers were designed as described by Evans et al. [17]. The SyberGreen II PCR Master Mix reagent (Applied Biosystems, Foster City, Calif., USA) was used for real-time PCR amplification of the ACE gene insertion/deletion allele. Genomic DNA (20 ng) was added to a reaction mixture containing 25 $\mu$l of 2×PCR universal mix, 50 pmol ACE1 (5'-CATCCTTTCTCCCATTTCTC-3'), 100 pmol ACE2 primer (5'-TGGGATTACAGGCGTGATACAG-3'), and 50 pmol ACE3 primer (5'-ATTTCAGAGCTGGAAT-AAAATT-3') in a final volume of 50 $\mu$l. Amplification and detection were performed with the GeneAmp 5700 sequence detection system. After initial activation of AmpliTaq Gold DNA polymerase at 95°C for 10 min, 40 cycles of 95°C for 15 s and 55°C for 1 min were performed. After amplification was complete, we performed melting curve analysis by cooling the reaction to 60°C and then heating slowly to 95°C, according to the manufacturer's instructions (Applied Biosystems). SyberGreen II fluorescence (F) was measured continuously during the heating period, and the signal was plotted against temperature (T) to produce melting curves for each sample. We then generated the melting peaks by plotting the negative derivative of the fluorescence with respect to temperature against temperature, and ACE I/D alleles were genotyped according to the melting curve pattern [18].

### Genotyping of Agt, AGTR1, and AGTR2 by specific real-time PCR

Amplification of 20 ng genomic DNA was performed in 25 $\mu$l reaction mixtures containing AmpliTaq Gold DNA polymerase, AmpErase UNG, dNTPs with dUTP, passive reference 1, and optimized buffer components (TaqMan universal PCR Master Mix, Applied Biosystems), with primers and probes (Table 1). After incubation for 2 min at 50°C and for 10 min at 95°C, 40 cycles of 95°C for 15 s and 55°C for 1 min were performed. The resultant signal was analyzed with the ABI PRISM 7900HT sequence detection system (Applied Biosystems).

### Statistics

The results were analyzed with SPSS 10.0 for Windows. Pearson's chi-square and Fisher's exact probability tests were used for allele frequency and paired genotype differences between patients and control group. Spearman's correlation coefficients were used for correlation between two genes. $P < 0.05$ was taken as the level of significance.

## Results

### ACE I/D, AGT, AGTR1, and AGTR2 genotypes in patients

Sequencing of the AGTR2 gene revealed that, among the 100 healthy Koreans, the frequency of the A and G alleles at the A–G transition site (position −1332 from the

**Table 2** Genotype distributions in HN and MCDK/RA group. The AGTR2 genotype distributions in HN and MCDK/RA group showed no difference (P=0.899), and those between disease group and control group showed significant difference (P=0.001)

| Genotype | HN | MCDK/RA | control |
|---|---|---|---|
| AA | 10 | 10 | 61 |
| AG | 3 | 3 | 17 |
| GG | 14 | 13 | 22 |
| Total | 27 | 26 | 100 |

**Table 3** Frequency of the angiotensinogen M235T, AGTR1 A1166C, and ACE I/D alleles in the general Korean population and patients with obstructive nephropathy

| Gene | Allele frequency | | P |
|---|---|---|---|
| | Patients | Control | |
| Angiotensinogen M235T | M 17/106 (16%) | M 45/200 (23%) | 0.181 |
| | T 89/106 (84%) | T 63/200 (77%) | |
| AGTR1 A1166C | A 104/106 (98%) | A 195/200 (98%) | 0.733 |
| | C 2/106 (2%) | C 5/200 (2%) | |
| ACE I/D | D 37/106 (35%) | D 83/200 (42%) | 0.261 |
| | I 69/106 (65%) | I 117/200 (68%) | |

translation initiation site) of intron 1 was 70% (140/200) and 30% (60/200), respectively (AA in 61 patients, AG in 17, GG in 22). In the patient group, the G allele frequency was 43% (46/106) and the A allele 57% (60/106) (AA in 27 patients, AG in 6, GG in 20), showing a significant increase in the G allele frequency in comparison with the general population (P=0.024). There was no significant difference of allele frequency between male and female, both in the patients and the control group. When the patient group was separated into HN and MCDK/RA, the genotype distribution was almost same (Table 2).

There was no difference in the A-1332G transition genotype between HN and MCDK/RA patients. In the 27 HN patients, genotypes were G/G, 10, A/G, 3, and A/A, 14, and, in the 26 MCDK/RA patients, G/G 10, A/G 3, and A/A 13, almost identical distributions (Table 2).

There were no significant differences in allele frequencies between patients and controls for ACE gene I/D, AGT M235T, and AGTR1 A1166C polymorphisms (Table 3).

We tried to find any combined mutations in association with obstructive nephropathy, but there were no correlations between AGTR2 genotype and the other three RAS genes, and when evaluating in two pairs (AGTR2–ACE, AGTR2–AGT, AGTR2–AGTR1), we found that no combined genotypes were associated with obstructive nephropathy.

## Discussion

An A–G transition in intron 1 of the *AGTR2* gene was more frequently found in the patient group than in the Korean general population. The transition is at the branch point motif within intron 1, which perturbs AGTR2 mRNA splicing efficiency and, therefore, might have a significant ontogenic role in the kidney and urinary system. Nishimura et al. observed an increased A–G transition in male Caucasian American and German patients with MCDK and UPJO [14], but Hiraoka et al. reported that there is no evidence for AGTR2 gene derangement in human urinary tract anomalies in Japanese patients [15]. In a recent study of Italian children, the G allele frequency in the *AGTR2* gene in patients showed statistical significance when compared with the general population [19]. The G allele frequency by ethnic group is approximately 30%, 40%, 30%, and 30.4% in Korean, American/German, Japanese and Italian populations, respectively. Except for the American and German groups, the G allele frequency showed no ethnic difference. Nishimura's subjects were selected through strict inclusion criteria, namely male UPJO/MCDK patients [14]. In contrast, the Japanese group [15] was comprised of patients with various diseases.

It is documented that in the late-stage wild-type embryo, intense expression of *Agtr2* mRNA is found, especially within the metanephros and around the ureter, and is absent in the urethral area: inactivation of *Agtr2* might hinder rapid growth in those areas [13]. Therefore, we speculated on upper urinary tract anomalies, and our patients were composed of MCDK and UPJO cases. VUR, UVJO, MU and PUV also produce dilation of the pelvis, but we thought that the pathogenesis of these diseases involve abnormalities of the lower urinary tract and excluded them from our study. Serial ultrasonographic examinations from the prenatal period demonstrate that MCDK is not a static disease, and, in some cases, organs increase only to subsequently regress and even involute completely. Mesrobian et al. suggested that unilateral renal agenesis might result from in utero regression of multicystic dysplasia [20]. Recently, Shukla et al. also reported "vanishing kidney" in a neonate with a sibling history of MCDK and suggested that the infant might have suffered from the prenatal involution of familial adysplasia [21]. From this, we suspected that at least some portion of unilateral RA might be part of the spectrum of MCDK and thus included RA cases in our study.

Our results show a G allele frequency of 43%, lower than that reported by Nishimura and similar to Rigoli's report of 47%. Our patient group's composition is more like the Caucasian group, although a difference lies in the inclusion of female subjects. However, in our data, no gender-related differences in the *AGTR2* polymorphism have been demonstrated, so we conclude that the *AGTR2* genotype is associated with UPJO/MCDK, and the frequency of the polymorphism shows ethnic variations. Although the Italian study viewed various diseases together, like the Japanese study, their results showed significant differences between patient and control groups. The results for each disease were not shown in that report, thus direct comparison with our results might be misleading; however, we suspect that this report also supports our conclusion.

The penetrance of Agtr2 in mice is reported as 21% in males and 5% in females [14], and the asymmetry of the CAKUT phenotype is well described, which suggests that *AGTR2* polymorphism is not the only cause of human CAKUT, and implies the existence of modifying genes and/or environmental factors. We hypothesized that interactions in RAS genes might play a role, and we analyzed the correlation of the four RAS genes and combined mutations of paired RAS genes, but failed to reach any results.

However, together with previous reports, our data strongly suggest a role for AGTR2 in kidney and urinary tract development. The number in the patients group is rather small for us to reach a definitive conclusion, but if one considers the rarity of severe obstructive nephropathy patients, our study suggests encouraging data.

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