Autosomal Recessive Renal Tubular Dysgenesis Caused by a Founder Mutation of Angiotensinogen

Min-Hua Tseng, Shih-Ming Huang, Jing-Long Huang, Wen-Lang Fan, Martin Konrad, Steven W. Shaw, Reyin Lien, Hui-Ping Chien, Jhao-Jhuang Ding, Tai-Wei Wu, Jeng-Daw Tsai, Ya-Chung Tian, Hwei-Jen Lee, Po-Jen Cheng, Jen-Fu Hsu and Shih-Hua Lin

1Division of Nephrology, Department of Pediatrics, Chang Gung Memorial Hospital and Chang Gung University, Taoyuan, Taiwan; 2Department of Biochemistry, National Defense Medical Center, Taipei, Taiwan; 3Division of Pediatric Allergy, Asthma, and Rheumatology, Department of Pediatrics, Chang Gung Memorial Hospital and Chang Gung University, Taoyuan, Taiwan; 4Genomic Medicine Core Laboratory, Chang Gung Memorial Hospital, Taoyuan, Taiwan; 5Department of General Pediatrics, University Children’s Hospital Münster, Münster, Germany; 6Department of Obstetrics and Gynecology, Taipei Chang Gung Memorial Hospital and Chang Gung University, Taoyuan, Taiwan; 7Division of Neonatology, Department of Pediatrics, Chang Gung Memorial Hospital and Chang Gung University, Taoyuan, Taiwan; 8Division of Pathology, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan; 9Department of Pediatrics, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan; 10Fetal and Neonatal Institute, Division of Neonatology Children’s Hospital Los Angeles, Department of Pediatrics, Keck School of Medicine, University of Southern California, Los Angeles, California, USA; 11Division of Nephrology, Department of Pediatrics, MacKay Children’s Hospital, New Taipei City, Taiwan; 12Division of Neonatology, Department of Pediatrics, MacKay Medical College, New Taipei City, Taiwan; 13Division of Nephrology, Department of Medicine, Chang Gung Memorial Hospital and Chang Gung University, Taoyuan, Taiwan; 14Department of Obstetrics and Gynecology, Chang Gung Memorial Hospital and Chang Gung University, Taoyuan, Taiwan; and 15Division of Nephrology, Department of Medicine, Tri-Service General Hospital, Taipei, National Defense Medical Center, Taiwan

Introduction: Autosomal recessive renal tubular dysgenesis (ARRTD) caused by inactivation mutations in AGT, REN, ACE, and AGTR is a very rare but fatal disorder with an unknown prevalence.

Methods: We report 6 Taiwanese individuals with ARRTD from 6 unrelated families diagnosed by renal histology. Clinical features, outcome, and prevalence of carrier heterozygosity were examined.

Results: All patients exhibited antenatal oligohydramnios, postnatal anuria, pulmonary hypoplasia, and profound hypotension refractory to interventions. Angiotensinogen (AGT) protein levels were diminished in the liver, along with reduced serum AGT, angiotensin I (Ang I) and angiotensin II (Ang II) levels. Neonatal demise occurred in all but 1 case. All individuals carried the same homozygous E3_E4 del:2870bp deletion+9bp insertion in AGT, which led to a truncated protein (1-292 amino acid). The allelic frequency of this heterozygous AGT mutation was approximately 1.2% (6/500), suggesting that ARRTD may not be exceedingly rare in Taiwan.

This mutation results in skipping of exons encoding the serpin domain of AGT, which is important for renin interaction and the generation of truncated protein. In silico modeling revealed a diminished interaction between mutant AGT and renin. One patient survived after responding to high-dose hydrocortisone therapy, with resolution of profound hypotension, accompanied by an increase in serum AGT, Ang I, and Ang II levels.

Conclusion: This AGT mutation may lead to the diminished interaction with renin and decreased Ang I and Ang II generation. Hydrocortisone may potentially rescue cases of ARRTD caused by this truncated AGT.

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KEYWORDS: angiotensinogen; founder effect; hydrocortisone; hypotension; renal tubular dysgenesis; renin

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Autosomal recessive renal tubular dysgenesis (ARRTD) is an inherited disorder characterized by the absence or poor differentiation of proximal convoluted tubules, profound arterial hypotension, pulmonary hypoplasia, and anuria, with a very high mortality. Nearly all affected individuals die either in utero or within the first few postnatal days, secondary to refractory hypotension and/or pulmonary hypoplasia. Because of its extreme rarity, its exact prevalence is unknown. In 2005, Gribouval et al described mutations in 4 different genes encoding
proteins of the renin–angiotensin system (RAS) resulting in ARRTD.\textsuperscript{10} These mutations were identified as AGT-encoding angiotensinogen, REN-encoding renin, ACE-encoding angiotensin-converting enzyme (ACE), or AGTR-encoding angiotensin II (Ang II) receptor. The RAS is critical in maintenance of blood pressure and blood perfusion of vital organs including the kidneys during fetal life and is also a key regulator of kidney development.\textsuperscript{11,12} A defect in RAS has been proposed to be responsible for the development of renal tubular dysgenesis and ARRTD via compromised renal perfusion\textsuperscript{13,14} and defective metanephric kidney development. Without a clear understanding of how mutations affecting the RAS can contribute to the pathogenesis of this disease, specific therapeutic strategies cannot be developed.

Recently, we identified 6 patients with ARRTD from 6 unrelated Taiwanese families. In this study, we examined their clinical features and outcomes, identified the mutations and calculated the estimated prevalence, and proposed a potential therapy.

**MATERIALS AND METHODS**

**Patients**

This study was approved by the ethics committee on human studies at Chang Gung Memorial Hospital in Taiwan (IRB105-6067C). Neonates with ARRTD caused by large deletion of AGT, their parents, and 500 healthy adult subjects (>18 years of age) were enrolled in this study. Written informed consent was obtained from all participants.

**Diagnosis of ARRTD**

Neonates with maternal oligohydramnios, normal fetal kidneys, and postnatal refractory hypotension underwent renal biopsy for histological evidence of renal tubular dysgenesis, and molecular sequencing was conducted for the diagnosis of ARRTD.

**Whole Exome Sequencing and Direct Sanger Sequencing**

Genomic DNA was isolated from peripheral venous blood sample. We performed exome capture using the Agilent SureSelect v6 and massively parallel sequencing using the HiSeq 4000 platform (Illumina, San Diego, CA) as previously reported.\textsuperscript{16,17} ANNOVAR was used to catalog the detected variations.\textsuperscript{18} Then, we filtered variations that had a homo-polymer length >6 (and synonymous substitutions) or that were common (>2\%) in dbSNP150 (http://www.ncbi.nlm.nih.gov/projects/SNP/), HapMap, the 1000 Genomes Project (http://www.1000genomes.org), Exome Aggregation Consortium (ExAC) database and the Genome Aggregation Database (gnomAD, https://gnomad.broadinstitute.org). Direct Sanger sequencing was performed for all patients and their parents to verify the genetic variants detected by whole exome sequencing (WES).

**Haplotype Analysis for Founder Effect**

Haplotype analysis using extragenic microsatellite markers (D1S2847, D1S2631, D1S2805, D1S3462, and D1S1656), intragenic single nucleotide polymorphism markers (D1S103), and coding single nucleotide polymorphisms from WES was performed in patients and their parents. Haplotype blocks were called using Haplovie version v4.1.

**Immunohistochemistry and Immunofluorescence Staining of Liver and Kidney Tissues**

The kidney specimens obtained from patients with ARRTD, normal controls (those with traumatic kidney injury), and disease controls (those with minimal change disease and acquired ARRTD) were fixed by paraffin-embedded tissue sections with paraformaldehyde. Deparaffinization and antigen unmasking were performed using Trilogy (cell marque, MilliporeSigma). A monoclonal anti-CD 10, anti-EMA, anti-renin, and anti-AGT antibodies (target epitope: amino acids 2–135) were used to recognize the proximal tubular cells, distal tubules/collection ducts, renin expression, and AGT expression, respectively. The biopsied liver specimens were fixed and prepared as paraffin-embedded tissue sections, and immunofluorescence staining for the expression of AGT obtained from patients with, normal controls (traumatic liver injury), and disease controls (hepatocellular carcinoma and ARRTD caused by ACE mutation) was performed.

**Analysis of Prevalence of AGT Heterozygosis**

Reverse transcription–polymerase chain reaction analysis using exon 2- to 5-specific primers (forward, TTCCATGGAGCTTTGAATCCA; reverse, AGCAGTTCGTGTGCAAGT) was performed to detect the heterozygosity of 500 healthy subjects.

**Determination of Levels of AGT, Ang I and II, and Renin**

Serum levels of AGT, renin, Ang I, ACE, and Ang II from patients, their parents, and healthy subjects were determined by enzyme-linked immunosorbent assay (ELISA; Cusabio Corporation).

**Simulation of the Complex Models**

The models of renin in complex with wild-type or mutant AGT were built using the Homology Modeling protocol (Biovia Discovery Studio 2017). The resolved
structures of human AGT in complex with renin, human, mouse, and rat AGT (PDB code: 2X0B, 2WXW, 2WXY, and 2WXZ) were used as templates. The model with the best probability density function energy and discrete optimized protein energy score was chosen for energy minimization using the CHARM (Chemistry at Harvard Macromolecular Mechanics) as the applied force field.

Statistical Analysis
The resulting mean data were compared with a Student 2-tailed t test. The coefficient of variation or the percent relative SD was calculated as 100 multiplied by SD divided by the mean.

RESULTS
Clinical Characteristics and Outcomes
Four neonates and 2 fetuses (4 male and 2 female) with ARRTD were studied. As shown in Table 1, patients were born between 22 and 35 weeks of gestation with a birth bodyweight ranging from 700 to 2205 g. Pregnancy was terminated in 2 cases because of severe oligohydramnios. All patients shared the same clinical features: oligohydramnios, pulmonary hypoplasia, joint contractures, skull ossification defects, wide fontanelles, and normal kidney size with increased echogenicity. In addition to profound hypotension refractory to fluid resuscitation, inotropic agents, and physiologic dosing of hydrocortisone, all neonates presented with respiratory failure and anuria after birth. Of note, infusion of fresh frozen plasma transiently improved the blood pressure to normotensive range (defined as mean blood pressure greater than that for gestational age). All but 1 neonate died at a postnatal age of 2 to 8 days because of severe pulmonary hypoplasia and profound hypotension.

Renal Histological Characteristics
Renal tissues were obtained by percutaneous fine needle biopsy in all cases. Immunolabeling with anti-CD10 and anti-EMA was used to identify proximal tubules and distal tubules, respectively. There was strong staining of proximal and distal tubules in disease controls with minimal change disease. Although there was intense staining of podocytes and diffuse tubular staining with anti-EMA of renal tissues, no discernible tubular labeling with anti-CD 10 antibody was observed. This is indicative of the absence of proximal tubular development (Figure 1a).

Molecular Analysis by Whole Exome Sequencing
Whole exome sequencing followed by validation with Sanger sequencing was performed in all 6 patients and their parents (Figure 2a). The WES did not reveal pathogenic mutations in the coding regions of REN, ACE, and AGTR1, but did identify a large homozygous deletion of exons 3 and 4 of AGT in all 6 patients that cosegregated with the disease. Further analysis using PCR primers flanking the possible deletion breakpoints revealed a 2800-bp deletion shared by all 6 patients and their parents (Figure 2b and c). Direct sequencing of the PCR product uncovered a novel deletion E3_E4 del:2870bp deletion (NM_000029.3:c.857-619_C1269+243del) +9bp insertion in AGT gene. This deletion included parts of intron 2 (619 bp), exon 3 (268 bp), intron 3 (1595 bp), exon 4 (145 bp), and intron 4 (243 bp). This mutation

| Table 1. Clinical characteristics and outcomes of patients with autosomal recessive renal tubular dysgenesis caused by AGT deletion |
|-------------------------------|---|---|---|---|---|---|
| Characteristic               | Patient 1 | Patient 2 | Patient 3 | Patient 4 | Patient 5 | Patient 6 |
| Parental consanguinity       | No         | No         | No         | No         | No         | No         |
| Sex                          | Male       | Female     | Male       | Female     | Male       | Male       |
| Gestational age (wk)         | 33         | 33         | 35         | 26         | 35         | 22         |
| Birth weight (g)             | 1615       | 2094       | 2205       | 804        | 2670       | 700        |
| Onset of oligohydramnios (wk)| 30         | 23         | 24         | 22         | 34         | 18         |
| Respiratory failure          | Yes        | Yes        | Yes        | -          | Yes        | -          |
| Hypotension (BP, mm Hg)      | Yes (29/13)| Yes (28/12)| Yes (34/23)| Not available| Yes (40/22)| Not available|
| Anuria at birth              | Yes        | Yes        | Yes        | Yes        | Yes        | Yes        |
| Joint contractures           | Yes        | Yes        | Yes        | No         | No         | Yes        |
| Wide fontanel                | Yes        | Yes        | Yes        | Yes        | Yes        | Yes        |
| Renal ultrasound             |           |           |           |           |           |           |
| Size                         | Normal     | Normal     | Normal     | Normal     | Normal     | Normal     |
| Echogenicity                  | Increased  | Increased  | Increased  | Increased  | Increased  | Increased  |
| Pulmonary hypoplasia          | Yes        | Yes        | Yes        | Not available| Yes        | Not available|
| Respond to fluid challenge   | No         | No         | No         | Not perform| No         | Not perform|
| Respond to inotropic agents  | No         | No         | No         | Not perform| No         | Not perform|
| Respond to plasma infusion   | Not perform| Yes        | Yes        | Not perform| Yes        | Not perform|
| Outcome (age at death)       | Death (8 days) | Death (3 days) | Death (2 days) | Death/ termination of pregnancy | Survival | Death/ termination of pregnancy |
causes a frame shift and a subsequent premature stop codon in exon 5 (NP_000020.1:p.Gly286Valfs*6), leading to a product shorter by 292 amino acids, containing intact N-terminal 285 amino acids of AGT (Figure 2c). This AGT deletion has not been reported previously and is absent from the gnomad and other databases. Using software (AnnotSV), prediction for this AGT mutation was severely pathogenic. We performed analysis of linkage disequilibrium and organization in haplotypes of single nucleotide polymorphisms adjacent to the AGT gene in all of the patients and their parents (Supplementary Figure S1). The probability that this haplotype occurred independently in all index cases was of \(1.52 \times 10^{-5}\), suggesting a founder effect.

**Prevalence of Heterozygous AGT Deletion**

Because of the nature of rare disease and identical deletion breakpoint of the AGT gene in patients and their parents, we assessed the frequency of the peculiar AGT deletion in 500 healthy and unrelated Taiwanese individuals. Six of 500 healthy subjects harbored this peculiar heterozygous AGT deletion in a heterozygous state, as detected by pulse field gel electrophoresis and confirmed by direct Sanger sequencing (Figure 3). Thus, in Taiwan, the heterozygous carrier rate was approximately 1.2% (6/500).

**Tissue AGT Expression**

We examined AGT expression in liver and kidney tissues obtained from autopsy of deceased patients 2
and 3, disease controls, and normal controls. As shown in Figure 1b, AGT expression in liver and kidney, as compared to control, was very faint in patients 2 and 3, indicating that the deletion of AGT diminished the expression of AGT both in liver and in the kidney.

Serum Levels of AGT, Renin, ACE, and Ang I and II
As the genetic defect of AGT resulted in decreased tissue expression of AGT, serum AGT, renin, Ang I, ACE, and Ang II levels were measured in affected individuals, their parents, and healthy subjects of similar age. Compared to healthy neonates, serum AGT, Ang I, and Ang II concentrations were markedly reduced in ARRTD patients (P = 0.042, P = 0.008, and P = 0.026, respectively) (Figure 4). In contrast, the serum renin levels of patients were much higher than those of healthy subjects and their parents. Relative increase in serum AGT and higher renin levels in their parents were observed. These findings indicated that the homozygous AGT deletion resulted in the reduction of serum AGT and its downstream end-product, serum Ang II.

Simulation and Measurement of Renin–AGT Interaction
As shown in Figure 5a, truncated AGT (AA 1–295) lacks the serpin domain of the AGT protein, which is critical for the interaction with renin for cleavage. The simulation models were first examined to evaluate whether this AGT deletion would abolish the interaction between AGT and renin. As shown in Figure 5b, the resolved structures of human AGT in complex with renin, human, mouse, and rat AGT (PDB code: 2X0B, 2WXW, 2WXY, and 2WXZ) were used as a template. A salt bridge...
(R:LYS286:HZ3 – A:GLU338:OE2) of wild-type AGT (amino acids 1–485) protein was not found in the 2 truncated AGT (AA 1–295 and AA 1–375/R375Q) proteins. Alternative hydrogen bonds were formed at the interface of the truncated AGT proteins and renin as compared to wild-type AGT. The calculated interaction energy at the interface of the truncated AGT (AA 1295 and AA R375Q) and renin was reduced by about 2-fold compared to that of the wild type.

Hydrocortisone May Exert Therapeutic Effects in ARRTD

Patient 5 was born after a pregnancy complicated by oligohydramnios at 34 weeks of gestational age. He presented with profound hypotension and primary anuria. Hypotension was refractory to i.v. saline challenges and vasopressors but transiently responsive to fresh frozen plasma. Peritoneal dialysis was initiated for his persistent anuria. Because of his refractory hypotension and in light of glucocorticoids as the

Figure 3. Prevalence of angiotensinogen (AGT) heterozygosity. Reverse transcription–polymerase chain reaction analysis using exon 2- to 5-specific primers detected comparatively small bands (6K) from 6 carriers of heterozygosity of AGT deletion (lanes 2–7) in contrast to relatively large bands (8K, lanes 8–14) in normal controls. Lanes 1 and 15 are positive and negative controls, respectively.

Figure 4. Serum angiotensinogen (AGT), renin, angiotensin I (Ang I), angiotensin-converting enzyme (ACE), and angiotensin II (Ang II) levels in patients, their parents, and healthy neonates.
regulators of hepatic AGT generation due to its stimulation of AGT mRNA transcription,\textsuperscript{21,22} we administered i.v. hydrocortisone (2 mg/kg every 6 hours) 40 hours after birth and continued hydrocortisone but not mineralocorticoid thereafter. His blood pressure, as shown in Figure 6, increased significantly after hydrocortisone treatment and the fresh frozen plasma infusion was discontinued at the age of 3 days. He remained normotensive until hydrocortisone was discontinued on day 5 of postnatal life. This was followed by hypotension, which was rescued again only by restarting hydrocortisone. Furthermore, we observed a much improved urinary output allowing cessation of renal replacement therapy on day 8 of postnatal life.

Further analysis demonstrated that serum Ang II level was significantly increased in parallel with serum AGT levels after administration of hydrocortisone (Figure 6). At the age of 28 days, parenteral hydrocortisone was switched to oral hydrocortisone. At last observation at the age of 12 months, he had chronic kidney disease, stage III, but still remained normotensive on oral hydrocortisone (1.5 mg/kg per day) treatment.

**DISCUSSION**

In this study, we identified a novel founder deletion mutation of AGT in 6 patients from 6 unrelated Taiwanese families. This homozygous deletion of AGT led to truncated protein (292 amino acids), less
detectable AGT protein in liver and kidneys, and diminished serum AGT, Ang I, and Ang II levels. The heterozygous carrier rate in Taiwan was approximately 1.2% (6/500). A simulation model demonstrated the attenuated interaction of this truncated protein with renin.

This novel AGT mutation is most likely pathogenic, based on aberrant protein expression (Figure 1), and also the absence of other genetic defects (ACE, REN, and AGTR) on WES that could cause ARRTD. The identification of this identical AGT mutation in our patients and parents urged us to assess the prevalence of this peculiar mutation in healthy individuals in Taiwan. Surprisingly, we found that the allelic frequency of this AGT mutations in a heterozygous state was 1.2%, and the expected incidence of patients with ARRTD would be approximately 3.6 per 100,000 pregnancies (1.2/100 × 1.2/100 × 1/4) is higher than expected. This indicates that the risk for ARRTD is higher in the Taiwanese population than in other parts of the world. Because heterozygous carriers of the AGT mutation are asymptomatic, molecular analysis of parents with offspring complicated by oligohydramnios and profound hypotension may help to uncover undiagnosed cases, enhance genetic counseling for heterozygous carriers, and provide earlier prenatal diagnosis of ARRTD for subsequent pregnancies.

In this study, ARRTD patients with truncation of AGT had low amounts of AGT protein in the liver and kidneys, resulting in decreased serum AGT and its downstream products of serum Ang I and Ang II levels. Lower expression of truncated AGT has been shown in an in vitro study. These findings resembled those in AGT null and hypomorph mice, and were consistent with a previous report in two patients with homozygous missense mutation (R375Q) in AGT. The source of AGT in the kidney is both liver and kidney derived, and a marked reduction in AGT expression in the liver and kidney indicates that the low hepatic expression of AGT might at least play an important role. The remarkably attenuated expression of truncated AGT protein in the liver caused by this AGT mutation may be related to either reduced hepatic production or enhanced hepatic degradation of truncated AGT.

Higher serum renin activity and enhanced tissue renin expression uniformly found in patients with ARRTD caused by mutations in RAS other than REN were thought to be secondary to the loss of negative Ang II-mediated regulation of renin synthesis. Of interest, our ARRTD patients with AGT truncation exhibited extraordinarily high serum renin levels (300-fold higher than normal control). Prior studies have demonstrated that the functional conserved sequences in the core serpin domain of AGT are necessary for AGT–renin interaction. The large deletion per se on AGT excluding this serpin domain of AGT may reduce renin cleavage and thus contribute to this markedly higher serum renin concentration. The results of a simulation model showing the diminishment of interaction between renin and truncated AGT may support this notion.

In parallel to an elevated serum AGT, the increased serum Ang II with simultaneous correction of
hypotension was notably found in patient 5 after persistent and higher-dose hydrocortisone treatment. The spontaneous recovery of hypotension and renal hypoperfusion in 2 cases of ARRTD caused by a nonhomozygous truncated AGT mutation have been reported previously.19,30 The presence of at least 1 nontruncating variant may allow survival.8 The rapidly fatal course in all other similar patients and the recurrence and resumption of profound hypotension after discontinuation and re-delivery of hydrocortisone in patient 5 was intriguing. Indeed, no ARRTD patients with homozygous truncating variants have survived to date.8 These findings argue against the possibility of spontaneous recovery in our surviving patient. Glucocorticoid has been shown to be the regulator of generation of AGT in liver by increasing the abundance mRNA of AGT in in vivo and in vitro studies.21,22 The mechanisms underlying the enhancement of serum AGT and Ang II concentrations by supraphysiological dosing of hydrocortisone require further elucidation.

Because glucocorticoid receptor is abundant in the proximal convoluted tubule during development,11,30 antenatal hydrocortisone may exert an effect on the expression of glucocorticoid receptor. The synthetic human Ang II might also be used for reversing the profound hypotension and might act as a growth factor during the development of proximal tubular cells.31–35 The creation of ARRTD-causing knock-in mice may help to clarify the effects of glucocorticoid and synthetic Ang II in patients with ARRTD.

In conclusion, we have identified a novel founder mutation of AGT in 6 ARRTD patients from 6 unrelated families and have discovered a higher frequency of this heterozygous mutation, suggesting that ARRTD may be underdiagnosed in Taiwan. This AGT E3_E4 del:2870bp deletion+9bp insertion mutation leads to the production of truncated AGT and a decreased detectable AGT protein in the liver with diminished serum AGT, Ang I, and Ang II levels. Prolonged and high-dose hydrocortisone may have a role in maintaining hemodynamic stability in ARRTD patients without severe pulmonary hypoplasia.

**DISCLOSURE**

All the authors declared no competing interests.

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**SUPPLEMENTARY MATERIAL**

**Supplementary File (PDF)**

**Figure S1.** Haplotype analysis of AGT deletion. The analysis of linkage disequilibrium and organization in haplotypes of single nucleotide polymorphisms adjacent to the AGT gene in all of the patients and their parents. The probability that this haplotype occurs independently in all index cases was of 1.52 x 10^-6, suggesting a founder effect.

**STREGA Checklist.**

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