Two novel deleterious variants of Angiotensin-I-converting Enzyme gene identified in a family with recurrent anhydramnios

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Abstract
Background: Anhydramnios results from the poor development of the placenta or problems with intrauterine development of the kidneys or urinary tract. Complete lack of amniotic fluid indicates a severe problem with the organs of the urinary system. The genes associated with anhydramnios show very diversity and are not yet well defined.

Methods: Whole-exome sequencing (WES) was used for an aborted male case around the 20th week of gestation diagnosed with anhydramnios. The resulted deleterious variants were verified by Sanger sequencing. Pathogenicity of deleterious variants was explored by in silico analysis.

Results: A maternally inherited deleterious frameshift variant, c.1454_1455insC, p.(S486Ffs29) in exon 9 and two paternally inherited missense variants c.1037C > G, p.(Ser346Trp) in exon 7 and c.1465A > G, p.(Asn489Asp) in exon 9 of Angiotensin-I-Converting Enzyme (ACE) gene were found and confirmed by Sanger sequencing. The resulted deleterious variants were verified by Sanger sequencing. c.1454_1455insC, p.(S486Ffs29) and c.1037C > G, p.(Ser346Trp) were identified as two novel compound heterozygous deleterious variants. The pathogenicity of these deleterious variants was determined by in silico analysis and both the deleterious variants disrupt the structure of the ACE protein.

Conclusion: Two novel compound heterozygous variants were identified in the case with anhydramnios, which may be associated with pathogenicity of anhydramnios. Our data also revealed that the WES approach may provide helpful information for genetic counseling of the families with anhydramnios.

KEYWORDS
ACE, anhydramnios, Autosomal recessive renal tubular dysgenesis, deleterious variants, WES

INTRODUCTION

Amniotic fluid is important for proper fetal development. It helps protect and cushion the fetus and plays an important role in the development of many of the fetal organs including the lungs, kidneys, and gastrointestinal tract (Abramovich, 1970; Dubil & Magann, 2013). The fluid is produced by the fetal lungs and kidneys. It is taken up with fetal swallowing.
and sent across the placenta to the mother’s circulation (Lind, Kendall, & Hytten, 1972). In the first trimester of pregnancy, amniotic fluid forms from filtration of maternal plasma. Once the fetal kidneys produce urine, around the 14–16th week of gestation, they become responsible for the production of amniotic fluid (Bronshtein, Yoffe, Brandes, & Blumenfeld, 1990). An adequate volume of amniotic fluid is pivotal in lung development (Grijseels et al., 2011). Too much or too little amniotic fluid is associated with abnormalities in development and pregnancy complications, which are referred to as hydramnios or oligohydramnios (Hamza, Herr, Solomayer, & Meyberg-Solomayer, 2013; Jeffcoate & Scott, 1959). If there is not any amniotic fluid around the fetus it is called anhydramnios, which will cause a number of potentially serious complications. Anhydramnios can be caused by a tear in the lining of the placenta or by problems with the developing fetus’s kidneys or urinary tract.

Autosomal recessive renal tubular dysgenesis (RTD, OMIM #267430) is a severe disorder of renal tubular development characterized by early onset and persistent fetal anuria and perinatal death, most likely due to pulmonary hypoplasia from early-onset oligohydramnios (the Potter phenotype)(Gubler, 2014). The disease is linked to mutations in the genes encoding several components of the renin–angiotensin system (RAS): such as AGT (Angiotensinogen, OMIM: 106150), REN (Renin, OMIM: 179820)(Al-Hamed et al., 2016), ACE (Angiotensin-I-Converting Enzyme, OMIM: 106180), and AGTR1 (Angiotensin II Receptor Type 1, OMIM:106165). Most of them are newly identified mutations and ACE mutations are the most frequently found and observed in two-thirds of families (64.6%) (Al-Hamed et al., 2016; Gribouval et al., 2005, 2012).

Here, we report c.1037C > G, p.(Ser346Trp) and c.1454_1455insC, p.(S486Fs29), two novel compound heterozygous deleterious variants of the ACE gene in a Chinese recurrent anhydramnios patient.

2 | MATERIALS AND METHODS

2.1 | Patients

A 33-year-old parous woman was cared for by our maternal medicine service due to a history of anhydramnios during the second trimester of a previous pregnancy. During her subsequent pregnancy ultrasounds were performed at 12 and 16 weeks with normal amniotic fluid volumes, and found have anhydramnios at the 20th week of gestation. Noninvasive prenatal testing and fetal cytogenomic SNP microarray showed no chromosomal abnormalities. The case was found to be appropriately grown. The kidneys appeared ultrasonographically normal with the positive end-diastolic flow on umbilical artery Doppler examinations. There was no history of rupture of membrane. The pathological anatomy of the case showed two kidneys with intact capsules, and the surface was visible with shallow grooves, like the brain. The right kidney is partially autolyzed. These findings were consistent with a normal amount of kidney tissue and a normal number of glomeruli, without any obvious abnormalities.

Written informed consent for genetic analysis and publication of personal photographs was obtained from each participant. This study was approved by the Medical Ethics Committee of Renmin Hospital, Wuhan University. All procedures were carried out in accordance with ethical guidelines for human subjects research. Family histories were determined, and pedigree charts were drawn to trace the inheritance model.

2.2 | Exome sequencing

Genomic DNA was extracted using a QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) from 400 μl peripheral blood. The DNA qualities and quantities were verified with a Nanodrop spectrometer (Nanodrop Technologies, Wilmington, DE, USA) and a Qubit fluorometer (Life Technologies, Grand Island, NY, USA). Amplicon libraries were prepared according to the manufacturer’s instructions using the Ion PI library kit plus an Ion PI Ampliseq Exome RDY 1 × 8 (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham, MA, USA), and an Ion PI Hi-Q OT2 200 kit was used for template preparation according to the manufacturer’s instructions. Sequencing was conducted with an Ion PI Hi-Q Sequencing 200 kit and an Ion PI Chip v3 in a Proton Semiconductor Sequencer (Life Technologies, Thermo Fisher Scientific Inc.).

2.3 | Bioinformatic analysis

Raw sequence data were processed to trim adapter sequences, align to the hg19 human reference genome (GRCh37), analyze coverage and call variants on the Ion Torrent Server using the Torrent Suite software version 5.4.11 and the Variant Caller plugin, with the default parameters suggested by the manufacturer. The variant analysis was performed with the Ion Reporter 5.10 software to classify variants as single-nucleotide variations (SNVs), multi-nucleotide variants (MNVs), insertions or deletions (indels). All found variants and regions below 20 × coverage were visually verified with the Integrative Genomics Viewer (IGV) v2.3.8 (Broad Institute). We then excluded the variants that were expected to have low pathogenicity (intergenic, intronic located away from the splice sites, and synonymous variants that did not interfere with the splice sites). The next step was to remove variants with...
minor allele frequencies (MAFs) in the Exome Aggregate Consortium (ExAC) database greater than 0.5% as well as the variants with a MAF greater than 0.5% in a local variant database to account for the local population-specific variants or systematic errors in sequencing or mapping methods. Finally, we filtered the variants found in the genes implicated inherited disorders of abnormal amniotic fluid metabolism, abnormal kidneys or urinary tract, or abnormal gastrointestinal tract and ranked them according to the presumed inheritance patterns in each family.

2.4 Pathogenic effect of the identified deleterious variants

In order to exclude genetic variants previously reported as polymorphisms, we compared the identified alleles with data reported in NCBI dbSNP build 37 (www.ncbi.nlm.nih.gov/projects/SNP/), ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/variation), HGMD (http://www.hgmd.cf.ac.uk/ac/index.php) as well as in 1000 Genomes Project (www.1000genomes.org/), NHLBI Exome Sequencing Project (ESP) (esp.gs.washington.edu), ExAC (http://exac.broadinstitute.org/), and Exome Variant Server (https://evs.gs.washington.edu/EVS/). The impact of the missense variants on protein structure was evaluated using several in silico prediction tools: InterVar (http://wintervar.wglab.org/) databases (Li & Wang, 2017), Mutation Taster (http://www.mutationtaster.org/), (Schwarz, Cooper, Schuelke, & Seelow, 2014), Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/) (Adzhubei, Jordan, & Sunyaev, 2013), Provean (http://provean.jcvi.org/index.php) (Choi & Chan, 2015), CADD (https://cadd.gs.washington.edu) (Rentzsch, Witten, Cooper, Shendure, & Kircher, 2015), REVEL (https://sites.google.com/site/revelgenomics/) (Ioannidis et al., 2016), VarCards (http://varcards.biols.ac.cn/) (Li et al., 2017), and SIFT (http://sift.jcvi.org) (Sim et al., 2012). Furthermore, PhyloP algorithm (http://compgen.bscb.cornell.edu/phast/background.php), GERP (http://mendel.stanford.edu/SidowLab/downloads/gerp/index.html), PhastCons (http://compgen.cshl.edu/phast/), or SiPhy (http://portals.broadinstitute.org/genome_bio/siphy/) allowed for the evaluation of the conservation of residues across the species. In order to exclude variants not segregating within the analyzed family, SNVs/INDELs were filtered by vcf tools (http://vcftools.sourceforge.net/) (Danecek et al., 2011). All variants were annotated according to the nomenclature recommended by the Human Genome Variation Society (HGVS, http://www.hgvs.org).

The 3D structures of the wild-type and the mutant forms of ACE were built with Swiss PDB viewer (Kaplan & Littlejohn, 2001). Both the wild-type and mutated ACE protein sequences were used to perform protein structure prediction using I-TASSER (http://zhanglab.ccmb.med.umich.edu/ITASSER/) as previously reported (Yang et al., 2015). The B-factor of I-TASSER, which indicates the extent of the inherent thermal mobility of residues/atoms in proteins, is calculated from threading template proteins from the Protein Data Bank along with sequence profiles derived from sequence databases. The normalized B-factor of the target protein was defined by \( B = \frac{B' - u}{s} \), where \( B' \) represents the raw B-factor value, and \( u \) and \( s \) represent the mean and standard deviation of the raw B-factors along the sequence, respectively.

2.5 Mutational analysis

The primers were designed using Primer-BLAST (Ye et al., 2012) online or using Primer Premier 5.0. Primers covered the sequences of the exon7 and exon9 of ACE (XM_006721737.3), including exon/intron junctions, and primer sequences are shown in Table S1. The primers were synthesized by Invitrogen (Shanghai, China). Each 20-μl PCR mixture contained 100 ng genomic DNA, 1 μl of 10 μM forward and reverse primers (with a final concentration of 100 nM), and 10 μl of 2 × Taq PCR MasterMix (Takara, Dalian, China). PCR was carried out in Veriti thermocycler (Applied Biosystems) using the following protocol: 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 60 s; and a final extension at 72°C for 10 min. The amplified products were purified with a QIAquick PCR Purification Kit (Qiagen, Shanghai, Germany) and sequenced using an ABI 3500 DNA sequencer (Applied Biosystems). DNA sequences were analyzed with a genome reference sequence on NCBI BLAST. The deleterious variant was named according to the recommendations of the Human Genomic Variation Society (HGVS: http://www.hgvs.org/).

3 RESULTS

3.1 Deleterious variants detection and sanger sequencing validation

After applying filtration process and annotation, WES revealed 3 heterozygous ACE variants in the patients. The resulted ACE variants were further subjected to a process for discovering pathogenic deleterious variants, and they are identified as an exonic missense variant c.1037C > G, p.(Ser346Trp) in exon 7, an exonic frameshift deleterious variant c.1454_1455insC, p.(S486Ffs29) and an exonic missense variant c.1465A > G, p.(Asn489Asp) in exon 9, respectively. Sanger sequencing of these loci was performed on all immediate family members as described previously, the results are shown in Figure 1a(Wang, Tang, Wang, Lu, &
Yang, 2019); and all the identified three mutants localized at the extracellular N-domain of the ACE protein (Figure 1b).

The ACE missense variants (c.1037C > G and c.1465A > G) of the case come from his unaffected father, the ACE insertion mutation (c.1454_1455insC) comes from his unaffected mother, whereas the unaffected elder sister of the proband harbored wild-type alleles. The deleterious variants are summarized in Table 1, and the family pedigree was drawn (Figure 2). Because the aborted fetus underwent pathological anatomy, it’s known that the case was male.

3.2 | The pathogenic effect of the identified deleterious variants

The missense variants were predicted to damage the protein function by online programs including SIFT, PolyPhen2, MutationTaster, PROVEAN, CADD, DANN, FATHMM, GenoCanyon, and REVEL (Table S2), the conservation analysis was assessed by online programs including GERP, phyloP, phastCons, and SiPhy (Table S3), and the variant nucleotides code for highly conserved amino acids throughout species as indicated in Figure 3.

3.3 | Protein structure prediction

The exonic frame-shift variant (c.1454_1455insC, p.(S486Ffs29) was absent from all public databases and our internal database. We applied SWISS-Model and I-TASSER to predict whether the mutation could alter the transcription in Figure 4a. We build 3D structures and compare the wild-type and the mutant forms of ACE with Swiss PDB viewer (Kaplan & Littlejohn, 2001). Our analysis showed that the deleterious variant, p.(S486Ffs29) cause theoretical truncated proteins and affect the secondary structures of the protein (Figure 4b).

The exonic deleterious variant c.1037C > G, p.(Ser346Trp) was absent from all public databases and our internal database, deleterious variant c.1465A > G, p.(Asn489Asp) was reported in ExAC with a frequency of 1.65E-05. We speculate the novel missense variant c.1037C > G, p.(Ser346Trp) contributed more to anhydramnios in this family. The chemical structure of the wild-type and mutated amino acids was drawn in Figure 5a, the wild-type serine (SER) has an uncharged polar hydrophilic amino acid, and the mutant tryptophan (Trp) has a nonpolar hydrophobic amino acid. This data indicate that the Ser-to-Trp will change the hydrophilicity of ACE protein. With Swiss PDB viewer, the 3D structures was built to show...
The switch by comparing the structure of the wild-type and the mutant protein (Figure 5b) (Kaplan & Littlejohn, 2001).

Taken together, our analysis showed that both deleterious variants, p.(S486Ffs29) and p.(Ser346Trp) cause a theoretical deleterious change in proteins and affect the secondary structures of the protein.

**4 | DISCUSSION**

The ACE gene plays a crucial role in the renin–angiotensin system during human kidney development, and inactivity of this enzyme will result in Autosomal recessive RTD (OMIM #267430) (Gribouval et al., 2012). Early death occurs in most cases from anuria, pulmonary hypoplasia, and refractory arterial hypotension.

The ACE gene is located at 17q23.3 and encodes an enzyme involved in catalyzing the conversion of angiotensin I into a physiologically active peptide angiotensin II. Angiotensin II is a potent vasopressor and aldosterone-stimulating peptide that controls blood pressure and fluid-electrolyte balance. This enzyme plays a key role in the renin–angiotensin system. Diseases associated with ACE include Microvascular Complications Of Diabetes 3 (OMIM #612624) and RTD (OMIM #267430). Pathways related with ACE gene are agents acting on the renin–angiotensin system pathway, pharmacodynamics, and Toll-like receptor signaling pathway. Homozygous or compound heterozygous mutations in the ACE gene may be involved in the pathogenesis of RTD. Missense and Indels account for the majority of the mutation types. In this case, novel compound heterozygous deleterious variants in the ACE gene (c.1037C > G and c.1454_1455insC) were identified in the abortion case. This deleterious variant was not found in the 1000G, EVS, ExAC or HGMD databases. Multiple lines of computational evidence support a deleterious effect on the gene. The ACE c.1454_1455insC variant resulted in frame-shift mutation, when it was mutated, the length of ACE Protein was truncated from 1366 amino acids (149.7kDa) to 513 amino acids (58.2kDa).

The ACE c.1037C > G deleterious variant resulted in a serine-to-tryptophan substitution at amino acid position 346 of angiotensin-converting enzyme. Various types of software predicted that this missense variant would have deleterious effects. Moreover, conservation analysis showed that the mutated nucleotide code the highly conserved amino acid throughout species. Protein structure analysis implied that this deleterious variant may affect the function of angiotensin-converting enzyme. Thus, ACE c.1037C > G (p.Ser346Trp) in trans with the ACE c.1454_1455insC;p.(S486Ffs29) may have caused RTD in this family and its cosegregation with this RTD in these family members. We conclude that they are pathogenic and led to anhydramnios in this family.
**FIGURE 3** Conservation analysis of ACE. The result indicated that the ACE amino acid sequence is partly evolutionarily conserved in most organisms. The arrow indicates the 346th, 486th, and 489th amino acid. Multiple alignments of indicated these mutated sites were highly conserved.

**FIGURE 4** Protein structure analysis of ACE c.1454_1455insC, p.(S486Fs29). (a) Both the wild-type (upper) and mutated (lower) ACE protein sequences were used to perform protein structure prediction using I-TASSER. (b) The wild-type (left) and mutant (right) structure of ACE protein was predicted by Swiss PDB viewer. The deleterious variant c.1454_1455insC, p.(S486Fs29) of ACE lead to premature termination of translation and result in a truncated protein as a result of nonsense-mediated mRNA decay. The truncating variant c.1454_1455insC result in a truncated ACE protein from 1,366 amino acids (149.7kDa) to 513 amino acids (58.2kDa).
According to ACMG guidelines (Richards et al., 2015), the de novo insertion variant c.1454_1455insC, p.(S486Ffs*29) (NM_000789.3) in \( \text{ACE} \) gene should be categorized as “pathogenic variant” which is evident by its frameshift, absent from control databases (Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium), potential deleterious effect on \( \text{ACE} \) protein identified by multiple computational protein structure analysis. The exonic missense variant, c.1037C > G, p.(Ser346Trp) (NM_000789.3) in the gene is categorized to be the “Likely pathogenic variant” because this variant is absent from the control databases, detected in trans with a pathogenic variant, showed a deleterious effect by the computational analysis, and the highly specific patient’s phenotype or family history for a disease with a single-genetic etiology. The exonic missense variant c.1465A > G, p.(Asn489Asp) is categorized as “Uncertain significance variant” for the disease owing to its extremely low frequency for a recessive disease in the control databases, observed in trans with a likely pathogenic variant in any inheritance pattern, and also the potential deleterious effect, and highly specific patient's phenotype or family history as variant c.1037C > G, p.(Ser346Trp).

In summary, novel compound heterozygous deleterious variants in the \( \text{ACE} \) gene (c.1454_1455insC and c.1037C > G) may be responsible for the clinical manifestations of the described recurrent anhydramnios patient with RTD. In this study, NGS (next-generation sequencing) provided a rapid and powerful approach for the genetic diagnosis of RTD. Our analyses indicated that the deleterious variants might constitute candidate disease-causing mutation associated with RTD.

Autosomal recessive RTD was regarded as a rare disorder, which may be more frequent than previously thought, especially in the stillborn case with abnormal amniotic fluid metabolism. It might be easily neglected in the absence of a detailed pathological examination of the renal parenchyma. Consideration of this severe disease in anuric cases with structurally normal kidneys at sonography is essential to allow for mutation analysis of \( \text{ACE} \) genes, genetic counseling and early prenatal diagnosis.

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CONFLICT OF INTEREST
The authors have declared no conflict of interest. The results presented in this paper have not been published previously in whole or in part, except for an abstract.

AUTHORS’ CONTRIBUTIONS
Clinical data collection/analysis and WES performance and data analysis (J.W., Q.B., B.C., L.X.); Manuscript writing

**FIGURE 5**  Protein structure analysis of \( \text{ACE} \) c.1037C > G, p.(Ser346Trp). (a) Chemical Structure of the original (left) and the mutant (right) amino acid. (b) Schematic structures of the original (left) and the mutant (right) amino acid. The uncharged polar hydrophilic wild-type amino acid serine (SER) was mutated to a non-polar hydrophobic amino acid tryptophan (Trp), the Ser-to-Trp will change the hydrophilicity of the subunit, which cause a theoretical deleterious change in proteins and affect the secondary structures of the protein.
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ETHICS APPROVAL AND CONSENT TO PARTICIPATE
This study has been approved by the Ethical Committee of Renmin Hospital of Wuhan University and written informed consent was obtained from all patients.

CONSENT FOR PUBLICATION
All participants signed a consent form for publication.

DATA AVAILABILITY STATEMENT
All data supporting the results reported in a published article can be found.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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