A Novel Mutation Pattern of Kidney Anion Exchanger 1 Gene in Patients With Distal Renal Tubular Acidosis in Iran

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Introduction. Mutations of the anion exchanger 1 (AE1) gene encoding the kidney anion exchanger 1 can result in autosomal dominant or autosomal recessive form of distal renal tubular acidosis (DRTA). This study aimed to report deletion mutations of the AE1 and its impact on Iranian children with DRTA.

Materials and Methods. Twelve children with DRTA referred to Ali Asghar Children Hospital were investigated for all AE1 gene exons through polymerase chain reaction amplification, DNA sequencing, and bioinformatics analysis.

Results. Eleven of 12 patients (91.7%) showed an alteration in AE1 gene with a real hot spot in its exons 11 or 15. Homozygote and heterozygote deletions were confirmed in exon 15 in 5 (41.7%) and 3 (25.0%), respectively. Two patients (16.7%) showed homozygote deletions in exon 11 of AE1 gene, and 1 patient (8.3%) showed point mutation in exon 11. The 3-dimensional structures of the native and these mutant kidney AE1 proteins were determined by the multitemplate method using the Phyre and Hidden Markov Model algorithms.

Conclusions. Parents’ consanguinity of these patients reveals that cousins are at a high risk for DRTA. This study is considered as a pilot study showing the importance of AE1 mutations in Iranian children with DRTA and further studies is recommended in this geographic region of the world. These models suggest that alteration in the structures leads to alteration in function and change in the current role of AE1.
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Table 1. List of Specific Primers Used for Amplification of Exons 11, 15, 17, 19, and 20 of Anion Exchanger 1 Gene in Patients With Distal Renal Tubular Acidosis

<table>
<thead>
<tr>
<th>Exon</th>
<th>Polymerase Chain Reaction Primer Sequence</th>
<th>Tm (°C)</th>
<th>Product Size</th>
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<tbody>
<tr>
<td>11</td>
<td>CCTCACCTCCTCCACGCTACTCC CAGAAGTTGGGGCTGAGACAGAG</td>
<td>62</td>
<td>318</td>
</tr>
<tr>
<td>15</td>
<td>AAGGCCAGGAGGTGGGGAGTGACTGGGAAATGAGGACCTGGGGGGTATC</td>
<td>70</td>
<td>201</td>
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<tr>
<td>17</td>
<td>TGAGAGGAGCGGGGAGACGGGGCAGGAGGATGGTGAAGGAGGTGCCCATGAACTTCTG</td>
<td>70</td>
<td>347</td>
</tr>
<tr>
<td>19</td>
<td>GGTACAGGACCGCTTTTCTGG GCTGCGCCTAGTCTGAGGAC</td>
<td>60</td>
<td>334</td>
</tr>
<tr>
<td>20</td>
<td>TCTCACCCCTGTCTCTCTGG TGAGTGCGCCATGACCTCCTG</td>
<td>65</td>
<td>198</td>
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</table>
with or without deafness. Children with acidosis at the time of pyelonephritis, obstructive uropathy, cystinosis, drug-induced tubulopathy, and vasculitis were not included. The study protocol was approved by the Ethics Committee of Iran University of Medical Sciences.

**Mutation Studies**

Genomic DNA was isolated from peripheral blood lymphocytes using standard phenol/chloroform DNA extraction method. To amplify *AE1* exons, exon-specific primers including exon/intron junctions were designed, synthesized, and utilized based on their coding sequence. The exon-11- and exon-15-specific primers and their sequences are listed in Table 1.

One microgram per microliter of each DNA sample was subjected to polymerase chain reaction. The samples were first denatured at 95°C for 2 minutes. Polymerase chain reaction reactions were carried out for 30 cycles consisting of denaturation at 94°C for 1 minute, annealing at specific temperature for each primer for 1 minute, and extension at 72°C for 1 minute. The polymerase chain reaction products were run on a 1.5% agarose gel electrophoresis, and the images were analyzed for mutation detections. For mutation screening, sequencing of selective exon polymerase chain reaction product of the human *AE1* gene was performed using the ABI sequencing system.

**In Silico and Statistical Analyses**

In silico analysis was performed in order to analyze the mutations and their effects on the *AE1* protein. Clustal W program (http://www.genome.jp/tools/clustalw/) and Modeller v9.10 were used, as well as Phyre 2 algorithm, HMM algorithm, Kyte-Doolittle scale (R), Needleman-Wunsch, and BLOSUM-62 alignment. All the sequences were derived from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/).

**RESULTS**

**Cases Reports and Clinical Follow-up**

Demographic information and clinical and paraclinical data of the patients are summarized in Table 2. Some of the patients had been referred while receiving bicarbonate or citrate solution. Most of the patients had suffered from hemolytic anemia (> 87%).
Figure 1. A, Number 1 is 100-bp DNA ladder fermentase; number 2, negative control; number 3, NL-1; number 4, 91-1; number 5, 91-2; number 6, NL-2; number 7, 91-3; number 8, 91-4; number 9, 91-5; number 10, 91-6; number 11, 91-7; number 12, 91-8; and number 13, 91-9. B, This alignment shows truncation of the anion exchanger 1 (AE1) proteins in cases 91-2 and 91-1 with deletion in exon 11. C, This alignment shows truncation of the AE1 proteins in cases 91-3 and 91-5, but in case 91-6 (that shows no frame shift) with deletion in exon 15. D, Deletion chromatography of AE1 exon 11 in case 91-3. E, Deletion sequence of AE1 exon 11 in case 91-3. F, Family analysis of AE1 exon 11 of patient 91-3. The ‘a’ is 100-bp DNA ladder; ‘b,’ negative control; 1, healthy control; 2, patient 91-2; 3, mother; 4, father; and 5, patient 91-3.
accompanying the DRTA criteria (Table 2), which was predictable as the most prevalent manifestation associated with DRTA in \textit{AE1} mutation.\textsuperscript{1}

**Polymerase Chain Reaction Products With Deletion Mutation**

Molecular characterization of the patients with DRTA referred to Ali Asghar Children Hospital was identified. Analysis of gel electrophoresis and sequencing results (Figures 1 and 2) confirmed that there were certain alterations in exons 11 and 15 of the \textit{AE1} among 11 of the 12 cases (91.7\%). Eight patients had alterations in exon 15, consisting of homozygote (5 cases) and heterozygote (3 cases) deletion mutations. Three patients showed alterations in exon 11, consisting of homozygote deletion (2 cases) and point mutation (1 case), the latter resulting in substitution of glycine with glutamic acid (c.G1097A). Deletion mutations included c.del 1103-1119 (16 bp), c.del 1165-1181 (16 bp), c.del1807-1848 (41 bp), c.del 1805-1866 (61 bp), c.del 1810-1859 (49 bp), and c.del1805-1832 (27 bp). These alterations likely affected the hydrophobicity of AE1. Table 3 shows DNA and corresponding protein alterations in DRTA patients. The major alterations occurred in the amino acids 368 (prolin) and 602/603 (R/V) which were located in the exons 11 and 15, respectively. Meanwhile, as 2 hotspots, these two exons of the \textit{AE1} could be the most common mutation sites leading to DRTA in our consanguineous Iranian patients.

**Table 3. DNA and Protein Change in Some Patients With Distal Renal Tubular Acidosis and Novel Mutations in Anion Exchanger 1 Gene**

<table>
<thead>
<tr>
<th>Patient</th>
<th>DNA Change</th>
<th>Amino Acid Change</th>
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<tbody>
<tr>
<td>91-1</td>
<td>c.del 1103-1119 (16 bp)</td>
<td>p.P368R, fs 20X</td>
</tr>
<tr>
<td>91-2</td>
<td>c.del 1165-1181 (16 bp)</td>
<td>p.P368A, fs 10X</td>
</tr>
<tr>
<td>91-3</td>
<td>c.del 1807-1848 (41 bp)</td>
<td>p.R603H, fs 19X</td>
</tr>
<tr>
<td>92-4</td>
<td>c.del 1805-1866 (61 bp)</td>
<td>p.R602P, fs 40 X</td>
</tr>
<tr>
<td>91-5</td>
<td>c.del 1810-1859 (49 bp)</td>
<td>p.V603W, fs 41 X</td>
</tr>
<tr>
<td>91-6</td>
<td>c.del 1805-1832 (27 bp)</td>
<td>p.del602-611,non fs</td>
</tr>
</tbody>
</table>

**Figure 2.** Top Left, Anion exchanger 1 (\textit{AE1}) exon 11. The ‘a’ is 100-bp DNA ladder; 1, patient 91-6; 2, mother, and 3, father. Top Right, Deletion chromatography of \textit{AE1} exon 11 in case 91-6. Bottom, Deletion sequence of \textit{AE1} exon 11 in case 91-6.
Protein Alignment, Mutation Detection, and Modeling

Our methods for detecting mutations are based on the alignment with the Clustal W program. To use clinical terminology, samples from the patients were compared to standard reference sequence for AE1 in the National Center for Biotechnology Information, NM_000342.3 (Figures 1B, 1C, 1E, and 2C). All detected mutations in this study altered the basolateral kidney transporter protein and have been modeled and submitted in protein model database (http://mi.caspur.it/PMDB; Table 4).

Table 4. Mutant Kidney Anion Exchanger 1 Proteins in Iranian Patients With Distal Renal Tubular Acidosis*

<table>
<thead>
<tr>
<th>Number</th>
<th>Length (Size) aa</th>
<th>Access Number</th>
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<tbody>
<tr>
<td>1</td>
<td>1599</td>
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<td>PM0078811</td>
</tr>
<tr>
<td>10</td>
<td>902</td>
<td>PM0078812</td>
</tr>
</tbody>
</table>

*Protein Model Database available from: http://mi.caspur.it/PMDB


The Phyre 2 algorithm which was used in this research project found similar folds. Any of the 20 identical amino acids between query and templates was a hit for this algorithm. After finding similar structures, this server predicted query model based on identical folds. In other words, by using this algorithm, alignment performed between folds instead of the entire template structure. Using this algorithm, we found 58%, 73%, and 62% identical folds for 3 patients (patients 7, 8, and 9). The rest of misaligned residues were modeled by the HMM algorithm. Finally, by using the Modeller v9.10 and multitemplate based prediction method, complete models were generated. The final predicted models minimized regarding energy level by choron algorithm.

Effect of Mutations on Protein Hydrophobicity

Because of frame shift mutations, structures of mutant models versus native one were different in conformation and amino acid type. Since AE1 is a transmembrane protein, any change in hydrophobicity properties may change position of the protein within cell membrane. Any alteration in the protein structure may influence protein function; therefore, to investigate hydrophobicity changes upon mutation the Kyte-Doolittle scale (R) was utilized. This scale is widely used for detecting hydrophobic regions in proteins and can be used for identifying both surface-exposed regions as well as transmembrane regions. Regions with a positive value are hydrophobic. Figure 3 indicates changes in hydrophobicity properties upon some mutations. Based on Figure 4, an increased hydrophobicity in the exon 11 was detected, while cases 91-3, 91-4, and 91-5 show significant decreased hydrophobicity. However, a change in hydrophobicity upon mutation in case 91-6 is not significant. The root-mean-square deviation between mutant structures and wild-type model were calculated by the Needleman-Wunsch alignment algorithm and blocks substitution matrix-62 as alignment matrix. Results indicated that root-mean-square deviation value between wild type and 91-1 was 1.223; for 91-2, it was 0.840; for 91-3 it was 0.472; for 91-4, it was 0.994; for 91-5, it was 0.787; and finally for 91-6, it was 1.088. These scores indicated that most of the changes in protein structure were imposed by mutations in 91-1 and 91-2, while 91-3 had the least influence on protein structure.

DISCUSSION

Our report is the first that identifies relatively large deletions, from 16 bp to 61 bp, in exons 11 and 15 of AE1 associated with ARDRTA with hemolytic anemia in some Iranian patients. Previously, some proton-ATPase mutations have been reported in DRTA associated with deafness in some other Iranian patients. Deletions mostly cause frame-shift and lead to the intracellular retention of AE1 transporter. We also presented a novel point mutation in exon 11 of AE1 that affected the hydrophobicity of the protein and likely prevented its placement in the basolateral membrane or trafficking. Our patients were mostly suffering from anemia, which is in line with the previous reports regarding ARDRTA. It should be noted that the recessive diseases are generally defined by clinical phenotypes, which may be variable in expressivity and penetrance and can...
also be modified by the effect of other genes.

It was first thought that AE1 mutations always cause autosomal dominant DRTA, although the first AE1 mutation associated with ARDRTA was reported in 2 Thai siblings. They were found to carry a homozygous AE1 mutation resulting in a substitution of glycine by aspartic acid at position 701 (exon 17) in the AE1 protein (G701D), namely band 3 Bangkok I, which also links to 2 polymorphisms, M31T and K56E. When expressed in Xenopus oocyte, both erythrocyte-AE1 and kidney-AE1 G701D showed lack of cell surface expression and anion transport activity. Three patients with ARDRTA with compound
heterozygous AE1 Southeast Asian ovalocytosis (SAO)/G701D mutations were later reported in 2 Malaysian families, and ARDRTA in another patient from a Malaysian family resulted from compound heterozygous AE1 SAO/A858D mutations (exon 19). In the same study, ARDRTA associated with other genotypes including SAO/ΔV850, ΔV850/ΔV850, and ΔV850/A858D was observed in patients from 6 Papua New Guinean families. Southeast Asian ovalocytosis is a morphological erythrocyte abnormality caused by a mutational deletion of 27 bp in exon 11 of AE1, leading to an inframe 9 amino acid deletion involving Ala400-Ala408 of eAE1, at the junction between the N-terminal domain and the first transmembrane span. Additionally, mutations in AE1 presenting in N-terminal and C-terminal domains, in exons 5 and 9, have shown to be associated in pH-dependent conformational changes. Other reports regarding ARDRTA have shown mutations in exons 5, 11, 13, 14, 15, and 16. A large proportion of these alterations are point mutations, and notably, some of them are shown to be compound heterozygotes, which result in dysfunctioning of AE1 in different manners, largely trafficking defect. However, a small number of deletion mutations are reported, including Human band 3 Walton, which is derived from deletion of 11 amino acids from the C-terminal of AE1. This condition is associated with the autosomal dominant DRTA in which intracellular retention of AE1 prevents its natural function. Another report regarding deletion mutation represents deletion of Valin 850 that showed an autosomal recessive pattern, though in combination with other mutation types (compound heterozygote) led to pseudo-dominant phenotype.

CONCLUSIONS
The results of our study clearly indicate that AE1 mutations in Iranian patients can cause ARDRTA, occurred in certain pattern of deletion or point mutation in exons 11 and 15. It shows that consanguineous marriage in Iran may put the siblings at high risk for this particular pattern of mutation resulting in DRTA among those with a history of this disease in close family members or relatives. Therefore, all these new findings regarding DRTA should be considered in genetic counseling in Iran. This consideration is recommended in a study of a large sample size of patients involved in DRTA in other Middle East countries. Also functional studies of the AE1 disrupted protein due to the reported mutations of the gene in this report and further translational studies are suggested. Consistent with our results, the most prevalent mutations in DRTA and hemolytic anemia is AE1 mutations, whereas deafness is an important feature in some proton-ATPase mutations. Therefore, we suggest a population-based approach in Iranian DRTA patients with hemolytic anemia to be examined exons 15 and 11, as one of the first actions toward genetic confirmation of the disease.

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CONFLICT OF INTEREST
None declared.

REFERENCES


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