Original Article

# Digenic Inheritance of Anion Exchanger 1 (SLC4A1) and Human Alpha Globin (HBA) Mutation Results in Distal Renal Tubular Acidosis and Hemolytic Anemia in a Thai Patient

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## Abstract

**Background:** Mutations of *SLC4A1* gene encoding anion ($Cl^-/HCO_3^-$) exchanger 1 *(AE1)* are responsible for majority of distal renal tubular acidosis (dRTA) in Thailand where thalassemias and hemoglobinopathies are also prevalent. It is possible that mutations in both genes may interact with each other and result in modification of hematologic phenotypes of the thalassemic patients and carriers.

**Objective:** To investigate *AE1* and alpha-globin gene mutations in patients with dRTA concomittent with anemia and hepatosplenomegaly

**Methods:** A patients with dRTA and anemia with hepatosplenomegly was examined by automated CBC, peripheral blood smear analysis, osmotic fragility test, hemoglobin typing, and serum iron study. Mutations of α- globin genes causing α- thalassemia were characterized by using standard method. *AE1* mutations were screened by using polymerase chain reaction and single-strand conformation polymorphism (PCR-SSCP), PCR and restriction digestion, and direct sequencing.

**Results:** A 9-year-old boy who manifested classical phenotypes of dRTA including hypokalemic metabolic acidosis and failure to thrive was found to have anemia (Hct 30%), mild anisopoikilosis (RDW 18%), macrocytosis (mean corpuscular volume (MCV) of 89.5 pg), stomatocytosis, decreased red cell osmotic fragility, normal hemoglobin typing (Hb A 96% and $A_2$ 3%), and normal iron study (serum ferritin 107 ng/mL). The mutational analyses demonstrated a single α-globin gene deletion ($-\alpha^{3.7}/\alpha\alpha$), and compound heterozygous *AE1* mutations, a 27-bp deletion in exon 11 and missense mutation in exon 17, designated as SAO/G701D.

**Conclusion:** The co-existence of heterozygous α-thalassemia ($-\alpha^{3.7}/\alpha\alpha$) and compound heterozygous SAO/G701D mutations of *AE1* gene results in hemolytic anemia and hepatosplenomegaly in a dRTA patient.

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## Introduction

Distal renal tubular acidosis (dRTA) is a disease characterized by an incapability of the distal nephron to secrete hydrogen ion into urine in the present of systemic metabolic acidosis.[1] The patient with dRTA has hyperchloremic metabolic acidosis in association with metabolic bone disease, failure to thrive, nephrocalcinosis and/or nephrolithiasis.[2] The anion ($Cl^-/HCO_3^-$) exchanger 1 *(AE1)* is found in the basolateral membrane of the type A intercalated cells of renal collecting ducts, which are involved in $H^+$ secretion.[3,4] The *AE1* is encoded by the solute carrier protein encoding gene, *SLC4A1* gene, located on chromosome 17q21-22[5] (MIM 109270). This *AE1* gene encodes both erythroid (e) and kidney (k) isoform of AE1 protein. The *AE1* mutation shows pleiotrophic effects resulting in two distinct disorders which are red cell abnormalities and dRTA. The defect in kAE1 of type A intercalated cells results in a failure to establish or maintain a cell-to-lumen H+ gradient, and leads to dRTA.[4] The mutations of the *AE1* gene associated with autosomal dominant (AD)[6-11] and autosomal recessive (AR) dRTA.[12-17]

Several *AE1* gene mutations have been described in Thai dRTA patients with Southeast Asian ovalocytosis (SAO) and G701D being the most two frequent mutations identified.[12,13,16]

The human eAE1 or band 3 is the major integral membrane protein of the red cell. The functions of band 3 protein are anion transport across membrane and stabilize the lipid bilayer membrane. The mutation of eAE1 results in hereditary spherocytosis, Southeast Asian ovalocytosis and hereditary acanthocytosis.[18] The majority of eAE1 mutation reported to date apparently cause only erythroid abnormalities without renal phenotype. However, there are some reports describing abnormalities of RBC accompanied with dRTA caused by *AE1* mutation.[12-15,19]

Thalassemias are prevalent in Thailand. Thus, mutations of *AE1* gene and globin gene may co-exist and could potentially increase the severity of the diseases in some individuals. Tanphaichitr *et al.* reported xerocytic hemolytic anemia in two siblings with dRTA who were found to be double homozygote for *AE1* gene mutation (G701D/G701D) and beta-globin gene mutation (Hb E/E).[12] Herein, we report the first case of a patient with hemolytic anemia and hepatosplenomegaly who was found to have α-thalassemia trait in addition to *AE1* gene mutations.

## Materials and methods

### Patient

A 9 year-old-Thai boy presented with polydipsia, polyuria, muscle weakness, and delayed growth. Physical examination revealed a height of 92 cm and a weight of 16 kg, pallor, liver span 9 cm, and spleen papated at 3 cm below left costal margin. There were hypokalemia and normal anion gap metabolic acidosis, serum Na 135, K 2.5, Cl 107, and $HCO_3$ 11.4 mEq/L, Urinary findings included pH 7.0 and decrease net acid excretion 20 μmol/1.73m$^2$/min (normal > 70), indicating urinary acidification defect. Urinary calcium excretion was 9.8 mg/kg/24 hours. Renal ultrasound showed bilateral nephrocalcinosis. The patient's hemotologic profiles are shown in Table 1. The peripheral blood smear shows ovalocytes, stomatocytes, and anisocytosis (Fig. 1). The patient was diagnosed with complete dRTA and responded well to treatment with potassium citrate. His hepatosplenomegaly resolved following normalization of the metabolic acidosis. However, the anemia still persisted (Table 1).

Family history was negative for consanguinity, growth retardation, renal or bone disease. Both parents and the patient's brother had no symptoms, and had normal values of serum electrolytes. Their hematologic and urinary values were shown in Table 2. This study was approved by

**Table 1** Hematologic studies of the patient

| Variables | Hb/Hct (gm.%/%) | MCV (fl) | Serum ferritin (ng/ml) | Serum $HCO_3$ (mmol/L) | Hepato-splenomegaly |
|---|---|---|---|---|---|
| Day 1 | 9.3/30 | 89.5 | 107 | 13 | Present |
| Day 60 | 9.2/30 | 87.2 | NA | 22 | Absent |

Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscle volume; NA, not applicable

**Table 2** Genotype and phenotype characteristic of family members

| | Genotype | | Phenotype | | |
|---|---|---|---|---|---|
| | *AE1* gene | Alpha-globin gene | Urine pH after acid loading test | Hemoglobin (g/dl) | Red cell morphology |
| Father | SAO/N | αα/αα | 5.4 | 16.2 | Ovalocyte Stomatocyte |
| Mother | G701D/N | $-\alpha^{3.7}/\alpha\alpha$ | 5.4 | 13.7 | Normal |
| Brother | SAO/N | αα/αα | 5.3 | 13 | Ovalocyte stomatocyte |
| Patient | SAO/G701D | $-\alpha^{3.7}/\alpha\alpha$ | NA | 9 | Ovalocyte stomatocyte |

*AE1*, anion exchanger 1; SAO, Southeast Asian ovalocytosis; N, normal; NA, not applicable

the Institutional Review Board of the Faculty of Medicine, Thammasat University.

**Short Acid-Loading Test**

Renal acidification was examined in the parents and the brother to identify incomplete distal renal tubular acidosis by using the short acid loading test. Briefly, 0.1 g/kg of $NH_4Cl$ was administered orally to subjects. Urine was sampled at the beginning of the acid load and at hourly intervals for the subsequent 6 hours. Urinary pH was measured. Results of the acid-loading test that indicated urinary acidification defect were the inability to decrease urine pH to less than 5.5 after acid loading.

**Screening for *AE1* Mutation by PCR and Single-Strand Conformation Polymorphism (SSCP)**

Genomic DNA was prepared from peripheral blood leukocytes, then initially screened for *AE1* mutation by PCR-SSCP as previously described.[13] Briefly, sequences in exons 4 to 20 and the kidney promoter sequence in intron 3 were amplified by PCR. SSCP analysis was performed by electrophoresis on nondenaturing 10% polyacrylamide gel and bands were visualized by silver staining. PCR products that showed mobility shift on the SSCP gel were then confirmed by second method of analysis, restriction digest or size differentiation on agarose gel.

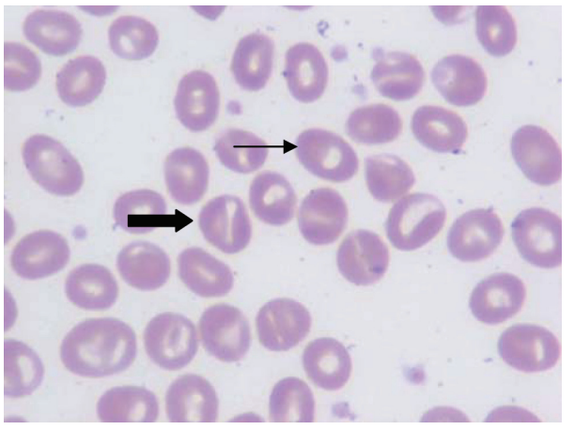

**Fig. 1** The red blood cell morphology of the patient shows ovalocytes (small arrow), stomatocytes (big arrow) and anisocytosis. Similar findings were observed in the father's and the brother's red cell (data not shown).

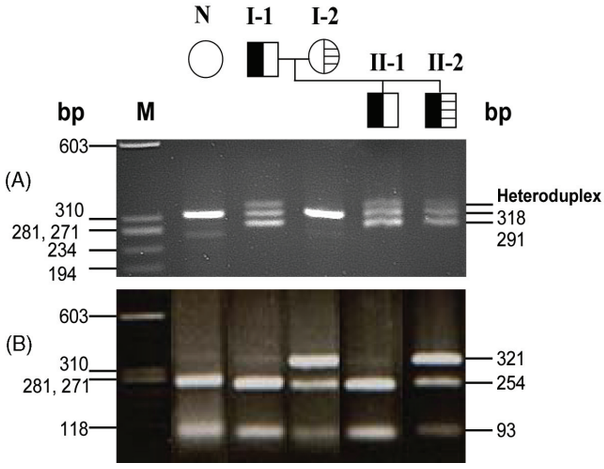


**Fig. 2** (A) Agarose-gel electrophoresis of PCR products from amplifications of exon 11 of *AE1* gene. Normal control sample (N) and mother's (I-2) showed only a PCR product with the size of 318 bp. DNA samples form the patient (II-2), father (I-1) and brother (II-1) showed PCR products with the sizes of 318 and 291 bp, and also their heteroduplexes, indicating 27 bp deletion in exon 11 in one allele of the *AE1* genes. (B) Analysis of the AE1 G701D mutation by restriction endonuclease Hpa*II* digestion of amplified exon 17 DNA fragments from a normal control sample, father and brother showed digested with the sizes of 254 and 93 bp. DNA sample from patient and mother who had exon 17 G701D mutation in one allele of the *AE1* gene revealed both digested (254 and 93 bp) and undigested (347 bp) PCR products.

**Analysis of *AE1* G701D Mutation by Restriction Endonuclease Hpa*II* Digestion**

The presence of the *AE1* G701D mutation abolishes the recognition site of restriction endonuclease Hpa*II*. Thus this mutation can be detected readily by digestion the amplified exon 17 fragment with Hpa*II* (Promega) and examining the fragments produced by agarose gel electrophoresis. A normal allele produces two fragments of 254 and 67bp, whereas the G701D allele results in a single fragment of 321bp (Fig. 2).

**Analysis of *AE1* SAO mutation by agarose-gel electrophoresis**

The SAO (or Ex11Δ27) mutation was analyzed by amplification in exon 11 region of AE1 by PCR. The amplified DNA fragment of normal exon 11 was 318 bp in length, whereas that of SAO was 291 bp (Fig. 2). The sample with the heterozygous SAO mutation showed fragments from the normal exon 11 (318 bp) and the deleted exon 11 (291 bp).

**DNA sequence analysis**

PCR products that showed mobility shift on the SSCP gel were reamplified for purification using QIAquick Gel Extraction Kit (Qiagen, GmbH, Germany). Purified PCR products then were sequenced using ABI-Prism BigDye Terminator Cycle Sequencing ready Reaction Kit (Applied Biosystems, Foster City, CA) and an automated sequence ABI-PRISM310 (Applied Biosystems)

## Results

The result of clinical and laboratory investigations showed that the patient had dRTA, while both parents and the brother had normal renal acidification (Table 2). The patient, brother and father had mobility shift of single stranded DNAs from exon 11 of *AE1*, while the patient and mother had mobility shift of single stranded DNAs from exon 17 of *AE1* (data not shown). The patient, his brother and father had one SAO allele on exon 11 as detected by agarose-gel elecrophoresis (Fig. 2A). The patient and his mother had one G701D allele on exon 17 as detected by restriction endonuclease Hpa*II* digestion (Fig. 2B).

The amplified DNAs of exon 11 and 17 from the patient were then analyzed by direct sequencing. The patient's exon 11 had a deletion of 27 bp corresponding to condons 400 to 408 (Fig. 3A) whereas exon 17 contained a nucleotide substitution of G to A in codon 701 (CGG→CAG), resulting in an amino acid change from glycine to aspartic acid (G701D) (Fig. 3B). Therefore the patient carried compound heterozygous SAO/G701D mutation of *AE1*. There were no mobility shift of single stranded DNAs from the promoter region and the other exons in all four members.

Hemoglobin electrophoresis revealed HbA 96%, $HbA_2$ 3%, and HbF 0.5% in the patient's and the mother's specimens. While both father and brother had HbA/$A_2$ ($A_2$ 2.9%). Red cell of patient showed reduced osmotic fragility. The serum ferritin was 107 ng/mL. The reticulocyte count was 7% indicating hemolytic anemia. The patient and his mother had $\alpha^+$-thalassemia trait due to a single α-globin gene deletion ($-\alpha^{3.7}/\alpha\alpha$), while the brother and the father had normal α- globin gene analysis (Table 2).

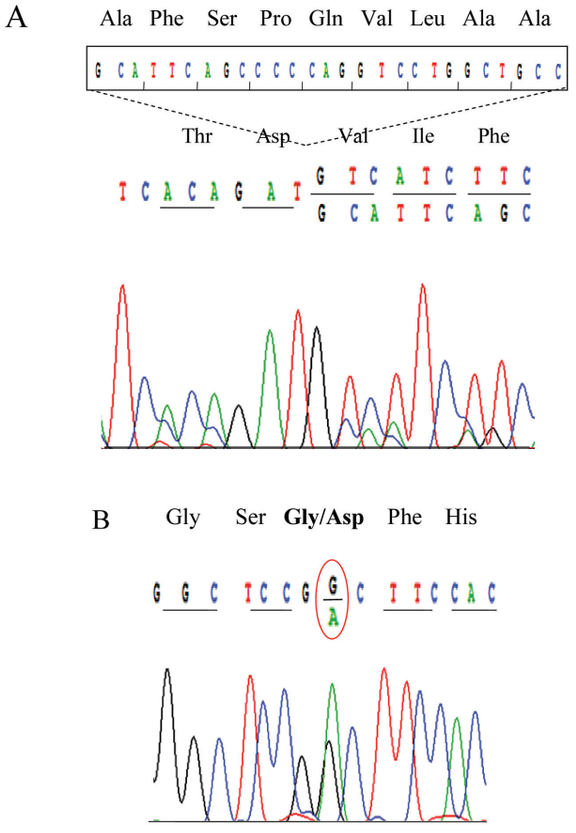


**Fig. 3** **(A)** Sequenogram of exon 11 of the *AE1* gene. Note 27 nucleotide deletion superimposing the normal sequence. **(B)** Sequenogram of exon 17 of *AE1* Gene. Note a single nucleotide substitution from G to A second base of condon 701.

## Discussion

This study described a dRTA patient who carried compound heterozygous (SAO/G701D) mutation of *AE1* gene and heterozygous $\alpha^+$-thalassemia presented with hemolytic anemia, and hepatosplenomegaly. The compound heterozygous (SAO/G701D) mutation was first described in Thai patients who had no anemia/hepatosplenomegaly, but ovalocytes and stomatocytes shown on blood smear.[13]

Several studies described dRTA patients with hemolytic anemia. Tanphaichit et al. reported two siblings with dRTA and xerocytic hemolytic anemia.[12] Both patients carried homozygous G701D mutation and homozygous HbE. Later, Yenchitsomanus et al. described homozygous G701D mutation in dRTA patients without xerocytic

hemolytic anemia.[16] They proposed that the presence of homozygous hemoglobin E which is unstable hemoglobin accompanied with homozygous G701D may compromise red cell membrane stability leading to xerocytic hemolytic anemia.[16]

Bruce et al. reported two dRTA patients from Malasia and Papua New Guinea who carried compound heterozygous (SAO/G701D) with hemolytic anemia and splenomegaly, but no hepatomegaly.[14] However, they did not report the iron study or hemoglobin typing in their patient. Recently, Choo KE et al. reported dRTA Sarawak patient with SAO/G701D mutation and hemolytic anemia with hepatosplenomegaly and normal hemoglobin typing, yet unknown status of ?-globin genotype.[20] To the best of our knowledge, there was no report case of dRTA patients with hemolytic anemia caused by *AE1* gene mutation and heterozygous state of α- or α-globin mutation.

Normally, heterozygous $\alpha^+$-thalassemic individuals are asymptomatic carrier.[21] The SAO erythrocyte is rigid.[14] The SAO/G701D erythrocyte is much more rigid than the SAO erythrocyte.[14] The molecular mechanism underlying SAO red cell membrane rigidity is not clear. SAO band 3 may binds abnormally and tightly to ankyrin and thus to the underlying skeleton.[18] Bruce et al. reported that the anion transport of SAO/G701D erythrocyte was 51% of normal erythrocyte.[14] The combination of SAO/G701D mutation of *AE1* protein and one alpha globin gene deletion of erythrocyte may compromise red cell membrane stability especially in acidic environment leading to hemolytic anemia and hepatosplenomegaly. The mechanism to explain this clinical manifestation is unknown. The study of red cell properties of this patient may elucidate underlying mechanism to explain his clinical manifestations.

In conclusion, we described G701D/SAO mutation of *AE1* gene accompanied with heterozygous $\alpha^+$-globin gene deletion in a Thai dRTA patient who had hemolytic anemia and hepatosplenomegaly. Given high prevalence of thalassemia gene among Thai population, the development of anemia and hepatosplenomegaly in dRTA Thai patient should alert clinician to consider investigation for hemoglobinopathies.

## Acknowledgements

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## บทคัดย่อ

**การกลายพันธุ์แบบยีนคู่ของยีนเออีวัน และยีนอัลฟาโกลบินในผู้ป่วยโรคดิสทอล-รีนอล-ทิวบูล่า-เอซิโดซีส ร่วมกับภาวะซีดจากเม็ดเลือดแดงแตกและตับม้ามโต**

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**วัตถุประสงค์** เพื่อศึกษาการกลายพันธุ์ของยีนเออีวันและยีนอัลฟาโกลบินในผู้ป่วยโรคดิสทอล-รีนอล-ทิวบูล่า-เอซิโดซีสที่มีภาวะซีดและตับม้ามโต

**วิธีการศึกษา** ผู้ป่วยโรคดิสทอล-รีนอล-ทิวบูล่า-เอซิโดซีส ที่มีภาวะซีดและตับม้ามโตได้รับการตรวจหาสาเหตุของภาวะซีด โดยการตรวจลักษณะรูปร่างและการแตกตัวของเม็ดเลือดแดง ปริมาณธาตุเหล็กในร่างกาย ตรวจหาการกลายพันธุ์ของยีนอัลฟาโกลบินโดยวิธีการมาตรฐาน ตรวจหาการกลายพันธุ์ของยีนเออีวันที่เป็นสาเหตุสำคัญของภาวะดิสทอล-รีนอล-ทิวบูล่า-เอซิโดซีส โดยวิธี PCR-single stranded conformation polymorphism (PCR-SSCP) วิธี PCR-restriction enzyme digestion และวิธี direct sequencing

**ผลการศึกษา** ผู้ป่วยเด็กชายไทยอายุ ๕ ปี มีภาวะการเจริญเติบโตช้าและภาวะเลือดเป็นกรด ได้รับการวินิจฉัยโรคดิสทอล-รีนอล-ทิวบูล่า-เอซิโดซีส และตรวจพบภาวะซีดและตับม้ามโต เม็ดเลือดแดงมีขนาดใหญ่ มีลักษณะ ovalocytes และ stomatocyte มีการลดลงของ osmotic fragility การตรวจฮีโมลโกลบิน พบ Hb A ๙๖% และ $A_2$ ๓% ปริมาณธาตุเหล็กในร่างกายอยู่ในเกณฑ์ปกติ พบการขาดหายไปของยีนอัลฟาโกลบินหนึ่งตำแหน่ง ($-\alpha^{3.7}/\alpha\alpha$) พบการกลายพันธุ์ของยีนเออีวันที่ตำแหน่ง exon 11 และ exon 17 (SAO/G701D)

**สรุปผลการศึกษา** ภาวะพาหะของยีนอัลฟาโกลบินร่วมกับการกลายพันธุ์ชนิด SAO/G701D ของยีนเออีวัน ทำให้เกิดอาการซีดและเม็ดเลือดแดงแตกและตับม้ามโตในผู้ป่วยดิสทอล-รีนอล- ทิวบูลล่า-เอซิโดซีส