HUMAN *ANION EXCHANGER1* MUTATIONS AND DISTAL RENAL TUBULAR ACIDOSIS

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Abstract. The human *anion exchanger 1 (AE1* or *SLC4A1)* gene encodes anion exchanger 1 (or band 3) protein in erythrocytes and in α-intercalated cells of the kidney. Thus, *AE1* mutations show pleiotrophic effects resulting in two distinct and seemingly unrelated defects, an erythrocyte abnormality and distal renal tubular acidosis (dRTA). Southeast Asian ovalocytosis (SAO), a well-known red blood cell (RBC) defect, which is widespread in Southeast Asian regions, is caused by *AE1* mutation due to a deletion of 27 base pairs in codons 400-408 (Δ400-408) leading to an in-frame 9 amino-acid loss in the protein. Co-existence of SAO and dRTA is usually not seen in the same individual. However, the two conditions can co-exist as the result of compound heterozygosities between Δ400-408 and other mutations. The reported genotypes include Δ400-408/G701D, Δ400-408/R602H, Δ400-408/ΔV850, and Δ400-408/A858D. The presence of dRTA, with or without RBC abnormalities, may occur from homozygous or compound heterozygous conditions of recessive *AE1* mutations (*eg* G701D/G701D, V488M/V488M, ΔV850/ΔV850, ΔV850/A858D, G701D/S773P) or heterozygous dominant *AE1* mutations (*eg* R598H, R589C, R589S, S613F, R901X). Codon 589 of this gene seems to be a 'mutational hot-spot' since repeated mutations at this codon occurring in different ethnic groups and at least two *de novo* (R589H and R589C) mutations have been observed. Therefore, *AE1* mutations can result in both recessive and dominant dRTA, possibly depending on the position of the amino acid change in the protein. As several mutant AE1 proteins still maintain a significant anion transport function but are defective in targeting to the cell surface, impaired intracellular trafficking of the mutant AE1 is an important molecular mechanism involved in the pathogenesis of dRTA associated with *AE1* mutations.

INTRODUCTION

The human kidney plays an important role in acid-base homeostasis. The human arterial blood pH is normally regulated at 7.4. This regulation is crucially maintained by the function of nephrons in the kidney, where bicarbonate is mainly reabsorbed at the proximal tubule and acid is secreted in the distal and collecting tubules. The defect of acid secretion in the distal nephron will result in distal renal tubular acidosis (dRTA).

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dRTA is a disorder characterized by inability of the kidney to acidify urine (to pH below 5.5) in the presence of systemic metabolic acidosis because of the failure of proton or hydrogen ion (H^+) secretion at the distal tubules of the nephrons (Batlle and Flores, 1996; Rodriguez-Soriano, 2000; Batlle *et al*, 2001). The patients present with hyperchloremic metabolic acidosis, frequently accompanied by hypokalemia, muscle weakness, metabolic bone disease, nephrocalcinosis and/or nephrolithiasis.

It is known that mutations of at least three human genes are responsible for dRTA. These genes are *ATP6V1B1* on chromosome 2 and *ATP6V0A4* on chromosome 7 encoding B1 and a4 subunits of H^+ ATPase, respectively (Karet *et al*, 1999; Smith *et al*, 2000; Stover *et al*, 2002), and *SLC4A1* on chromosome 17 encoding anion exchanger 1 (AE1) or band 3 (Bruce *et al*, 1997,

2000; Jarolim *et al*, 1998; Karet *et al*, 1998; Tanphaichitr *et al*, 1998; Vasuvattakul *et al*, 1999). Therefore, dRTA has genetic (or locus) heterogeneity since it may result from mutations of at least three different genes. In this review article, only the mutations of *SLC4A1* or *AE1* involved in the pathogenesis of dRTA will be discussed in detail.

HUMAN *AE1* GENE AND PROTEIN

The human *AE1* gene is located on chromosome 17 at the region q21-22, spanning about 20 kb of DNA region (Sahr *et al*, 1994; Schofield *et al*, 1994). The same gene encodes both erythroid (eAE1) and kidney (kAE1) isoforms, using two different promoters. The promoter for the eAE1 isoform is present upstream of exon 1 of the gene, whereas the promoter for the kAE1 isoform locates within intron 3 (Fig 1). The eAE1 polypeptide contains 911 amino acids while the kAE1 polypeptide has 65 amino acids at the N-terminus shorter than the eAE1 polypeptide. eAE1 is an integral membrane glycoprotein consisting of a long cytoplasmic N-terminus of approximately 400 amino acids, 12-13 transmembrane domains, and a short cytoplasmic C-terminus of about 35 amino acids. A glycosylation site is at asparagine 642 in the extracellular region.

In erythrocytes, AE1 is found as dimers, tetramers or higher oligomers on the cell membrane. Beside having anion exchange function, it serves as an anchor protein of the cytoskeleton network, binding to ankyrin, bands 4.1 and 4.2, and cytoplasmic proteins (Tanner, 1993; 1997). In the α-intercalated kidney cells, AE1 locates at the basolateral membrane and functions in chloride (Cl^-) and bicarbonate (HCO^-_3) exchange (Fig 2). Defects of AE1 may affect this chloride and bicarbonate exchange function. The accumulation of bicarbonate may lead to reduction of carbonic acid (H_2CO_3) dissociation and hydrogen (H^+) ion secretion at the apical side of the α-intercalated cells. Since *AE1* has expression in two different cells with distinct functions, its mutation may show a pleiotrophic effect; the mutations of this gene are responsible for two distinct and seemingly unrelated phenotypes. *AE1* mutations may result in hereditary spherocytosis, or other forms of red blood cell (RBC) abnormalities, and in

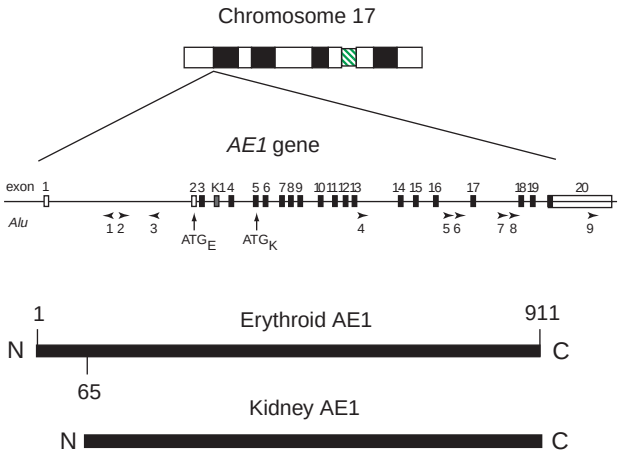

Fig 1–Human *anion exchanger 1 (AE1)* gene. The gene is located on chromosome 17 at the region 17q21-22, spanning 20 kb and consisting of 20 exons and 19 introns. Exons are indicated by vertical filled and unfilled bars while introns by lines between the bars. The exon numbers are enumerated above the bars. Arrow heads and numbers under the gene represent *Alu* sequences. The same gene encodes both erythroid (eAE1) and kidney (kAE1) protein isoforms using different promoters and start codons, resulting in different lengths of polypeptides. The eAE1 contains 911 amino acids whereas kAE1 has a truncated 65 amino acids at the N-terminus.

dRTA. It was found that *AE1* mutations account for approximately 20% of spherocytosis and almost all ovalocytosis in Southeast Asia (Bruce and Tanner, 1996; Tanner, 1997; Tse and Lux, 1999). The mutations of this gene have also recently been shown to cause dRTA in many ethnic groups, especially in children in the Southeast Asian populations.

SOUTHEAST ASIAN OVALOCYTOSIS (SAO) AND DRTA

The association between hereditary elliptocytosis and dRTA was first recognized in 1968 in a Fillipino family by Baehner and colleagues (Baehner *et al*, 1968). The propositus in this family was reported to have both elliptocytosis and dRTA with the presence of hyperchloremic metabolic acidosis, hypokalemia, rickets, and nephrocalcinosis. The authors of this report could not

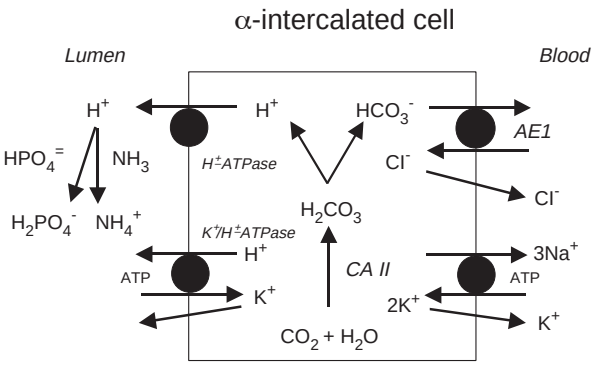

Fig 2–Schematic picture of the acid-secreting, α-intercalated, cell present in the distal and collecting tubules of kidneys. In this cell, carbon dioxide is hydrated by catalytic activity of carbonic anhydrase II (CAII) to carbonic acid, which is dissociated to hydrogen ion (H^+) and bicarbonate (HCO^-_3). The hydrogen ion is excreted into the tubular lumen by $H^±$ATPase and $K^+/H^±$ ATPase locating at the apical side of the cell while the bicarbonate is extruded the basolateral side of the cell in exchange of chloride by the action of anion exchanger 1 (AE1). Defect of $H^±$ATPase or AE1 will cause distal renal tubular acidosis (dRTA).

suggest reason for the co-existence of the two disorders in the same individual and considered that they had occurred by chance. The RBC abnormality of the Fillipino child with dRTA was the same as that typically found in a person with Southeast Asian ovalocytosis (SAO), clearly different from that of hereditary elliptocytosis (Wrong *et al*, 2002). It is most likely that the child had SAO and dRTA.

SAO is a RBC defect which is widespread and observed in high frequencies in regions of southern Thailand, Malaysia, Indonesia, the Phillipines, and Papua New Guinea (PNG). Its prevalence is high in the Indonesian islands and parts of PNG (Nurse *et al*, 1992). On the north coast of Madang Province in PNG, its prevalence is as high as 35% (Mgone *et al*, 1996). SAO is caused by an *AE1* mutation due to a 27 base-pair (bp) deletion in exon 11, resulting in an in-frame deletion of 9 amino acids at positions 400-408 (Δ400-408), the junction between the N-terminal domain and the first transmembrane span of the AE1 protein (Jarolim *et al*, 1991). The mutant protein seems to have an increased propensity to form oligomers, which appear as longitudinal strands of intramembrane particles and exhibit an increased association with the membrane skeleton (Liu *et al*, 1995). The mutant protein oligomerization underlies the increase in membrane rigidity by precluding membrane skeletal extension, leading to membrane deformation. A polymorphism due to substitution of lysine by glutamic acid at position 56 (K56E), band 3 Memphis I, is invariably linked to the SAO mutation (Jarolim *et al*, 1991; Vasuvattakul *et al*, 1999).

SAO is the heterozygous state of this mutation. The homozygous state of this mutation has never been observed, suggesting that it is a lethal condition (Liu *et al*, 1994; Mgone *et al*, 1996). The fetus carrying homozygous SAO mutation may not survive *in utero*. The high SAO mutation frequency in Southeast Asia is believed to result from its selective survival advantage against malaria infection. The patient with SAO is less susceptible to cerebral malaria (Allen *et al*, 1999).

SAO, DRTA AND *AE1* MUTATIONS

Wrong and co-workers were the first to suspect a common molecular link between SAO and dRTA (Wrong *et al*, 1996) but this had not been unraveled. Our group was interested in studying molecular defects of the combined SAO and dRTA condition. We performed short and three-day acid (NH_4Cl) loading tests in 20 individuals with SAO and in two subjects, including their families, with both SAO and dRTA, and studied mutations of *AE1* in individuals with SAO and members of the two families by single strand conformation polymorphism (SSCP) and DNA sequencing analysis (Vasuvattakul *et al*, 1999). Renal acidification was normal in the 20 individuals with SAO who carried heterozygous *AE1* Δ400-408. Therefore, individuals with SAO do not generally have dRTA. However, the two clinically affected individuals with SAO and dRTA had compound heterozygosity of *AE1* Δ400-408 and missense mutation in exon 17. The latter occurred from a single nucleotide change (GGC>GAC) at codon 701, resulting in a substitution of glycine by aspartic acid (G701D). It was demonstrated for the first time by our group

that compound heterozygosity between Δ400-408 mutation typical of SAO and a missense G701D mutation of *AE1* (Δ400-408/G701D genotype) causes the combined defect of SAO and dRTA. Thus, the disease is inherited in an autosomal recessive mode.

This finding was later confirmed by other groups (Bruce *et al*, 2000; Wrong *et al*, 2002) and extended to include other compound heterozygosity genotypes, with and without the SAO mutation, including (Δ400-408/ΔV850, Δ400-408/A858D, ΔV850/ΔV850, ΔV850/A858D) in Malaysian and Papua New Guinean dRTA patients . The heterozygous A858D mutation was also noted to be associated with an impaired ability to acidify the urine but with no acidosis (incomplete syndrome of dRTA) in subjects from two families (Bruce *et al*, 2000).

OTHER *AE1* MUTATIONS AND DRTA

Before we reported the result of our study on SAO and dRTA, Tanphaichitr and colleagues (Tanphaichitr *et al*, 1998) had reported the homozygous state of the *AE1* G701D mutation (G701D/G701D) in two siblings of the same family who had severe anemia and dRTA. This mutation was named 'Band 3 Bangkok I'. The two patients who had both the homozygous *AE1* G701D mutation and homozygous Hb E were severely anemic with the presence of xerocyte-like dumbbell forms of RBCs in their blood pictures. This typical RBC abnormality might result from the presence of both the homozygous *AE1* G701D and homozygous Hb E mutations. The chloride transport functions of the wild-type AE1 and mutant *AE1* G701D were tested in *Xenopus* oocytes. The transport function of the mutant *AE1* G701D protein was low in the absence of glycophorin A (GPA), the known chaperonin of eAE1. However, its transport function in the oocytes could be rescued in the presence of GPA. GPA is present in RBC but absent in the α-intercalated cells of distal nephron. The kAE1 G701D mutant protein may have impaired targeting to the basolateral membrane of the α-intercalated cells and the absence of kAE1 for chloride/bicarbonate exchange will lead to dRTA.

Since two cases each with the homozygous *AE1* G701D mutation and with the compound heterozygous Δ400-408/G701D mutations were detected in Thai patients, we thought that the *AE1* G701D mutation might be commonly found in Thai pediatric patients with dRTA. Therefore, we performed an analysis of *AE1* in another twelve Thai children with dRTA and have identified seven patients from five families homozygous for the *AE1* G701D mutation (Yenchitsomanus *et al*, 2002). Their parents or siblings heterozygous for the *AE1* G701D mutation were clinically normal and did not have abnormal urinary acidification, although a heterozygous sibling in one family did have abnormal urinary acidification without a known reason. This homozygous *AE1* G701D condition, could be simply detected by PCR and *Hpa* II digestion. The mutant *AE1* could not be digested with *Hpa* II but the wild-type *AE1* could be digested (Fig 3). The mutant homozygote shows only undigested DNA fragments while the wild-type homozygote has only digested DNA fragment. In the heterozygote, there are both digested and undigested fragments.

The results of this as well as previous studies demonstrated that the homozygous *AE1* G701D mutation causes autosomal recessive dRTA and is a common molecular defect among Thai pediatric patients with dRTA studied. None of the seven patients with homozygous *AE1*

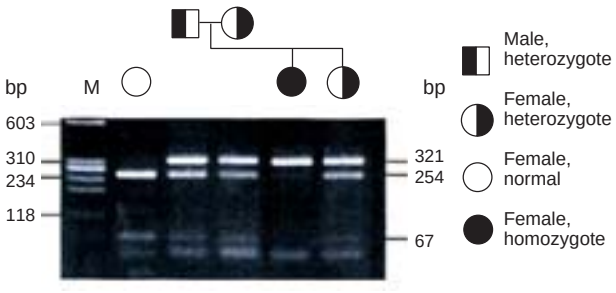

Fig 3–Detection of *AE1* G701D mutation by PCR and *Hpa* II digestion. The PCR product of wild-type *AE1* could be digested with *Hpa* II but that of the *AE1* G701D mutation could not be digested. The wild-type homozygote has only digested DNA fragments (254 and 67 bp) while the mutant homozygote shows only one undigested DNA fragment (321 bp). In the heterozygote, there are both digested (254 and 67 bp) and undigested fragments (321 bp). M is standard DNA size markers, PhiX174 DNA/*Hae*III.

Table 1
AE1 mutations associated with autosomal recessive dRTA.

Author	Disease	Mutation
Tanphaichitr *et al*, 1998	dRTA + xerocytosis (+HbE)	G701D/G701D
Vasuvattakul *et al*, 1999	dRTA + SAO	Δ400-408/G701D
Ribeiro *et al*, 2000	dRTA + Spherocytosis	V488M/V488M
Bruce *et al*, 2000	dRTA + SAO	Δ400-408/G701D
		Δ400-408/ΔV850
		Δ400-408/A858D
	dRTA + Abnormal RBC	ΔV850/A858D
	dRTA	ΔV850/ΔV850
Yenchitsomanus *et al*, 2000	dRTA	G701D/G701D
Sritippayawan *et al*, 2003, in preparation	dRTA + SAO	Δ400-408/R602H
	dRTA	G701D/S773P

G701D mutation that were studied had anemia and xerocyte-like dumbbell forms of RBC as were observed in the previous report (Tanphaichitr *et al*, 1998). This is probably because of the absence of homozygous Hb E in the patients we studied.

A summary of *AE1* mutations resulting in autosomal recessive (AR) and dominant (AD) dRTA reported by several groups are shown in Tables 1 and 2. Seven mutations are now known to be associated with AR dRTA and five with AD dRTA. Seven mutations associated with AR dRTA might be found in homozygous states or compound heterozygous conditions (Table 1). Our group recently identified additional novel compound heterozygosities (Δ400-408/R602H and G701D/S773P) associated with AR dRTA (Thuwajit, 1999; Wilairat, 2000; Sritippayawan *et al*, 2003, in preparation). In a recent review article, the *AE1* R602H mutation discovered by our group was mentioned without quoting a reference (Alper, 2002) and the later self-citation (Alper *et al*, 2002) has potentially misled readers regarding the first discoverer.

The *AE1* arginine 589 (R589) mutations have been reported in multiple families with AD dRTA (Bruce *et al*, 1997; Jarolim *et al*, 1998; Karet *et al*, 1998; Weber *et al*, 2000). The arginine at this position could be altered to histidine (R589H), serine (R589S), or cysteine (R589C). Altogether *AE1* R589 mutations have been identified in 10 of 12 families with AD dRTA, including one family found by our group

Table 2
AE1 mutations associated with autosomal dominant dRTA.

Author	Mutation
Bruce *et al*, 1997	R589H
	R589C
	S613F
Jarolim *et al*, 1998	R589H
Karet *et al*, 1998	R589H
	R589H (*de novo*)
	R589S
	R901X (Band 3 Walton)
Weber *et al*, 2000	R589C
Sritippayawan *et al*, 2002	R489C (*de novo*)

(Sritippayawan *et al*, 2002, revised). The R589H mutation is the most common, it was observed in 6 families. The high prevalence of mutations at this position is unlikely to result from the founder effect because of its occurrences in different populations and the presence of allelic heterogeneity. A *de novo* R589H mutation has been observed in a sporadic case with AD dRTA (Karet *et al*, 1998). Our group has also recently discovered a *de novo* heterozygous R589C mutation in one patient with AD dRTA (Sritippayawan *et al*, 2002, revised). The presence of *de novo AE1* R589 mutations strongly indicates recurrent mutations. A high prevalence of *AE1* R589 mutations and the presence of at least two *de novo* mutations at this position lead us to propose that

codon 589 (CGC) is a "mutational hotspot" of *AE1*. The mechanism of recurrent mutations probably involves methylation and deamination altering cytosine (C) to thymine (T) in the CpG dinucleotides.

The normal triplet-nucleotide for arginine at the codon 589 is CGC; its complementary triplet-nucleotide is GCG. Thus, the first two nucleotides are CpG dinucleotides present on both DNA strands (5′CGC3′ and 3′GCG5′). R589C occurred from the alteration of CGC to TGC while R589H resulted from the change of CGC to CAC, while its complementary change was from GCG to CTC. Therefore, both R589C and R589H mutations (CGC>TGC and GCG>CTG) are essentially the alteration from cytosine to thymine (C>T), likely to occur from the same mutational mechanism. Methylation and deamination are well-known chemical modifications producing this C>T alteration (Duncan and Miller, 1980). The CpG dinucleotide is a 'mutational hotspot' in the human genome (Perry and Carrel 1989; Bottema *et al*, 1993; Pfeifer, 2000). Cellular DNA methyltransferases converts cytosine (C) to 5-methyl cytosine (5mC) and after a spontaneous deamination it is changed to thymine (T).

MOLECULAR PATHOGENESIS OF DRTA ASSOCIATED WITH AE1 DEFECTS

It has been shown that there is little reduction of chloride transport activity of mutant kAE1 R589H or R589C protein in *Xenopus* oocytes (Bruce *et al*, 1997; Jarolim *et al*, 1998). The defect of mutant AE1 transport activity does not seem to be the explanation for these mutations in association with AD dRTA. However, impaired trafficking of mutant kAE1 R589H as well as R589C and R589S proteins in human embryonic kidney (HEK 293) cells was recently demonstrated (Quilty *et al*, 2002a). These mutant kAE1 proteins were retained in the cytoplasm as shown by immunofluorescence staining. This was not the case for the wild-type and mutant eAE1 proteins or the wild-type kAE1 protein, which could normally migrate to the cell surface. The wild-type kAE1 protein when co-expressed with the mutant kAE1 protein also failed to locate at the cell surface, indicating dominant negative effect caused by the interaction between the two proteins. Thus, impaired trafficking of the protein is probably an important molecular mechanism of AD dRTA associated with AE1 mutations. A similar finding was also observed for band 3 Walton (R901X), which has normal chloride transport activity in the *Xenopus* oocyte, either in the absence or presence of GPA (Toye *et al*, 2002). When transfected into the Madin-Darby canine kidney (MDCK) or HEK 293 cells, kAE1 Walton failed to express at the cell surface but it was retained in the cytoplasm (Quilty *et al*, 2002b; Toye *et al*, 2002). Similar to the situation with with mutant kAE1 R589H, the wild-type kAE1 protein also failed to locate at the cell surface when co-expressed with the mutant kAE1 R901X protein, indicating a dominant negative mechanism (Quilty *et al*, 2002b, submitted).

SUMMARY

AE1 mutations can result in both AR and AD dRTA. The work from our and other groups in Thailand has demonstrated that the homozygous *AE1* G701D mutation is a common genotype in Thai pediatric patients with dRTA studied. dRTA, associated with SAO results from compound heterozygosities between *AE1* Δ400-408 and G701D or other mutations. Impaired trafficking of mutant AE1 protein is probably an important molecular mechanism involved in the pathogenesis of dRTA. And finally, the explanation for AR and AD dRTA due to *AE1* mutations may be that the mutant protein from AR mutation does not interfere with the wild-type protein while the mutant protein from the AD mutation interacts with the wild-type protein and causes retention of both wild-type and mutant proteins in the cytoplasm, the dominant negative effect due to hetero-oligomer formation.

ACKNOWLEDGEMENTS

This article was presented as an invited paper at the 4th HUGO Pacific Meeting and 5th Asia Pacific Conference on Human Genetics, October 27-30, 2002, Pattaya, Chonburi, Thailand.

This work was financially supported by a Siriraj Grant for Research Development, the National Center for Genetic Engineering and Biotechnology (Biotec), NSTDA and the Thailand Research Fund (TRF).

I am grateful to Dr Prida Malasit, Professor Prapon Wilairat, and Professor Sumalee Nimmannit, for their kind and continuous support. The collaboration of Drs Somkiat Vasuvattakul, Suchai Sritippayawan, Sukachart Kirdpon, Sirijitta Wasanawatana, Watanachai Susaengrat, Prayong Vachuanichsanong, Charoen Kaitwatcharachai, Vichai Laosombat, and the technical and other assistance of Miss Nunghathai Sawasdee, Atchara Paemanee, Thitima Keskanokwong, Duangporn Chuawatana, and Sumitra Mingkum are greatly appreciated.

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