Final Report

Research Project
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Genetics and Molecular Biology of Diseases Relevant to Thais
(พันธุศาสตร์และอณูชีววิทยาของโรคที่สำคัญในคนไทย)

By

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Executive Summary

Human genomes are subject to alteration causing phenotypic variability, leading to human evolution in changing environments. As the result of diverse environments, human genomic alterations causing disease phenotypes in each human population are likely to be different, especially the alterations causing complex diseases caused by interactions of multiple genes and environments. Based on this hypothesis, we chose to study certain complex diseases relevant to Thai population by employing genetic/genomic and molecular biology approaches to understand their underlying genetic/genomic bases and molecular mechanisms, which will guide future diagnostic and therapeutic development. These diseases include kidney stone – a prevalent disorder and a public health problem in the Northeastern Thai population, distal renal tubular (dRTA) – a rare but distinct genetic disorder in Thai and Southeast Asian populations, and diabetes mellitus (DM) – a threatening disease in the 21st century which its prevalence rapidly increases in Thai and other populations worldwide. Additionally, dengue virus infection, an import infectious disease in Thailand and the countries in tropical and subtropical regions, were also investigated by molecular and cellular biology techniques with the emphasis on aspects of virus and host protein interactions involving in cellular pathogenesis.

The result of genetic epidemiological study of kidney stone disease in the Northeastern Thai population demonstrated the predominance of calcium salt stone and familial aggregation with relative risk (λR) of 3.18 among family members, supporting the role of genetic factor in its pathogenesis. By candidate-gene association study, our group originally discovered that genetic variations of *prothrombin (F2)* encoding a urinary stone inhibitor, prothrombin fragment 1, are associated with kidney stone risk in Northeastern Thai female patients. Genome-wide association and genome-wide linkage studies using DNA microarray are currently being conducted to identify the causative genes for kidney stone disease.

Mutations of the human *solute carrier family 4, anion exchanger, member 1 (SLC4A1)* gene, encoding erythroid and kidney isoforms of anion exchanger 1 (AE1, band 3), result in erythrocyte abnormalities or distal renal tubular acidosis (dRTA). The coexistence of homozygous or compound heterozygous *SLC4A1* mutations and hemoglobinopathies (e.g. thalassemia or hemoglobin E) has been observed in Thai population, presenting a combined effect of red cell morphological changes and hemolytic anemia aggravating by acidosis. Novel compound heterozygous mutations (G701D/A858D) of human *SLC4A1* were identified in two pediatric Thai patients with dRTA. The wild-type and two mutant kAE1 proteins (G701D and A858D) were expressed and co-expressed in human embryonic kidney (HEK) 293T and Madin-Darby canine kidney (MDCK) epithelial cells to examine their interaction, trafficking, and cellular localization. Impaired trafficking and intracellular retention of the mutant kAE1 G701D and A858D may lead to decrease functional kAE1 at the basolateral membrane of the kidney α-intercalated cells in the distal nephron. A novel *SLC4A1* mutation, *Band 3 Edmonton I* (C479W), which causes dominant hereditary spherocytosis (HS) and recessive dRTA was described in a patient from Edmonton, Alberta, Canada, with compound heterozygous (C479W/G701D) mutations. *SLC4A1* G701D mutation was first described outside Southeast Asia as a new mutation. The C479W mutant is a novel trafficking mutant of AE1, which causes HS due to a decreased cell surface AE1 protein and results in dRTA due to its intracellular retention in kidney.

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. It was estimated that 9.6% (2.4 million) of Thai adults were affected with DM and 5.4% (1.4 million) had impaired fasting glucose. Since the disease is very heterogeneous, abnormality at different biological pathways can lead to hyperglycemia and subtypes of the disease requiring precise diagnostic criteria. Several studies have concentrated on identifications of diabetic susceptibility genes. These efforts facilitate a better understanding in molecular pathogeneses and pathophysiologies underlying DM subtypes, thereby leading to the development of appropriate and effective therapeutic approaches. Six known genes responsible for maturity-onset diabetes of the young (MODY), including *HNF-4alpha, GCK, HNF-1alpha, IPF-1, HNF-1beta,* and *NeuroD1/beta2,* were analyzed to evaluate the prevalence of their mutations in Thai patients with MODY and early-onset type 2 diabetes. We found that mutations of the six known MODY genes account for a small proportion of classic MODY (19%) and early-onset type 2 diabetes (10%) in Thais. Thus, mutations of the six known MODY genes may not be a major cause of MODY and early-onset type 2 diabetes in Thais. Five mutations identified are novel including GCK R327H, HNF-1alpha P475L, HNF-1alphaG554fsX556, NeuroD1-1972 G > A and NeuroD1 A322N. A novel frameshift mutation encoding a truncated HNF-1α (G554fsX556) with 76-amino acid deletion at its carboxyl terminus was attributable to 14-nucleotide insertion in *HNF-1α*. The wild-type and mutant HNF-1α could similarly bind to the sequence of human *GLUT2* promoter but transactivation activities of mutant HNF-1α on human *GLUT2* and rat *L-PK* promoters reduced to approximately 55-60% of the wild-type protein. The functional defect of novel truncated HNF-1α (G554fsX556) on the transactivation of its target-gene promoters is likely to account for the β-cell dysfunction associated with the pathogenesis of MODY.

Dengue fever (DF) and dengue hemorrhagic fever (DHF) are important public health problems in Thailand and other tropical countries. Abnormal hemostasis and plasma leakage are the main patho-physiological changes in DHF but hepatomegaly, hepatocellular necrosis and fulminant hepatic failure are occasionally observed in patients with DHF. Dengue virus-infected liver cells undergo apoptosis but the underlying molecular mechanism remains unclear. We found that dengue virus capsid protein (DENV C) physically interacts with the human death domain-associated protein Daxx, a Fas-associated protein. The two proteins were predominantly co-localized in the cellular nuclei. Fas-mediated apoptotic activity in liver cells constitutively expressing DENV C was induced by anti-Fas antibody, indicating that the interaction of DENV C and Daxx involves in apoptosis of dengue virus-infected liver cells. Stable HepG2 cells constitutively expressing DENV C, DENV C (Delta85-100) and DENV C (Delta73-100) were constructed to clarify whether nuclear translocation of DENV C affected apoptosis in liver cell line. While the wild-type DENV C could translocate into the nuclei of HepG2 cells, the mutant DENV Cs were restricted to the cytoplasm. The loss of nuclear localization of both mutant DENV Cs resulted in the disruption of their interactions with the apoptotic protein Daxx. Interestingly, upon treatment with anti-Fas antibody, the HepG2 cells expressing the wild-type DENV C showed significantly more apoptosis compared with the HepG2 cells expressing either mutant DENV C. Our results also demonstrate that (73)KK and (85)RK of DENV C are important for its nuclear localization, interaction with DAXX and induction of apoptosis.

The glycosylated envelope protein of dengue virus, DENV E, is processed in the endoplasmic reticulum of host cells and therefore reliant on host processing functions.

We found that domain III of DENV E interacts with human immunoglobulin heavy chain binding protein (BiP). The association of DENV E with two other chaperones, calnexin and calreticulin was also observed. Knocking-down expression of BiP, calnexin, or calreticulin by siRNA significantly decreased the production of infectious dengue virions. These results indicate that the interaction of these three chaperones with DENV E plays an important role in virion production, likely facilitating proper folding and assembly of dengue proteins. Dengue virus nonstructural protein 1 (NS1) is a key glycoprotein involved in the production of infectious virus and the pathogenesis of dengue diseases. However, very little is known how NS1 interacts with host cellular proteins and functions in dengue virus-infected cells. We identified human heterogeneous nuclear ribonucleoprotein (hnRNP) C1/C2 as NS1-interacting host cellular proteins in dengue virus-infected cells by employing co-immunoprecipitation, two-dimensional gel electrophoresis, and mass spectrometry. Further investigation by co-immunoprecipitation and co-localization confirmed the association of hnRNP C1/C2 and dengue virus NS1 proteins in dengue virus-infected cells. Their interaction may have implications in virus replication and/or cellular responses favorable to survival of the virus in host cells.

In conclusion, our group is employing genetic/genomic and molecular biology approaches to investigate into genetic factors and molecular mechanisms of four certain diseases (kidney stone, dRTA, DM, and dengue-virus infection) relevant to Thai population and made significant discoveries and progresses in the studies of these diseases. However, further investigations of these diseases are required to gain complete understanding and to develop appropriate methods for prevention, control, and intervention.

During three years of this project, our group published altogether 17 articles in peer-reviewed international journals, 7 articles in peer-reviewed national journals, and 5 abstracts in international proceeding. Thirty-three oral and 39 poster presentations were given in the international and national conferences with 22 awards obtained. Three students within our group graduated with Ph.D. degrees and 8 with M.Sc. degrees. Another 10 Ph.D. and 8 M.Sc. students are currently working within our group under this and continued projects.

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Abstract

Human enomic alterations causing disease phenotypes in Thai population are proposed to be different from those found in other populations. Our group is thus employing genetic/genomic and molecular biology approaches to study certain complex diseases relevant to Thais. These diseases include kidney stone, distal renal tubular (dRTA), diabetes mellitus (DM) and dengue virus infection.

Kidney stone disease in the Northeastern Thai population shows familial aggregation with relative risk (λR) of 3.18 among family members, supporting the role of genetic factor in its pathogenesis. Genetic variations of *prothrombin (F2)* encoding a urinary stone inhibitor, prothrombin fragment 1, were first shown to be associated with kidney stone risk in the female patients. Genome-wide association and genome-wide linkage studies using DNA microarray are being conducted to identify the causative genes for kidney stone disease.

Mutations of the human *SLC4A1* gene encoding erythroid and kidney anion exchanger 1 (eAE1 and kAE1) result in erythrocyte abnormalities or distal renal tubular acidosis (dRTA). *SLC4A1* mutations and hemoglobinopathies (e.g. thalassemia or hemoglobin E) were observed in Thai population, presenting a combined effect of red cell morphological changes and hemolytic anemia aggravating by acidosis. Novel *SLC4A1* mutations were discovered in Thai and other populations. The wild-type and mutant kAE1 proteins were expressed and co-expressed in cultured epithelial cells to examine their interaction, trafficking, and cellular localization. Impaired trafficking and intracellular retention of the mutant kAE1 may lead to decrease functional kAE1 at the basolateral membrane of the kidney α-intercalated cells in the distal nephron resulting in dRTA.

The causes of diabetes mellitus (DM) are heterogeneous; abnormality at different biological pathways can lead to hyperglycemia. Six known genes responsible for maturity-onset diabetes of the young (MODY), including *HNF-4alpha, GCK, HNF-1alpha, IPF-1, HNF-1beta,* and *NeuroD1/beta2,* were analyzed in Thai patients with MODY and early-onset type 2 diabetes. Mutations of these six known MODY genes account for a small proportion of classic MODY (19%) and early-onset type 2 diabetes (10%) in Thais. A novel frameshift mutation of *HNF-1alpha* was identified in a Thai family with MODY. The functional defect of this mutant on the transactivation of its target-gene promoters is likely to account for the β-cell dysfunction associated with the pathogenesis of MODY.

Dengue virus-infected liver cells undergo apoptosis but the underlying molecular mechanism remains unclear. We found that dengue virus capsid protein (DENV C) physically interacts with the human death domain-associated protein Daxx, a Fas-associated protein. The two proteins were predominantly co-localized in the cellular nuclei. Fas-mediated apoptotic activity in liver cells constitutively expressing DENV C was induced by anti-Fas antibody, indicating that the interaction of DENV C and Daxx involves in apoptosis of dengue virus-infected liver cells. Upon treatment with anti-Fas antibody, the liver cells expressing the wild-type DENV C showed significantly more apoptosis compared with the cells expressing mutant DENV C that could not enter the nucleus.

The glycosylated envelope protein of dengue virus, DENV E, is processed in the endoplasmic reticulum of host cells and therefore reliant on host processing functions. Our group found that domain III of DENV E interacts with human immunoglobulin heavy chain binding protein (BiP). The association of DENV E with two other chaperones, calnexin and calreticulin was also observed. Knocking-down expression of BiP, calnexin, or calreticulin by siRNA significantly decreased the production of infectious dengue virions, indicating that the interaction of these three chaperones with DENV E plays an important role in virion production.

Dengue virus nonstructural protein 1 (NS1) is a key glycoprotein involved in the production of infectious virus and the pathogenesis of dengue diseases. However, very little is known how NS1 interacts with host cellular proteins and functions in dengue virus-infected cells. Human heterogeneous nuclear ribonucleoprotein (hnRNP) C1/C2 was identified as NS1-interacting host cellular proteins in dengue virus-infected cells. Their interaction may have implications in virus replication and/or cellular responses favorable to survival of the virus in host cells.

In conclusion, our group is employing genetic/genomic and molecular biology approaches to investigate into genetic factors and molecular mechanisms of four certain diseases (kidney stone, dRTA, DM, and dengue-virus infection) relevant to Thai population and made significant discoveries and progresses in the studies of these diseases. However, further investigations of these diseases are required to gain complete understanding and to develop appropriate methods for prevention, control, and intervention.

Keyword: kidney stone, distal renal tubular (dRTA), diabetes mellitus (DM), dengue virus infection, genetics, genomics, molecular biology

บทคัดย่อ

ความผันแปรของจีโนมมนุษย์ที่ทำให้เกิดโรคในประชากรไทย ได้รับการสันนิฐานว่าแตกต่างจากประชากรอื่นๆ คณะผู้วิจัยจึงใช้วิธีทางพันธุศาสตร์/จีโนมิกส์และอณูชีววิทยา เพื่อศึกษาโรคที่ซับซ้อนและสำคัญบางโรคในคนไทย ได้แก่ โรคนิ่วในไต โรคไตผิดปกติในการขับกรด โรคเบาหวาน และโรคติดเชื้อไวรัสเด็งกี

โรคนิ่วในไตในคนภาคอิสาน พบในคนที่อยู่ในครอบครัวเดียวกัน โดยมีความเสี่ยงของการเกิดโรคในพี่น้องสูงกว่าประชากร 3.18 เท่า สนับสนุนว่าปัจจัยทางพันธุกรรมมีผลต่อพยาธิกำเนิดของโรค คณะผู้วิจัยได้ค้นพบเป็นครั้งแรกว่า ความผันแปรของยีนโปรทรอมบิน *(F2)* ซึ่งควบคุมการสังเคราะห์โปรตีนยับยั้งการเกิดนิ่วในปัสสาวะ คือ prothrombin fragment 1 มีความสัมพันธ์กับความเสี่ยงในการเกิดนิ่วในไตของคนไทยเพศหญิงในภาคอิสาน งานวิจัยที่กำลังดำเนินการ คือ การค้นหายีนที่ทำให้เกิดโรคนิ่วในไตโดยวิธีศึกษาจีโนม ทั้งแบบหาความสัมพันธ์ระหว่างยีนกับโรคในกลุ่มผู้ป่วยและกลุ่มเปรียบเทียบ และแบบตรวจการถ่ายทอดของยีนในสมาชิกครอบครัว โดยการใช้ดีเอ็นเอไมโครอาร์เรย์

มิวเตชันของยีน *SLC4A1* ซึ่งควบคุมการสร้างโปรตีน anion exchanger 1 ของเม็ดเลือดแดงและของไต (eAE1 และ kAE1) ทำให้เกิดความผิดปกติของเม็ดเลือดแดงและโรคไตผิดปกติในการขับกรด (dRTA) คณะผู้วิจัยได้พบมิวเตชันของยีน *SLC4A1* ร่วมกับความผิดปกติของการสังเคราะห์ฮีโมโกลบิน (ได้แก่ ธาลัสซีเมีย หรือฮีโมโกลบินอี) ในคนไทย ทำให้มีความผิดปกติของเม็ดเลือดแดงและการแตกของเม็ดเลือดแดง ซึ่งรุนแรงขึ้นเมื่อร่างกายมีภาวะเป็นกรด และพบมิวเตชันของยีน *SLC4A1* ชนิดใหม่ในประชากรไทยและประชากรอื่น จึงทำการสังเคราะห์โปรตีนจากยีนปกติและที่เกิดมิวเตชัน ในเซลล์เพาะเลี้ยง เพื่อศึกษาปฏิสัมพันธ์ การเคลื่อนย้าย และตำแหน่งของโปรตีนภายในเซลล์ ความผิดปกติในการเคลื่อนย้ายและการค้างภายในเซลล์ของโปรตีน (kAE1) จากยีนที่เกิดมิวเตชัน อาจจะทำให้โปรตีนที่เยื้อหุ้มของเซลล์ลดลง ซึ่งทำหน้าที่ขับกรดที่ท่อฝอยของเนฟฟอนในไต ซึ่งจะส่งผลให้เกิดโรคไตผิดปกติในการขับกรด

สาเหตุของโรคเบาหวานมีความหลากหลาย อาจจะเกิดจากความผิดปกติในวิถีทางชีวภาพต่างๆ ซึ่งทำให้เกิดภาวะน้ำตาลในเลือดสูง ยีนที่ทำให้เกิดโรคเบาหวานชนิดที่พบในผู้ป่วยที่มีอายุน้อย (MODY) 6 ยีน ได้แก่ *HNF-4alpha, GCK, HNF-1alpha, IPF-1, HNF-1beta* และ *NeuroD1/beta2* ในผู้ป่วยไทยที่เป็นโรคเบาหวานชนิด MODY และในผู้ป่วยโรคเบาหวานชนิดที่สองที่มีอายุน้อย ได้ถูกวิเคราะห์ คณะผู้วิจัยพบว่ามิวเตชันของ 6 ยีนนี้ เป็นสาเหตุส่วนน้อยของ MODY (19%) และโรคเบาหวานชนิดที่สองที่มีอายุน้อย (10%) ในผู้ป่วยไทย มิวเตชันชนิดใหม่ของยีน *HNF-1alpha* ได้ถูกค้นพบในครอบครัวไทยซึ่งเป็นโรคเบาหวานชนิด MODY ความผิดปกติในหน้าที่ของโปรตีนจากยีนที่เกิดมิวเตชัน ในการควบคุมการทำงานของยีนเป้าหมาย อาจจะทำให้เกิดความผิดปกติของเบต้า-เซลล์ ซึ่งเกี่ยวข้องกับพยาธิกำเนิดของโรคเบาหวานชนิด MODY

การติดเชื้อเด็งกีไวรัสของเซลล์ตับทำให้เกิดการตายของเซลล์แบบ apoptosis แต่กลไกที่เกิดขึ้นในระดับอณูยังไม่เป็นที่เข้าใจชัดเจน คณะผู้วิจัยได้ค้นพบว่าโปรตีน capsid ของไวรัสเด็งกีจับกับโปรตีน Daxx

ของคน ซึ่งเป็นโปรตีนในกลุ่ม Fas-associated protein ที่เกี่ยวข้องกับการตายของเซลล์ โปรตีนทั้งสองปรากฏร่วมกันในนิวเคลียส เมื่อทำให้เซลล์ตับสร้างโปรตีน capsid ของไวรัสเด็งกีและเหนี่ยวนำด้วย anti-Fas antibody จะทำให้เกิดการตายของเซลล์แบบ apoptosis ได้ แสดงว่าปฏิสัมพันธ์ของโปรตีน capsid ของไวรัสเด็งกีและโปรตีน Daxx เกี่ยวข้องกับการตายแบบ apoptosis ของเซลล์ตับที่ติดเชื้อเด็งกีไวรัส เมื่อมีการกระตุ้นด้วย anti-Fas antibody เซลล์ตับที่สังเคราะห์โปรตีน capsid ของไวรัสเด็งกีที่ปกติ จะเกิดการตายแบบ apoptosis มากกว่าเซลล์ที่สังเคราะห์โปรตีนซึ่งถูกเปลี่ยนแปลงให้ไม่สามารถเข้านิวเคลียสได้

โปรตีนเปลือกหุ้มของไวรัสเด็งกี จะผ่านกระบวนการที่เกิดขึ้นใน endoplasmic reticulum ของโฮสเซลล์ จึงขึ้นกับกระบวนการทำงานของโฮสเซลล์ คณะผู้วิจัยพบว่าโดเมนที่สาม (domain III) ของโปรตีนเปลือกหุ้มของไวรัสเด็งกี จับกับโปรตีน BiP ของมนุษย์ นอกจากนี้ยังพบว่าโปรตีนเปลือกหุ้มของไวรัสเด็งกีเกี่ยวข้องกับโปรตีน calnexin และ calreticulin การทำให้โปรตีน BiP, calnexin และ calreticulin สังเคราะห์ลดลงด้วย siRNA จะทำให้มีการสร้างไวรัสเด็งกีลดลง แสดงว่าปฏิสัมพันธ์ของโปรตีนทั้งสามชนิดกับโปรตีนเปลือกหุ้มของไวรัสเด็งกีมีบทบาทสำคัญในการสร้างไวรัส

โปรตีน NS1 ของไวรัสเด็งกี เป็นไกโคโปรตีนที่เกี่ยวข้องกับการสร้างไวรัสและพยาธิกำเนิดของโรคไข้เลือดออก แต่ยังรู้ค่อนข้างน้อยว่าโปรตีน NS1 จะมีปฏิสัมพันธ์กันโปรตีนภายในเซลล์ของโฮสและทำงานอย่างไร คณะผู้วิจัยได้พบว่าโปรตีน hnRNP C1/C2 มีปฏิสัมพันธ์กับโปรตีน NS1 ในเซลล์ที่ติดเชื้อไวรัสเด็งกี่ การมีปฏิสัมพันธ์กันของโปรตีนทั้งสองอาจจะเกี่ยวข้องกับการแบ่งตัวของไวรัส และ/หรือการตอบสนองต่อเซลล์เพื่อช่วยให้ไวรัสอยู่รอดในโฮสเซลล์

โดยสรุป คณะผู้วิจัยได้ใช้วิธีทางพันธุศาสตร์/จีโนมิกส์และอณูชีววิทยาเพื่อศึกษาปัจจัยทางพันธุกรรมและกลไกในระดับอณูของโรค 4 ชนิด (ได้แก่ โรคนิ่วในไต โรคไตผิดปกติในการขับกรด โรคเบาหวาน และโรคไข้เลือดออก) ซึ่งมีความสำคัญในคนไทย โดยทำให้เกิดการค้นพบและความก้าวหน้าหลายอย่างในการศึกษาโรคเหล่านี้ อย่างไรก็ตาม ยังมีความจำเป็นที่จะต้องทำการศึกษาโรคเหล่านี้ต่อไป เพื่อให้ได้องค์ความรู้ที่สมบูรณ์และเพื่อที่จะพัฒนาวิธีที่เหมาะสมในการควบคุม ป้องกันและรักษาโรคเหล่านี้

คำหลัก: โรคนิ่วในไต, โรคไตผิดปกติในการขับกรด, โรคเบาหวาน, โรคติดเชื้อไวรัสเด็งกี, พันธุศาสตร์, จีโนมิกส์, อณูชีววิทยา

Background/Rationale of the Problem for Research and Its Significance

Human health and illness are fundamentally determined by genetic make-up and environmental factor, and the interplay between these two components. Although the result from the Human Genome Project has demonstrated that human beings contain a high degree of genetic similarity, human populations have evolved divergently in differently changing environments. Thus, a majority of genetic mutations causing human diseases independently occurs in individual populations leading to 'genetic heterogeneity'. It has been shown by studies of genetic disorders that a certain disease has a remarkable phenotypic diversity reflecting heterogeneity of mutations (1) and same diseases can be caused either by different mutations of the same gene (allelic heterogeneity) or by mutations of different genes (locus heterogeneity) (1-14). The examples of genetic (allelic and locus) heterogeneities have been observed both in monogenic and complex (multifactorial) disorders (1-14). Based on these findings, it is conceivable that genetic and genetic-related diseases in Thais, although clinical phenotypes are similar to those found in other populations, would also possess genetic (allelic and locus) heterogeneity, occurring from different mutations of the same genes or from mutations of the genes different from those found in other populations. This situation will particularly be observed in complex (polygenic or multifactorial) disorders resulted from mutations (or variations) of several or many genes. The mutations (or variations) of different sets of genes will lead to dissimilar molecular pathogeneses and pathological mechanisms, consequently affecting correct prognosis and treatment. This is truly important especially when the genetic variations are taken into consideration to design effective therapeutic intervention. It is thus crucial to identify the genes that directly cause or lead to susceptibility to the diseases in Thai population to gain information on their genetic mutations and variations, and also important to study their molecular/cellular biology and pathology. These will be beneficial not only to provide insight into their molecular pathogeneses and pathological processes but also to develop efficient methods for diagnosis, treatment, prevention and control of the diseases.

The rapid advancement of knowledge and recent development of technologies in genetics/genomics and molecular/cellular biology have led to the innovation of powerful techniques and tools for the study of human diseases. Our group has a long-term experience in the study of genetics and molecular biology of kidney diseases (15-37), diabetes (38-45), and dengue virus infection in Thais (46-55). These diseases are therefore taken as models for further studies. We proposed to study these diseases by applying different technological approaches and by emphasizing on different aspects of the diseases in the context of their relevance and of our ongoing studies. Four research projects proposed to be carried out include:

Project I: Genetics and Molecular Biology of Kidney Stone

Kidney stone is public health problem and endemic in the population in the northeast of Thailand, causing a great healthcare burden and economic loss. Its prevalence is as high as 5-10% (56). Each year, several thousands of new cases are admitted for treatment in local hospitals and a large number of patients undergo treatment by surgery with 40% of recurrent rate within two years (57). The etiology and pathogenesis of kidney stone in the northeastern Thai population is unknown. In the past few decades, environmental factors and diets as the cause of kidney stone in this population were extensively studied but the cause has not been identified. A number of genetic abnormalities resulting in kidney stone diseases have been reported in the literatures (58-62) but they are unlikely to cause the kidney stone disease in the northeastern Thai patients because the phenotypes associated with these genetic abnormalities, particularly hypercalciuria, hyperoxaluria,

and hyperuricosuria, are not present in the northeastern Thai patients (56). Our group has conducted clinical studies and collected clinical and genetic data as well as samples from patients and their family members at Khon Kaen and Sappasitthiprasong (Ubon Ratchathani) Hospitals, making us in an advantageous position to conduct genetics and molecular biology studies of kidney stone in this population.

In this project, we propose to continue the study in order to identify genes involving in kidney stone disease in the northeastern Thai population by genome-wide linkage analysis together with genome-wide association study using a high-throughput single nucleotide polymorphism (SNP) genotyping by DNA microarray, in parallel with candidate gene association study that is being carried out in our group (partly supported by BIOTEC and Mahidol University grants), to further investigate into its molecular pathogenesis and pathological mechanism. The understanding of its genetic and molecular pathogenesis and the potential availability of method for molecular testing for its genetic defect or susceptibility will lead to the improvement of prevention, control, and management of kidney stone in this affected population.

Project II: Genetics and Molecular Biology of Distal Renal Tubular Acidosis

Distal renal tubular acidosis (dRTA) is a kidney disease characterized by an inability of the kidney to secrete acid (or hydrogen ion, H^+) into urine resulting in systemic metabolic acidosis. Its clinical manifestations include muscle weakness, growth retardation, metabolic bone disease, nephrocalcinosis, nephrolithiasis, chronic pyelonephritis, and renal failure (63-64). Defects in the function of either H^+-ATPase or kidney anion exchanger 1 (kAE1) results in impairment of acid excretion or bicarbonate reabsorption by the α-intercalated cells leading to dRTA (65-67). Genetic studies by our and other groups have revealed that mutations of human *SLC4A1* encoding both erythroid AE1 (eAE1 or band 3) and kAE1 lead to the development of both autosomal dominant (AD) and autosomal recessive (AR) forms of dRTA (15-16, 20-23, 25-27, 32, 34, 36). These mutations affect the intracellular trafficking of kAE1 to the basolateral surface, either mis-targeting to inappropriate destinations or accumulation of proteins within intracellular compartments, demonstrated by the studies in transfected cells (25, 27, 29-30, 36).

To investigate the trafficking process of kAE1 and why mutant kAE1 fail to transport to the cell surface, we aim to identify binding proteins of kAE1 (kAE1-BPs) in human kidney cells using yeast two hybrid (Y2H) screening method. In our initial attempt (supported by TRF grant), proteins that specifically interact with kAE1 (either N-terminus or C-terminus) have been screened from a human kidney cDNA library by Y2H system. In this continuing study, the interactions between kAE1 and kAE1-BPs isolated will be further verified by expermental assays in human kidney cell lines. The interactions between mutant kAE1 and kAE1-BPs and the effect of over-expression or knock-down of kAE1-BPs to the cell surface transport of wild-type and mutant kAE1 will be examined to understand the molecular defects of kAE1 transport caused by dRTA-associated AE1 mutations.

Project III: Genetics and Molecular Biology of Diabetes Mellitus

Diabetes mellitus (DM) is a chronic metabolic disease characterized by hyperglycemia, resulting in long-term complications including retinopathy, nephropathy, neuropathy, and cardiovascular disease (68). Type 1 diabetes (T1D) is caused by immunological destruction of pancreatic β-cells that synthesize and secret insulin. Type 2 diabetes (T2D), resulting from defects in insulin secretion, insulin action, or combination of both (69), accounting for 90% of all types of diabetes mellitus, and affecting more than 170 million individuals worldwide, is a genetically heterogeneous disease occurring from defects or variations of single or multiple genetic loci, in addition to the effect of

environmental factors and lifestyle. Genetic studies have demonstrated that T2D is inherited as a non-Mendelian (multifactorial) fashion but a small proportion (~10%) of T2D segregating in families as a Mendelian (monogenic) disorder is classified as maturity onset diabetes of the young (MODY), characterized by young age at onset with autosomal dominant inheritance (70). Defects of at least 6 different genes (*HNF-4*α, *GCK*, *HNF-1*α, *IPF-1*, *HNF-1*β, and *NeuroD1*) result in MODY (71-72) but a number of MODY families have no mutations in these six genes, known as MODY-X.

Our research group has recruited the patients with MODY and early-onset T2D as well as the patients with adult-onset T2D to investigate their genetic variations. The preliminary result showed that the six known MODY genes account for a small proportion of MODY and early-onset T2D patients, suggesting that MODY-X is more common in this group of Thai patients. Novel genes that cause MODY-X in Thai patients are being searched by candidate-gene approach. In this project, we propose to apply the genome-wide linkage method to identify novel candidate genes that cause MODY and early-onset T2D in Thai patients. Additionally, T2D-susceptibility genes in adults will also be investigated by the candidate-gene approach. The genetic mutations and polymorphism identified will be further examined into their biologically functional impact by molecular/cellular biology techniques. This molecular genetics as well as molecular/cellular biology information is valuable for understanding molecular mechanisms underlying pathogeneses of MODY and T2D in Thai patients and would facilitate more appropriate measures for prevention, control, and treatment of diabetes in Thai.

Project IV: Molecular Biology of Human Host and Virus Protein Interactions

Dengue fever (DF) and dengue hemorrhagic fever (DHF) caused by dengue virus (DEN) infection are important public health problems in Thailand and tropical regions (73-76). DHF presents with continuous fever (for 2 to 7 days), hemorrhagic tendencies, and thrombocytopenia with hemoconcentration (77). DEN infection causes alterations in the transcriptional and translational patterns, cell cycle, cytoskeleton, and apoptosis pathways of the host cells (78). These together with host immune responses lead to pathogenesis and clinical manifestations of the disease. Both viral and cellular proteins are known to be required for virus replication. However, it is not clear how virus and host proteins interact to alter host cellular pathways. Our research interest in this project is to investigate DEN and host protein interactions and alterations of host molecular and cellular pathways.

Yeast two-hybrid (Y2H) system was used to screen the DEN E-binding proteins (E-BP) from a human HeLa cDNA library by using DEN E III as bait. Human glucose regulated protein 78 (GRP78) or immunoglobulin binding protein (BiP) is an isoform 5 of the heat shock protein 70 (HSP70) has been identified. GRP78 primarily locates in the endoplasmic reticulum (ER) and functions as a molecular chaperone involving in the folding and assembly of cellular and viral membrane proteins. This molecular chaperone may be beneficial for virus replication. However, its role in DEN replication is unknown. Further study will be performed to demonstrate the role of GRP78 in DEN replication. We also identified a human Fas-associated protein, Daxx, binding to DEN capsid (C) protein. The roles of Daxx and DEN C in apoptosis of the infected cells will also be investigated. These studies will help to elucidate molecular/cellular pathogenesis and mechanism of DEN infection, beneficial for designing novel treatment and intervention.

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Objectives

(1) To identify genes causing and/or proteins involving in some diseases relevant to Thais.
(2) To elucidate the molecular pathogeneses and mechanisms of these diseases.
(3) To gain experience and develop expertise in advanced genetic/genomic and molecular/cellular biological technologies.
(4) To develop molecular methods for testing disease-associated or susceptibility genes for the diseases studied.
(5) To produce research graduates and train researchers in the fields of human genetics and medical molecular biology.

Research Activities and Results

Project I: Genetics and molecular biology of kidney stone

Evidence suggesting a genetic contribution to kidney stone in northeastern Thai population.

By Suchai Sritippayawan, Sombat Borwornpadungkitti, Atchara Paemanee, Chagkrapan Predanon, Wattanchai Susaengrat, Duangporn Chuawatana, Nunghathai Sawasdee, Sirintra Nakjang, Suttikarn Pongtapaditep, Choochai Nettuwakul, Nanyawan Rungroj, Somkiat Vasuvattkul, Prida Malasit and Pa-thai Yenchitsomanus

Genetic factor may play a role in the pathogenesis of kidney stone that is found in the northeastern (NE) Thai population. Herein, we report initial evidence suggesting genetic contribution to the disease in this population. We examined 1,034 subjects including 135 patients with kidney stone, 551 family members, and 348 villagers by radiography of kidney-ureter-bladder (KUB) and other methods, and also analyzed stones removed by surgical operations. One hundred and sixteen of 551 family members (21.05%) and 23 of the 348 villagers (6.61%) were affected with kidney stone. The relative risk (lambda(R)) of the disease among family members was 3.18. Calcium stones (whewellite, dahllite, and weddellite) were observed in about 88% of stones analyzed. Our data indicate familial aggregation of kidney stone in this population supporting that genetic factor should play some role in its pathogenesis. Genetic and genomic studies will be conducted to identify the genes associated with the disease.
(Published in Urological Research 2009;37:141-6.)

Prothrombin haplotype associated with kidney stone disease in Northeastern Thai patients.

By Nanyawan Rungroj, Suchai Sritippayawan, Wanna Thongnoppakhun, Atchara Paemanee, Nunghathai Sawasdee, Choochai Nettuwakul, Nirinya Sudtachat, Duangporn Ungsupravate, Pairao Praihirunkit, Duangporn Chuawattana, Varaporn Akkarapatumwong, Sombat Borvornpadungkitti, Wattanachai Susaengrat, Somkiat Vasuvattakul, Prida Malasit and Pa-thai Yenchitsomanus

To evaluate genetic variations associated with kidney stone disease in Northeastern Thai patients, altogether, 67 single nucleotide polymorphisms (SNP) distributed within 8 candidate genes, namely *TFF1*, *S100A8*, *S100A9*, *S100A12*, *AMBP*, *SPP1*, *UMOD*, and *F2*, which encode stone inhibitor proteins, including trefoil factor 1, calgranulin (A, B, and C), bikunin, osteopontin, tamm-Horsfall protein, and prothrombin, respectively, were initially genotyped in 112 individuals each and in additional subjects to consist of 164 patients and 216 control subjects in total. We found that minor allele and homozygous genotype frequencies of 8 of 10 SNPs distributed within the *F2* gene were significantly higher in the control group than in the patient group. Two *F2* haplotypes were found to be dually associated with kidney stone risk, one (TGCCGCCGCG) with increased disease risk and the other (CGTTCCGCTA) with decreased disease risk. However, these 2 haplotypes were associated with the disease risks in only the female, not the male, group. The results of our study indicate that genetic variation of *F2* is associated with kidney stone risk in Northeastern Thai female patients.
(Published in Urology 2011;77:249.e17-23.)

Project II: Genetics and molecular biology of distal renal tubular acidosis

Hematological abnormalities in patients with distal renal tubular acidosis and hemoglobinopathies

By Sookkasem Khositseth, Apiwan Sirikanaerat, Siri Khoprasert, Sauwalak Opastirakul, Pornchai Kingwatanakul, Wanna Thongnoppakhun and Pa-thai Yenchitsomanus

Mutations of the human *SLC4A1* gene encoding erythroid and kidney isoforms of anion exchanger 1 (AE1, band 3) result in erythrocyte abnormalities or distal renal tubular acidosis (dRTA) and such mutations are observed in Southeast Asia, where hemoglobinopathies are prevalent. Genetic and hematological studies in 18 Thai patients with dRTA have shown that 12 of them (67%) carried *SLC4A1* mutations (7 G701D/G701D, 3 SAO/G701D, and 2 G701D/A858D). Of these 12 patients, three had homozygous G701D/G701D and heterozygous Hb E; one compound heterozygous SAO/G701D and heterozygous a α-thalassemia; and one compound heterozygous G701D/A858D and heterozygous Hb E. Of 6 patients without *SLC4A1* mutation, two each carried heterozygous or homozygous Hb E and one of the latter also had Hb H disease ($--^{SEA}/-\alpha 4.2$). The blood smears of patients with homozygous G701D/G701D showed ~25% ovalocytes. Strikingly, the patients with coexistence of homozygous G701D/G701D and heterozygous Hb E had 58% ovalocytes. Similarly, the patients who had compound heterozygous SAO/G701D showed 49% ovalocytes, but the patient with coexistence of compound heterozygous SAO/G701D and heterozygous a α-thalassemia had 70% ovalocytes. Our previous study has shown that under metabolic acidosis, the patients with homozygous G701D/G701D or compound heterozygous SAO/G701D had reticulocytosis, indicating compensated hemolysis. A patient with compound heterozygous SAO/G701D and heterozygous a α-thalassemia presented with hemolytic anemia and hepatosplenomegaly which was alleviated by alkaline therapy. Taken together, the coexistence of both homozygous or compound heterozygous *SLC4A1* mutations and hemoglobinopathy has a combined effect on red cell morphology and degree of hemolytic anemia, which is aggravated by acidosis.
(Published in American Journal of Hematology 2008;83:465-71.)

Rapid genotyping of solute carrier family 4, member 1 (SLC4A1) mutations and polymorphisms by high-resolution melting analysis

By Choochai Nettuwakul, Nunghathai Sawasdee and Pa-thai Yenchitsomanus

We developed and tested a new genotyping method for *SLC4A1* mutations and polymorphisms by high-resolution melting (HRM) analysis. Eighteen known *SLC4A1* variants were used for optimization of the HRM analysis. The HRM analysis was then used for genotyping 16 blind DNA samples highly enriched with two common mutations, Southeast Asian ovalocytosis (SAO) and band 3 Bangkok 1 (G701D), to compare its results with that of the conventional procedures. The HRM analysis could detect all 18 *SLC4A1* variants. In the samples that the difference plots of homozygous wild-type and homozygous variant could not be distinguished, they were successfully analyzed by spiking with a sample with known homozygous genotype. The results of the HRM analysis of the blind samples showed 100% compatible with that of the conventional methods. HRM analysis is an efficient and accurate genotyping method for *SLC4A1* mutations and polymorphisms.
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Impaired trafficking and intracellular retention of mutant kidney anion exchanger 1 proteins (G701D and A858D) associated with distal renal tubular acidosis

By Duangporn Ungsupravate, Nunghathai Sawasdee, Sookkasem Khositseth, Jing li, Wandee Udomchaiprasertkul, Siri Khoprasert, Reinhart A. F. Reithmeier and Pa-thai Yenchitsomanus

Novel compound heterozygous mutations (*G701D/A858D*) of human *solute carrier family 4, anion exchanger, member 1 (SLC4A1)* were identified in two pediatric patients with distal renal tubular acidosis (dRTA). To examine the interaction, trafficking, and cellular localization of the wild-type and two mutant kidney AE1 (kAE1) proteins, we expressed and co-expressed them in human embryonic kidney (HEK) 293T and Madin-Darby canine kidney (MDCK) epithelial cells. In individual expressions, wild-type kAE1 was localized at the surface of HEK 293T and MDCK cells, kAE1 G701D was mainly retained in the cytoplasm while kAE1 A858D was observed both in the cytoplasm and at the cell surface. In co-expressions, the wild-type kAE1 could form heterodimers with either kAE1 G701D or kAE1 A858D and rescued the mutant proteins to express on the cell surface. The co-expressed kAE1 G701D and A858D could also form heterodimer but showed intracellular retention in both HEK 293T and MDCK cells. kAE1 G701D might partially be rescued by kAE1 A858D to the cell surface. Impaired trafficking and intracellular retention of the mutant kAE1 G701D and A858D would lead to decrease functional kAE1 at the basolateral membrane of the kidney α-intercalated cells in the distal nephron – this molecular defect is likely to occur in the patients with the autosomal recessive dRTA associated with the novel compound heterozygous *SLC4A1 G701D/A858D* mutations.
(Published in Mol Membr Biol. 2010;27:92-103.)

Band 3 Edmonton I, a novel mutant of the anion exchanger 1 causing spherocytosis and distal renal tubular acidosis

By Carmen Chu, Naomi Woods, Nunghathai Sawasdee, Naomi Glick, Sabin Shurraw, Pa-thai Yenchitsomanus, Sandra Cockfield, Manjula Gowrishankar and Emmanuelle Cordat

Distal renal tubular acidosis (dRTA) and hereditary spherocytosis (HS) are two diseases that can be caused by mutations in the gene encoding the anion exchanger 1 (AE1, Band 3). dRTA is characterized by defective urinary acidification, leading to metabolic acidosis, renal stones and failure to thrive. HS results in anemia, which may require regular blood transfusions and splenectomy. Mutations in the gene encoding AE1 rarely cause both HS and dRTA. Here, we describe a novel *AE1* mutation, *Band 3 Edmonton I*, which causes dominant HS and recessive dRTA. The patient is a compound heterozygote with the new mutation, C479W and the previously described mutation, G701D. Red blood cells from the patient presented a reduced amount of AE1. Expression in a kidney cell line showed that kAE1 C479W is retained intracellularly. As kAE1 is a dimer, we performed co-expression studies and found that in kidney cells, kAE1 C479W and G701D proteins traffic independently from each other despite their ability to form heterodimers. Therefore, the patient carries one kAE1 mutant that is retained in the Golgi (G701D) and another kAE1 mutant (C479W) located in the endoplasmic reticulum of kidney cells, and is thus likely unable to reabsorb bicarbonate into the blood. We conclude that the C479W mutant is a novel trafficking mutant of AE1, which causes HS due to a decreased cell surface AE1 protein and results in dRTA due to its intracellular retention in kidney.
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Project III: Genetics and molecular biology of diabetes mellitus

Molecular genetics of diabetes mellitus

By Nattachet Plengvidhaya, Watip Boonyasrisawat, Prapaporn Jungtrakoon, Jatuporn Sujjitjon and Pa-thai Yenchitsomanus

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels. DM is a threat to the world population and a global burden in the 21st century. Its prevalence in adults worldwide was estimated to be 4.0% in the year 1995 and will have risen to 5.4% by the year 2025. There will be a 42% increase, from 51 to 72 million people affected, in the developed countries and a 170% increase, from 84 to 228 million people affected, in the developing countries. It was estimated in 2000 that 9.6% (2.4 million) of Thai adults were affected with DM and 5.4% (1.4 million) had impaired fasting glucose. These have indicated that DM is an enormous global public health problem and also an increasingly significant public health problem in Thailand. Since the disease is very heterogeneous, abnormality at different biological pathways can lead to hyperglycemia and subtypes of the disease requiring precise diagnostic criteria. Several research studies have concentrated on identifications of diabetic susceptibility genes. These efforts facilitate a better understanding in molecular pathogeneses and pathophysiologies underlying DM subtypes, thereby leading to the development of appropriate and effective therapeutic approaches.
(Published in Siriraj Medical Journal 2008;60(5):273-278.)

Molecular genetics of diabetes mellitus

By Prapaporn Jungtrakoon, Watip Boonyasrisawat, Nattachet Plengvidhya, Napatawn Banchuin and Pa-thai Yenchitsomanus

Diabetes mellitus (DM) is a disease that causes major public health problem worldwide. In Thailand, it was estimated that 9.6% or 2.4 million adults were affected with DM and the prevalence is increasing. The unawareness of having the disease leads to delayed treatment and development of chronic complications. The cost for management of DM and its complications is increasing enormously, causing a great economic and healthcare burden. DM is caused by both environmental and genetic factors. Although type 1 diabetes (T1D) is not a genetically predestined disease, an increased susceptibility to the disease can be inherited. Genetic factor plays a crucial role in pathogenesis and complications of type 2 diabetes (T2D) while environmental factors are also required for the disease development. Even if modifications of life-style are important for controlling T2D, the identification of susceptibility genes will lead to understanding of its complex pathogenesis and development of more effective treatment. Up to date, a number of diabetic susceptible genes are identified in Western populations. It is now the time to identify the causative and susceptibility genes of diabetes in Thai. This review aims to provide a current overview of molecular genetics of DM and some available information in Thais.
(Published in Thai Journal of Genetics 2008;1:17-31.)

Molecular genetics of monogenic beta-cell diabetes

By Jatuporn Sujjitjoon, Prapaporn Jungtrakoon, Watip Boonyasrisawat, Nalinee Chongjaroen, Titikan Chukijrungroat, Suwattanee Kooptiwut, Nattachet Plengvidhya, Napatawn Banchuin and Pa-thai Yenchitsomanus.

Monogenic β-cell diabetes – a rare form of diabetes mellitus (DM) is caused by defects in a group of genes controlling pancreatic β-cell development and function. The diabetic symptoms are manifested within a short period after birth as neonatal diabetes mellitus (NDM), in childhood or early adulthood as maturity-onset diabetes of the young (MODY) and mitochondrial diabetes. Several etiologic genes for this form of DM have been identified in many patients. The common etiologic genes encode β-cell transcription factors and proteins involving in glucose-stimulated insulin secretion. Owning to their nature of genetic heterogeneity, monogenic β-cell diabetes presents the characteristics of variable age at onset, degree of severity, and occurrence of diabetic complications. The study of this form of diabetes has provided new knowledge and a better insight into the molecular mechanism controlling normal and pathological states of β-cells as reviewed in this article.
(Published in Thai Journal of Genetics 2008;1:93-108.)

Mutations of maturity-onset diabetes of the young (MODY) genes in Thais with early-onset ypye 2 diabetes mellitus

By Nattachet Plengvidhya, Watip Boonyasrisawat, Nalinee Chongjaroen, Prapaporn Jungtrakoon, Sutin Sriussadaporn, Sathit Vannaseang, Napatawn Banchuin and Pa-thai Yenchitsomanus

Object: Six known genes responsible for maturity-onset diabetes of the young (MODY) were analysed to evaluate the prevalence of their mutations in Thai patients with MODY and early-onset type 2 diabetes.
Patients and Methods: Fifty-one unrelated probands with early-onset type 2 diabetes, 21 of them fitted into classic MODY criteria, were analysed for nucleotide variations in promoters, exons, and exon-intron boundaries of six known MODY genes, including HNF-4alpha, GCK, HNF-1alpha, IPF-1, HNF-1beta, and NeuroD1/beta2, by the polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) method followed by direct DNA sequencing. Missense mutations or mutations located in regulatory region, which were absent in 130 chromosomes of non-diabetic controls, were classified as potentially pathogenic mutations.
Results: We found that mutations of the six known MODY genes account for a small proportion of classic MODY (19%) and early-onset type 2 diabetes (10%) in Thais. Five of these mutations are novel including GCK R327H, HNF-1alpha P475L, HNF-1alphaG554fsX556, NeuroD1-1972 G > A and NeuroD1 A322N. Mutations of IPF-1 and HNF-1beta were not identified in the studied probands.
Conclusions: Mutations of the six known MODY genes may not be a major cause of MODY and early-onset type 2 diabetes in Thais. Therefore, unidentified genes await discovery in a majority of Thai patients with MODY and early-onset type 2 diabetes.
(Published in Clin Endocrinol. 2009;70:847-53.)

Construction of a mutation due to fourteen base-pair insertion in HNF-1α gene causing maturity-onset diabetes of the young (MODY) in Thai patients

By Suwattanee Kooptiwut, Jatuporn Sujjitjon, Titikan Chukijrungroat, Nattachet Plengvidhaya, Napatawn Banchuin, Pa-thai Yenchitsomanus

The aim of this study is to generate a mutation causing maturity-onset diabetes of the young (MODY) in Thai patients by insertion of fourteen base-pair (bp) into *HNF-1α* gene using a modified site-directed ligase-independent mutagenesis (SLIM) method. Two pairs of long- and short-tailed primers were designed to amplify a plasmid construct containing *HNF-1α* and to insert 14-bp at a desired position. Long-tailed primers contained the overhanging 14-nucleotide (nt) insert at their termini which were complimentary to each other. Polymerase chain reactions (PCR) were performed in two separated tubes using different pairs of primers. After amplifications, PCR products from both tubes were pooled together, denatured and then re-annealed to allow formation of double stranded DNA molecules containing the 14-bp insert within *HNF-1α*. The pooled and reannealed PCR products without ligation were transformed into competent *E.coli* cells to generate ligated recombinant plasmid with 14-bp insertion. Five of 14 bacterial colonies contained the desired recombinant plasmid with14-bp insertion within *HNF-1α̃* The efficiency of method for generation of recombinant plasmid was about 36 percent. This method is simple and rapid to insert a long stretch of nucleotides into a plasmid construct containing a gene of interest at a desired position. A recombinant plasmid containing an insertion mutation in *HNF-1α* gene was successfully generated, allowing an opportunity to perform functional study of the mutated gene.
(Published in Siriraj Medical Journal 2008;60:175-179.)

Functional defect of truncated heptocyte nuclear factor-1α (G554fsX556) associated with maturity-onset diabetes of the young

By Suwattanee Kooptiwut, Jatuporn Sujjitjoon, Nattachet Plengvidhya, Watip Boonyasrisawat, Nalinee Chongjaroen, Prapapron Jungtrakoon, Hiroto Furuta, Kishio Nanjo, Napatawn Banchuin, and Pa-thai Yenchitsomanus

Mutations of *hepatocyte nuclear factor-1α* (*HNF-1α*) result in maturity-onset diabetes of the young (MODY). We recently identified a novel frameshift mutation attributable to 14-nucleotide insertion in *HNF-1α*, encoding a truncated HNF-1α (G554fsX556) with 76-amino acid deletion at its carboxyl terminus, in a Thai family with MODY. To examine functional defect of the truncated HNF-1α (G554fsX556) associated with the pathogenesis of MODY. A recombinant plasmid containing *HNF-1α* G554fsX556 cDNA was constructed. The wild-type and mutant HNF-1α proteins were expressed by *in vitro* transcription and translation (TNT) assay and by HeLa cell transfection. Binding of wild-type and mutant proteins to the sequence of human glucose-transporter 2 (*GLUT2*) promoter containing HNF-1α binding site was examined by electrophoretic mobility shift assay (EMSA). Transactivation activities of wild-type and mutant HNF-1α on human *GLUT2* and rat L-type pyruvate kinase (*L-PK*) promoters in HeLa cells were examined by luciferase reporter assay. The wild-type and mutant HNF-1α produced by TNT assay and HeLa cell transfection could similarly bind to the sequence of human *GLUT2* promoter as examined by EMSA. However, transactivation activities of mutant HNF-1α on human *GLUT2* and rat *L-PK* promoters in HeLa cells reduced to approximately 55-60% of the wild-type protein. The functional defect of novel truncated HNF-1α (G554fsX556) on the

transactivation of its target-gene promoters is likely to account for the β-cell dysfunction associated with the pathogenesis of MODY.
(Published in Biochem Biophys Res Commun. 2009;383:68-72.)

Project IV: Molecular biology of human host and virus protein interactions

Sensitization to Fas-mediated apoptosis by dengue virus capsid protein

By Thawornchai Limjindaporn, Janjuree Netsawang, Sansanee Noisakran, Somchai Thiemmeca, Wiyada Wongwiwat, Sangkab Sudsaward, Panisadee Avirutnan, Chunya Puttikhunt, Watchara Kasinrerk, Rungtawan Sriburi, Nopporn Sittisombut, Pa-thai Yenchitsomanus and Prida Malasit

Dengue fever (DF) and dengue hemorrhagic fever (DHF) are important public health problems in tropical regions. Abnormal hemostasis and plasma leakage are the main patho-physiological changes in DHF. However, hepatomegaly, hepatocellular necrosis and fulminant hepatic failure are occasionally observed in patients with DHF. Dengue virus-infected liver cells undergo apoptosis but the underlying molecular mechanism remains unclear. Using a yeast two-hybrid screen, we found that dengue virus capsid protein (DENV C) physically interacts with the human death domain-associated protein Daxx, a Fas-associated protein. The interaction between DENV C and Daxx in dengue virus-infected liver cells was also demonstrated by co-immunoprecipitation and double immunofluorescence staining. The two proteins were predominantly co-localized in the cellular nuclei. Fas-mediated apoptotic activity in liver cells constitutively expressing DENV C was induced by anti-Fas antibody, indicating that the interaction of DENV C and Daxx involves in apoptosis of dengue virus-infected liver cells.
(Published in Biochem Biophys Res Commun. 2007;362:334–339)

Cloning and expression of human apoptotic Daxx gene in human kidney cells

By Thawornchai Limjindaporn and Janjuree Netsawang

The Fas/Fas ligand system is a key signal pathway involved in the regulation of apoptosis, which is a genetically controlled process for the elimination of the cells. Human Daxx was first identified as a Fas-binding protein and acts as a pro-apoptotic ptotein that enhances Fas-mediated aopotosis. However, the anti-aopototic function of Daxx is suggested. Therefore, the precise role of Daxx still needs further investigation. The purpose of this study was to clone, and verify the expression of human Daxx in human kidney HEK293T cells. Total RNA was extracted from human lymphocytes and converted to cDNA by RT-PCR. Daxx was then amplified with primers designed according to the published sequence of Daxx in Genbank. The PCR product was inserted into pcDNA3.1Hygro expression vector and the recombinant plasmid, namely pcDNA3.1Hygro-Daxx, was further verified by restriction endonuclease analysis and DNA sequencing. Furthermore, plasmid pcDNA3.1Hygro-Daxx was transfected into HEK293T cells and the protein expression was verified by flow cytometry analysis. The results showed that the entire coding region of human Daxx gene was cloned, and expression in HEK293T cells. The clone will be used to investigate the detailed molecular mechanisms of human Daxx in apoptotic process of the cells.
(Published in Thai Journal of Genetics. 2008;1:57-62)

Cloning of JNK activation domain and Fas-binding domain of human apoptiotic Daxx gene by homologous recombination in yeast

By Thawornchai Limjindaporn, Siwimon Sawaitbud, Janjuree Netsawang, Dumrong Mairiang, Sasiprapa Khunchai and Wiyada Wongwiwat

The death domain-associated protein (Daxx) was originally cloned as a Fas-interacting protein. It is ubiquitously expressed and highly conserved in mammals. Currently, the role of Daxx is controversy between apoptotic or anti-apoptotic molecules. To understand the precise function of human Daxx, yeast two hybrid screening will be used as a tool to identify interactions between human Daxx and other human proteins. The first step in a yeast two hybrid method is to express a bait plasmid in the recombinant form in yeast. We utilized the *in vivo* homologous recombination in yeast to insert a JNK activation domain and a Fas-binding domain of human Daxx, into the bait plasmid, pEG-NRT. Furthermore, the expression of a JNK activation domain and a Fas-binding domain of human Daxx was demonstrated by Western blot analysis. Yeast expressing a JNK activation domain and a Fas-binding domain of human Daxx will be used as bait strains to screen for human proteins interacting with Daxx, which may provide us the clue to uncover the mysterious function of human Daxx.
(Published in Thai Journal of Genetics. 2008;1:63-68)

Nuclear localization of dengue virus capsid protein is required for DAXX interaction and apoptosis.

By Janjuree Netsawang, Sansanee Noisakran, Chunya Puttikhunt, , Watchara Kasinrerk, Wiyada Wongwiwat, Prida Malasit, Pa-thai Yenchitsomanus, and Thawornchai Limjindaporn

Dengue virus capsid protein (DENVC) localizes to both the cytoplasm and nucleus of dengue virus-infected cells. DENV C contains three nuclear localization signals (NLS), (6)KKAR(9), (73)KKSK(76), and the bipartite signal (85)RKeigrmlnilnRRRR(100). Stable HepG2 cells constitutively expressing DENV C, DENV C (Delta85-100) and DENV C (Delta73-100) were constructed to clarify whether nuclear translocation of DENV C affected apoptosis in liver cell line. While the wild-type DENV C could translocate into the nuclei of HepG2 cells, the mutant DENV Cs were restricted to the cytoplasm. The loss of nuclear localization of both mutant DENV Cs resulted in the disruption of their interactions with the apoptotic protein Daxx. Interestingly, upon treatment with anti-Fas antibody, the HepG2 cells expressing the wild-type DENV C showed significantly more apoptosis compared with the HepG2 cells expressing either mutant DENV C. To identify the amino acids required for DAXX interaction and apoptosis, substitution mutations either (K73A/K74A) or (R85A/K86A) were introduced into the C-terminal region of DENV C, and tested whether these mutations affected its interaction with Daxx and apoptosis. The results demonstrate that (73)KK and (85)RK of DENV C are important for its nuclear localization, interaction with DAXX and induction of apoptosis. This work is the first to demonstrate that nuclear localization of DENV C is required for DAXX interaction and apoptosis.
(Published in Virus Res. 2010;147:275-83.)

Interaction of dengue virus envelope protein with endoplasmic reticulum-resident chaperones facilitates dengue virus production.

By Thawornchai Limjindaporn, Wiyada Wongwiwat, Sansanee Noisakran, Janjuree Netsawang, Chunya Puttikhunt, Watchara Kasinrerk, Panisadee Avirutnan, Somchai Thiemmeca, Rungtawan Sriburi, Nopporn Sittisombut, Prida Malasit and Pa-thai Yenchitsomanus

Dengue virus infection is an important mosquito-borne disease and a public health problem worldwide. A better understanding of interactions between human cellular host and dengue virus proteins will provide insight into dengue virus replication and cellular pathogenesis. The glycosylated envelope protein of dengue virus, DENV E, is processed in the endoplasmic reticulum of host cells and therefore reliant on host processing functions. The complement of host ER functions involved and nature of the interactions with DENV E has not been thoroughly investigated. By employing a yeast two-hybrid assay, we found that domain III of DENV E interacts with human immunoglobulin heavy chain binding protein (BiP). The relevance of this interaction was demonstrated by co-immunoprecipitation and co-localization of BiP and DENV E in dengue virus-infected cells. Using the same approach, association of DENV E with two other chaperones, calnexin and calreticulin was also observed. Knocking-down expression of BiP, calnexin, or calreticulin by siRNA significantly decreased the production of infectious dengue virions. These results indicate that the interaction of these three chaperones with DENV E plays an important role in virion production, likely facilitating proper folding and assembly of dengue proteins.
(Published in Biochem Biophys Res Commun. 2009;379:196-200.

Identification of human hnRNP C1/C2 as a dengue virus NS1-interacting protein.

By Sansanee Noisakran, Suchada Sengsai, Visith Thongboonkerd, Rattiyaporn Kanlaya, Supachok Sinchaikul, Shui-Tein Chen, Chunya Puttikhunt, Watchara Kasinrerk, Thawornchai Limjindaporn, Wiyada Wongwiwat, Prida Malasit and Pa-thai Yenchitsomanus.

Dengue virus nonstructural protein 1 (NS1) is a key glycoprotein involved in the production of infectious virus and the pathogenesis of dengue diseases. Very little is known how NS1 interacts with host cellular proteins and functions in dengue virus-infected cells. This study aimed at identifying NS1-interacting host cellular proteins in dengue virus-infected cells by employing co-immunoprecipitation, two-dimensional gel electrophoresis, and mass spectrometry. Using lysates of dengue virus-infected human embryonic kidney cells (HEK 293T), immunoprecipitation with an anti-NS1 monoclonal antibody revealed eight isoforms of dengue virus NS1 and a 40-kDa protein, which was subsequently identified by quadrupole time-of-flight tandem mass spectrometry (Q-TOF MS/MS) as human heterogeneous nuclear ribonucleoprotein (hnRNP) C1/C2. Further investigation by co-immunoprecipitation and co-localization confirmed the association of hnRNP C1/C2 and dengue virus NS1 proteins in dengue virus-infected cells. Their interaction may have implications in virus replication and/or cellular responses favorable to survival of the virus in host cells.
(Published in Biochem Biophys Res Commun. 2007;372:67-72)

Others

Molecular Genetics and Genomics in Medicine

By Pa-thai Yenchitsomanus

In the past few decades, a remarkable progression occurred in the field of Human Genetics – the discipline that provides essential knowledge to understand human biology in normal and abnormal conditions. This was importantly attributable to the strong impetus of the Human Genome Project (HGP), the internationally collaborative effort to sequence the whole human genome comprising about 3.2 gigabases (Gb) and to identify all about 25,000 human genes. This project announced its success on April 14, 2003 – two years ahead its original schedule which was coincidentally the 50th anniversary of Watson and Crick's discovery of the double helical structure of DNA.

Since the HGP produced enormous data of human and model-organism genomes, during the project 'Bioinformatics' was originated from the necessity to manage, analyze, and understand the myriad amount of data using informatics tools. These data were deposited in public databases freely accessible via the World Wide Web. Before the end of HGP, the International Haplotype Mapping (HapMap) Project – an effort to produce a genome-wide map of common human genetic variations with the aim to speed the search for genes that contribute to common diseases – was launched in November 2002. This project has successfully generated the data of over 3 million human single nucleotide polymorphisms (SNPs) from geographically diverse populations.

The HGP has a great impact to the research and application in human molecular genetics, which finally leads to the origination of 'Genomics' – a new discipline that studies functions and interactions of all the genes in the genome. Functional Genomics is its division that involves the examination of global gene expression (transcriptome) and overall proteins (proteome) in the cells or their extrracellular milieu. It extension to the understanding of genetic contributions to human health gives rise to 'Genomic Medicine'. This new discipline not only provides an important insight into the biology of health and disease but also plays an increasingly important role in the development of new methods for prevention, diagnosis, monitoring, and treatment of diseases. It has started to fundamentally change the practice of medicine in the way that is not possible before and will revolutionize medicine in the 21st century. The importance of 'Genomic Revolution' has been emphasized in two series of review articles recently published in The New England Journal of Medicine (November 2002 – September 2003) and Mayo Clinic Proceeding (August 2002 – May 2004).
(Published in Siriraj Medical Journal. 2008;60:270-272.)

Variable-length haplotype construction for gene-gene interaction studies: a nonparametric classification approach.

By Anunchai Assawamakin, Nachol Chaiyaratana, Chanin Limwongse, Saravudh Sinsomros, Pa-thai Yenchitsomanus and Prakarnkiat Youngkong.

Genetic epidemiology is a research field that aims to identify genetic polymorphisms that are involved in disease susceptibility. In this article, a variable-length haplotype construction for gene--gene interaction (VarHAP) technique is proposed. The technique will involve nonparametric classification where haplotypes inferred from multiple single

nucleotide polymorphism (SNP) data are the classifier inputs. Usual candidate polymorphisms include restriction fragment-length polymorphisms (RFLPs), variable number of tandem repeats (VNTRs), and SNPs. In recent years, SNPs are the most common choices because of its simplicity and cost reduction in identification protocols. SNPs in diploid organisms are excellent biallelic genetic markers for various studies including genetic association, gene-gene interaction, and gene-environment interaction. The availability of multiple SNPs on the same gene can also lead to haplotype analysis where genotypes of interest can be phased into pairs of haplotypes. In this article, a VarHAP technique is proposed. The technique involves nonparametric classification where haplotypes inferred from multiple SNP data are the classifier inputs for case-control studies.
(Published in IEEE Eng Med Biol Mag. 2009;28:25-31.)

Simple, Efficient and Cost-Effective Multiplex Genotyping with MALDI-TOF Mass Spectrometry of Human Beta-Globin (HBB) Gene Mutations

By Wanna Thongnoppakhun, Surasak Jiamsap, Suganya Yongkiettrakul, Chompunut Karnjanakorn, Chanin Limwongse, Prapon Wilairat, Anusorn Vanasant, Nanyawan Rungroj and Pa-thai Yenchitsomanus

A number of common mutations in the hemoglobin beta (*HBB*) gene cause beta-thalassemia, a monogenic disease with high prevalence in certain ethnic groups. As there are 30 *HBB* variants that cover more than 99.5% of *HBB* mutant alleles in the Thai population, an efficient and cost-effective screening method is required. Three panels of multiplex primer extensions, followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry were developed. The first panel simultaneously detected 21 of the most common *HBB* mutations, while the second panel screened nine additional mutations, plus seven of the first panel for confirmation; the third panel was used to confirm three *HBB* mutations, yielding a 9-Da mass difference that could not be clearly distinguished by the previous two panels. The protocol was both standardized using 40 samples of known genotypes and subsequently validated in 162 blind samples with 27 different genotypes (including a normal control), comprising heterozygous, compound heterozygous, and homozygous beta-thalassemia. Results were in complete agreement with those from the genotyping results, conducted using three different methods overall. The method developed here permitted the detection of mutations missed using a single genotyping procedure. The procedure should serve as the method of choice for *HBB* genotyping due to its accuracy, sensitivity, and cost-effectiveness, and can be applied to studies of other gene variants that are potential disease biomarkers.
(Published in J Mol Diagn. 2009;11(4):334-46.)

Thailand mutation and variation database (ThaiMUT).

By Uttapong Ruangrit, Metawee Srikummool, Anunchai Assawamakin, Chumpol Ngamphiw, Suparat Chuechote, Vilasinee Thaiprasarnsup, Gallissara Agavatpanitch, Ekawat Pasomsab, Pa-thai Yenchitsomanus, Surakameth Mahasirimongkol, Wasun Chantratita, Prasit Palittapongarnpim, Bunyarit Uyyanonvara, Chanin Limwongse and Sissades Tongsima.

With the completion of the human genome project, novel sequencing and genotyping technologies had been utilized to detect mutations. Such mutations have continually been produced at exponential rate by researchers in various communities. Based on the

population's mutation spectra, occurrences of Mendelian diseases are different across ethnic groups. A proportion of Mendelian diseases can be observed in some countries at higher rates than others. Recognizing the importance of mutation effects in Thailand, we established a National and Ethnic Mutation Database (NEMDB) for Thai people. This database, named Thailand Mutation and Variation database (ThaiMUT), offers a web-based access to genetic mutation and variation information in Thai population. This NEMDB initiative is an important informatics tool for both research and clinical purposes to retrieve and deposit human variation data. The mutation data cataloged in ThaiMUT database were derived from journal articles available in PubMed and local publications. In addition to collected mutation data, ThaiMUT also records genetic polymorphisms located in drug related genes. ThaiMUT could then provide useful information for clinical mutation screening services for Mendelian diseases and pharmacogenomic researches.
(Published in Hum Mutat. 2008;29:E68-75.)

Metabolic enzymes, antioxidants, and cytoskeletal proteins are significantly altered in vastus lateralis muscle of K-depleted cadaveric subjects.

By Ratree Tavichakorntrakool, Pote Sriboonlue, Vitoon Prasongwattana, Anucha Puapairoj, Pa-thai Yenchitsomanus, Supachok Sinchaikul, Shui-Tein Chen, Chaisiri Wongkham and Visith Thongboonkerd.

Molecular mechanisms underlying myopathy caused by prolonged potassium (K) depletion remain poorly understood. In the present study, we examined proteome profile of vastus lateralis muscle obtained from cadaveric subjects who had K depletion (KD) (muscle K<80 micromol/g wet weight) compared to those who had no KD (NKD) (muscle K>or=80 micromol/g wet weight) (n=6 per group). Muscle proteins were extracted, resolved by 2-DE, and visualized with CBB-R250 stain. Spot matching and intensity analysis revealed significant changes in levels of 11 (6 increased and 5 decreased) protein spots in the KD group. Q-TOF MS and MS/MS analyses identified these altered proteins as metabolic enzymes (aldehyde dehydrogenase 1A1, uridine diphosphoglucose pyrophosphorylase, enolase 1, cytosolic malate dehydrogenase, and carbonic anhydrase III), antioxidants (peroxiredoxin-3 isoform b), cytoskeletal proteins (slow-twitch skeletal troponin I and myosin light chain 2), and others. These altered proteins are involved in many cellular functions, including bioenergetics, acid-base regulation, oxidative stress response, and muscle contractility. Validation was done by Western blot analysis, which confirmed the increased level of peroxiredoxin-3 and decreased level of troponin-I in the KD muscle. Linear regression analysis also revealed a significant negative correlation between peroxiredoxin-3 level and muscle K content ($r=-0.887$; $p<0.001$), as well as a significant positive correlation between troponin-I level and muscle K content ($r=0.618$; $p<0.05$). Our results implicate the important roles these altered proteins play in the development of KD-associated myopathy.
(Published in J Proteome Res. 2009;8:2586-93.)

K^+, Na^+, $Mg2^+$, $Ca2^+$, and water contents in human skeletal muscle: correlations among these monovalent and divalent cations and their alterations in K^+-depleted subjects.

By Ratree Tavichakorntrakool, Vitoon Prasongwattana, Pote Sriboonlue, Anucha Puapairoj, Chaisiri Wongkham,Thitichai Wiangsimma,Wattana Khunkitti, Pa-thai Yenchitsomanus and Visith Thongboonkerd.

None of previous studies had simultaneously analyzed the $K(^+)$, $Na(^+)$, $Mg(^{2+})$, and $Ca(^{2+})$ contents in human skeletal muscle. We examined extensively and simultaneously the levels of all these cations and examined water content in vastus lateralis and pectoralis major muscles in 30 northeastern Thai men who were apparently healthy but died from an accident. Specimen collection was performed within 6 h of death. We used atomic absorption or flame photometry to measure the level of muscle cation. Histopathology of muscle and kidney was also evaluated. $K(^+)$, $Na(^+)$, $Mg(^{2+})$, and $Ca(^{2+})$ contents in vastus lateralis were 84.74 +/- 1.50, 38.64 +/- 0.77, 7.58 +/- 0.17, and 0.94 +/- 0.06 micromol/g wet weight, respectively, whereas K(+), Na(+), and $Mg(^{2+})$ contents in pectoralis major were 82.83 +/- 1.54, 37.57 +/- 0.72, and 7.30 +/- 0.17 micromol/g wet weight, respectively. The water component was comparable in vastus lateralis and pectoralis major (78.66 +/- 0.41 and 78.09 +/- 0.56 %, respectively). Based on muscle $K(^+)$ levels, we divided the subjects into 2 main groups: $K(^+)$-depleted (KD) group ($K(^+)$ < 80 micromol/g wet weight; n = 7) and non-$K(^+)$-depleted (NKD) group ($K(^+)$ > or = 80 micromol/g wet weight; n = 23). In the KD muscle, $Na(^+)$ and $Ca(^{2+})$ levels were significantly higher, whereas the level of $Mg(^{2+})$ was significantly lower. Linear regression analysis showed significant correlations of $K(^+)$ and $Mg(^{2+})$ levels and between $Na(^+)$ and $Ca(^{2+})$. However, $K(^+)$ and $Mg(^{2+})$ had the negative correlation with $Na(^+)$ and $Ca(^{2+})$. Histopathologic examination showed no change in the KD muscles, whereas 29% (2 of 7) of the KD kidneys had vacuolization in proximal renal tubular cells. Our study not only provided the descriptive data but also implied the balance or homeostasis of these monovalent and divalent cations in their muscle pools.

(Published in Transl Res. 2007;150:357-66.)

Outputs

1. Publications

In Peer-reviewed International Journals

1. *Duangporn Ungsupravate, Nunghathai Sawasdee, Sookkasem Khositseth, Jing li, Wandee Udomchaiprasertkul, Siri Khoprasert, Reinhart A. F. Reithmeier, Pa-thai Yenchitsomanus. Impaired Trafficking and Intracellular Retention of Mutant Kidney Anion Exchanger 1 Proteins (G701D and A858D) Associated with Distal Renal Tubular Acidosis. Mol Membr Biol. 2010;27:92-103*
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27. *Janjuree Netsawang, Sansanee Noisakran, Sangkrab Sudsaward, Wiyada Wongwiwat, Somchai Thiemmeca, Panisadee Avirutnan, Chunya Puttikhunt, Watchara Kasinrerk, Rungtawan Sriburi, Nopporn Sittisombat, Pa-thai Yenchitsomanus, Prida Malasit, Thawornchai Limjindaporn. Dengue Virus Capsid Protein Potentiates Fas-mediated Apoptosis. การประชุมวิชาการ โครงการปริญญาเอกกาญจนาภิเษก ครั้งที่ 9 (RGJ-Ph.D. Congress IX). ชลบุรี, 4-6 เมษายน 2551.*
28. *Nattachet Plengvidhya, Watip Boonyasrisawat, Nalinee Chongjaroen, Prapaporn Jungtrakool, Sutin Sriussadaporn, Sathit Vannasaeng, Napatawn Banchuin, Pa-thai Yenchitsomanus. Mutations of Maturity-Onset Diabetes of the Young (MODY) Genes I Thais with Early-Onset Type 2 Diabetes Mellitus. Siriraj Scientific Congress – The 120th Anniversary of Siriraj Hospital: A New Era of Best Practice and Innovation. กรุงเทพ, 17-20 มีนาคม 2551.*
29. *Napat Songtawee, Nattachet Plengvidhya, Watip Boonyasrisawat, Napatawn Banchuin, Pa-thai Yenchitsomanus. Genetic Variabilities of Nkx6.1 and Nkx2.2 in Thais with Maturity-Onset Diabetes of the Young (MODY). Siriraj Scientific Congress – The 120th Anniversary of Siriraj Hospital: A New Era of Best Practice and Innovation. กรุงเทพ, 17-20 มีนาคม 2551.*
30. *Wanisa Salaemae, Nattachet Plengvidhya, Napatawn Banchuin, Pa-thai Yenchitsomanus. Association Analysis of Peroxisome Proliferator-Activated Receptor Gamma (PPARγ) Polymorphisms and Type 2 Diabetes Mellitus in Thai Patients. Siriraj Scientific Congress – The 120th Anniversary of Siriraj Hospital: A New Era of Best Practice and Innovation. กรุงเทพ, 17-20 มีนาคม 2551.*
31. *Nunghathai Sawasdee, Sookkasem Khositseth, Wandee Udomchaiprasertkul, Siri Khoprasert, Jing Li, Reinhart A.F. Reithmeier, Pa-thai Yenchitsomanus. Distal Renal Tubular Acidosis Caused by a Novel Compound Heterozygous A858D/G701D Mutations in Human Solute Carrier Family 4, Anion Exchanger, Member 1 (SLC4A1) Gene. Siriraj Scientific Congress – The 120th Anniversary of Siriraj Hospital: A New Era of Best Practice and Innovation. กรุงเทพ, 17-20 มีนาคม 2551.*
32. *Jatuporn Sujjitjoon, Suwattanee Kooptiwut, Titikan Chukijrungroat, Wiwit Tantibhedhyangkul, Naomoiy Semprasert, Nattachet Plengvidhya, Napatawn Banchuin, Pa-thai Yenchitsomanus. A Novel Hepatocyte Nuclear Factor-1α Frameshift (G554fsX556) Mutation Identified in a Thai MODY Family Decrease Transcriptional Activity on Human GLUT2 and Rat L-PK Promoters in Hela*

Cell Line. Siriraj Scientific Congress – The 120th Anniversary of Siriraj Hospital: A New Era of Best Practice and Innovation. กรุงเทพ, 17-20 มีนาคม 2551.

33. *Titikan Chukijrungroat, Suwattanee Kooptiwut, Jatuporn Sujjitjoon, Napat Songtawee, Namoiy Semprasert, Nattachet Plengvidhya, Napatawn Banchuin, Pa-thai Yenchitsomanus. Decreased Repressor Activity of Paired Box 4 (PAX4) R192 H Polymorphism Associated with Maturity-onset Diabetes of the Young (MODY) in Thai Patients. Siriraj Scientific Congress – The 120th Anniversary of Siriraj Hospital: A New Era of Best Practice and Innovation. กรุงเทพ, 17-20 มีนาคม 2551.*
34. *Nonglucksanawan Ritthisuntorn, Kanjana Leejinda, Nattachet Plengvidhya, Napatawn Banchuin, Pa-thai Yenchitsomanus. Genetic Variability of Visceral Adipose Tissue-derived Serpin (vaspin) associated with clinicalcharacteristics of type 2 diabetes in Thai patients. Siriraj Scientific Congress – The 120th Anniversary of Siriraj Hospital: A New Era of Best Practice and Innovation. กรุงเทพ, 17-20 มีนาคม 2551.*

3. Awards

1. *ผศ.ดร.นพ.ถาวรชัย ลิ้มจินดาพร ได้รับรางวัลผลนวัตกรรมดีเด่น ประเภททีมสหสาขาวิชาชีพ ชื่อผลงาน "การจัดการเรียนการสอนรายวิชาสร้างเสริมสุขภาพและจิตวิญญาณความเป็นมนุษย์ SIID 204" โครงการติดดาว ประจำปี 2552 (โล่รางวัล)*
2. *รศ.ดร.พญ.สุวัฒณี คุปติวุฒิ ได้รับรางวัลผลนวัตกรรมดีเด่น ประเภทหน่วยงาน ชื่อผลงาน "แบบจำลองช่วยในการศึกษาแบบตรวจการตั้งครรภ์ โดยวิธี Two-site sandwich immunoassay" โครงการติดดาว ประจำปี 2552 (โล่รางวัล)*
3. *ผศ.ดร.นพ.ถาวรชัย ลิ้มจินดาพร ได้รับรางวัลผลงานวิจัยดีเด่นทางปรีคลินิก คณะแพทยศาสตร์ศิริราชพยาบาล ประจำปี 2552 ผลงานวิจัยเรื่อง "Nuclear localization of dengue virus capsid protein is required for Daxx interaction and apoptosis." สนง. รองคณบดีฝ่ายวิจัย คณะแพทยศาสตร์ศิริราชพยาบาล (โล่รางวัล)*
4. *รศ.ดร.พญ.สุวัฒณี คุปติวุฒิ ได้รับรางวัลผลงานวิจัยดีเด่นทางปรีคลินิก คณะแพทยศาสตร์ศิริราชพยาบาล ประจำปี 2552 ผลงานวิจัยเรื่อง "Functional defect of truncated hepatocyte nuclear factor-1 alpha (G554fsX556) associated with maturity-onset diabetes of the young" สนง. รองคณบดีฝ่ายวิจัย คณะแพทยศาสตร์ศิริราชพยาบาล (โล่รางวัล)*
5. *ศ.ดร.เพทาย เย็นจิตโสมนัส ได้รับรางวัลอาจารย์ที่ปรึกษาวิทยานิพนธ์หลักของนักศึกษา นางสาววทิพย์ บุณยศรีสวัสดิ์ ประเภทรางวัลวิทยานิพนธ์ (ระดับปริญญาเอก) ระดับดี ประจำปี 2552 เรื่อง "ยีนของมนุษย์ที่เกี่ยวข้องกับการเกิดโรคเบาหวานชนิดที่พบในผู้ป่วยที่อายุน้อยและการเกิดภาวะ แทรกซ้อนของหลอดเลือดหัวใจในโรคเบาหวาน" สภาวิจัยแห่งชาติ, 2553 (โล่รางวัล).*
6. *นางสาววทิพย์ บุณยศรีสวัสดิ์ ได้รับรางวัลวิทยานิพนธ์ (ระดับปริญญาเอก) ระดับดี ประจำปี 2552 เรื่อง "ยีนของมนุษย์ที่เกี่ยวข้องกับการเกิดโรคเบาหวานชนิดที่พบในผู้ป่วยที่อายุน้อยและการเกิดภาวะแทรกซ้อนของหลอดเลือดหัวใจในโรคเบาหวาน" สภาวิจัยแห่งชาติ, 2553 (ประกาศนียบัตร).*
7. *ผศ.ดร.นพ.ถาวรชัย ลิ้มจินดาพร ได้รับรางวัลผลงานวิจัยดีเยี่ยมแบบโปสเตอร์ เรื่อง Interaction of Dengue Virus Envelop Protein with Endoplasmic Reticulum-Resident*

Chaperones Facilitates Dengue Virus Production. ในการประชุมนักวิจัยรุ่นใหม่..พบ..เมธีวิจัยอาวุโส สกว. ระหว่างวันที่ 15-17 ตุลาคม 2552 (ประกาศนียบัตร).

8. *ดร.มุทิตา จุลกิ่ง ได้รับรางวัลที่ 2 จากการประกวดผลงานวิจัย Oral Presentation กลุ่ม อาจารย์ข้าราชการและบุคคลทั่วไป เรื่อง Determination of Genetic Variation of Galectin-3 in Cholangiocarcinoma ในงานประชุมวิชาการ ครั้งที่ 25 ประจำปี 2552 ระหว่างวันที่ 13-16 ตุลาคม 2552 (ประกาศนียบัตร).*
9. *นางสาวอำภา ยาสมุทร์ นำเสนอผลงานและได้รับการประเมินให้เป็นผลงานวิจัยดี กลุ่มสาขาวิทยาศาสตร์สุขภาพ เรื่อง การแสดงออกของยีนที่เกี่ยวข้องกับกระบวนการออโตเฟจีในเซลล์ตับที่ติดเชื้อไวรัสเด็งกี่ ในการประชุมเสนอผลงานวิจัยระดับบัณฑิตศึกษาแห่งชาติครั้งที่ 14 ที่มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าพระนครเหนือ ระหว่างวันที่ 10-11 กันยายน 2552 (ประกาศนียบัตร).*
10. *นางสาวสุชาดา เสียงใส นักศึกษาปริญญาโท ของ ศ.ดร. เพทาย เย็นจิตโสมนัส ได้รับรางวัลวิทยานิพนธ์ชมเชย เรื่อง "Identification of Host Cellular Proteins Interacting with Dengue Viral Nonstructural Protein 1 in Dengue Virus Infected Human Kidney Cell Line" ในโครงการรางวัลวิทยานิพนธ์ดีเด่น ระดับปริญญาโท ประจำปี 2551, บัณฑิตวิทยาลัย มหาวิทยาลัยมหิดล, 2552 (ประกาศนียบัตร).*
11. *ศ.ดร.เพทาย เย็นจิตโสมนัส ได้รับรางวัลอาจารย์ที่ปรึกษาวิทยานิพนธ์หลักของนักศึกษา นางสาวสุชาดา เสียงใส ประเภทรางวัลวิทยานิพนธ์ชมเชย เรื่อง "Identification of Host Cellular Proteins Interacting with Dengue Viral Nonstructural Protein 1 in Dengue Virus Infected Human Kidney Cell Line" ในโครงการรางวัลวิทยานิพนธ์ดีเด่น ระดับปริญญาโท ประจำปี 2551, บัณฑิตวิทยาลัย มหาวิทยาลัยมหิดล, 2552 (โล่รางวัล).*
12. *ผศ.ดร.นพ.ถาวรชัย ลิ้มจินดาพร ได้รับรางวัลผลงานวิจัยดีเด่นปรีคลินิก เรื่อง "Interaction of dengue virus envelope protein with endoplasmic reticulum-resident chaperones facilitated dengue virus production" คณะแพทยศาสตร์ศิริราชพยาบาล, 2552 (โล่รางวัล)*
13. *ผศ.ดร.นพ.ถาวรชัย ลิ้มจินดาพร ได้รับรางวัลผลนวัตกรรมดีเด่น ประเภททีมฯ ชื่อการจัดการเรียนการสอนรายวิชาสร้างเสริมสุขภาพและจิตวิญญาณความเป็นมนุษย์ SIID 204, งานพัฒนาคุณภาพ คณะแพทยศาสตร์ศิริราชพยาบาล, 2552 (โล่รางวัล).*
14. *นางสาวประภาพร จึงตระกูล นักศึกษาระดับปริญญาเอก ของ ศ.ดร. เพทาย เย็นจิตโสมนัส ได้รับรางวัลดีเด่นประเภทการนำเสนอผลงานแบบปากเปล่า เรื่อง "Genetic Variations of Adiponectin in Thais with Type 2 Diabetes" ในการประชุมวิชาการโครงการปริญญาเอกกาญจนาภิเษก ครั้งที่ 10 (RGJ-Ph.D. Congress X) ปี 2552, โครงการปริญญาเอกกาญจนาภิเษก สำนักงานกองทุนสนับสนุนการวิจัย, 2552 (ประกาศนียบัตร).*
15. *นายณฐพล ดวงธรรม นักศึกษาระดับปริญญาโท ของ ผศ.ดร.นพ.ถาวรชัย ลิ้มจินดาพร ได้รับรางวัลดีเด่นประเภทการนำเสนอผลงานแบบ Poster presentation กลุ่ม 2 สาขาวิทยาศาสตร์ชีวภาพ เทคโนโลยีชีวภาพ และวิทยาศาสตร์ศึกษา ในการประชุมวิชาการวิทยาศาสตร์วิจัยครั้งที่ 2 วิทยาศาสตร์สร้างปัญญา (Science Research Conference 2nd), คณะวิทยาศาสตร์ มหาวิทยาลัยนเรศวร, 2552 (ประกาศนียบัตร).*
16. *นางสาวหนึ่งหทัย สวัสดี ผู้ช่วยนักวิจัย ของ ศ.ดร. เพทาย เย็นจิตโสมนัส ได้รับรางวัลดีเด่น ลำดับที่ 2 ประเภทการนำเสนอผลงานแบบ Poster Presentation เรื่อง "Visualization of Interaction*

between Kidney Anion Exchanger 1 (kAE1) and Adaptor-related Protein Complex 1 μ1A Subunit (AP1M1) by Protein Fragment Complementary Assay (PCA)" ในงานประชุมวิชาการ "Third Annual Symposium of the Protein Society of Thailand", สถาบันวิจัยจุฬาภรณ์, 2551 (ประกาศนียบัตร).

17. *นางสาวฐิติการต์ ชูกิจรุ่งโรจน์ ได้รับรางวัลชนะเลิศ ประเภท Poster Presentation เรื่อง "Decrease repressor activity of paired box 4 (PAX4) R192H polymorphism associated with maturity-onset diabetes of the young (MODY) in Thai patients" ในการประกวดเสนอผลงานวิจัยระดับบัณฑิตศึกษาศิริราช (Siriraj Graduate Research conference) ในการประชุมวิชาการ 120 ปีศิริราช ประจำปี 2551 (ครั้งที่ 47) (ประกาศนียบัตร).*
18. *นางสาวแจนจุรีย์ เนตรสว่าง นักศึกษาระดับปริญญาเอก โดยมี ศ.ดร. เพทาย เย็นจิตโสมนัส เป็นอาจารย์ที่ปรึกษาร่วม ได้รับรางวัลดีเด่นประเภทการนำเสนอผลงานแบบโปสเตอร์ เรื่อง "Dengue Virus Capsid Protein Potentiates Fas-Mediated Apoptosis" ในการประชุมวิชาการโครงการปริญญาเอกกาญจนาภิเษก ครั้งที่ 9 (RGJ-Ph.D. Congress IX) ปี 2551, โครงการปริญญาเอกกาญจนาภิเษก สำนักงานกองทุนสนับสนุนการวิจัย, 2551 (ประกาศนียบัตร).*
19. *นางสาวหนึ่งหทัย สวัสดี นักศึกษาปริญญาโท ของ ศ.ดร. เพทาย เย็นจิตโสมนัส ได้รับรางวัลวิทยานิพนธ์ชมเชย เรื่อง "Interaction, trafficking, and subcellular localization of mutant kidney anion exchanger 1 (kAE1) proteins in cultured human embryonic kidney 293 (HEK 293) cells" ในโครงการรางวัลวิทยานิพนธ์ดีเด่น ระดับปริญญาโท ประจำปี 2550, บัณฑิตวิทยาลัย มหาวิทยาลัยมหิดล, 2551 (ประกาศนียบัตร).*
20. *ศ.ดร. เพทาย เย็นจิตโสมนัส ได้รับรางวัลอาจารย์ที่ปรึกษาวิทยานิพนธ์หลักของนักศึกษา นางสาวหนึ่งหทัย สวัสดี ประเภทรางวัลวิทยานิพนธ์ชมเชย เรื่อง "Interaction, trafficking, and subcellular localization of mutant kidney anion exchanger 1 (kAE1) proteins in cultured human embryonic kidney 293 (HEK 293) cells" ในโครงการรางวัลวิทยานิพนธ์ดีเด่น ระดับปริญญาโท ประจำปี 2550, บัณฑิตวิทยาลัย มหาวิทยาลัยมหิดล, 2551 (โล่รางวัล).*
21. *ศ.ดร. เพทาย เย็นจิตโสมนัส ได้รับรางวัลบุคลากรดีเด่น ประจำปี 2550, คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล, 2551 (โล่รางวัล).*
22. *คณะผู้วิจัย และ ศ.ดร. เพทาย เย็นจิตโสมนัส ได้รับรางวัลผลงานวิจัย ระดับดีเยี่ยม ประจำปี 2550 เรื่อง "งานวิจัยไข้เลือดออกและไวรัสเด็งกี" สภาวิจัยแห่งชาติ, 2551 (ประกาศนียบัตร).*
23. *ศ.ดร. เพทาย เย็นจิตโสมนัส และคณะผู้วิจัย ได้รับรางวัลผลงานวิจัย ระดับชมเชย ประจำปี 2550 เรื่อง "การศึกษาอณูพันธุศาสตร์ของโรคไตผิดปกติในการขับกรด"สภาวิจัยแห่งชาติ, 2551 (ประกาศนียบัตร).*

4. Student Graduation

Doctoral degree

1. *Student name:* *Miss Wiyada Wongwiwat*
   Thesis title: *The Roles of an Endoplasmic Reticulum Chaperoning Protein - immunoglobulin Binding Protein (BiP) in Dengue Virus Infection*
   Date of graduation: *April 7, 2010*
   Advisory committee: *Prida Malasit, M.D., F.R.C.P., UK*
   Thawornchai Limjindaporn, MD. Ph.D.
   Pa-thai Yenchitsomanus, Ph.D.

2. *Student name:* *Miss Janjuree Netsawang*
   Thesis title: *The Role of Dengue Virus Capsid Protein in Apoptosis*
   Date of graduation: *February 25, 2010*
   Advisory committee: *Prida Malasit, M.D., F.R.C.P., UK*
   Thawornchai Limjindaporn, MD., Ph.D.
   Pa-thai Yenchitsomanus, Ph.D.

3. *Student name:* *Miss Watip Boonyasrisawat*
   Thesis title: *Novel Human Genes Involving in Maturity Onset Diabetes of the Young and in Diabetic Cardiovascular Complication*
   Date of graduation: *January 31, 2008*
   Advisory committee: *Pa-thai Yenchtisomanus, Ph.D.*
   Napatawn Banchuin, M.D., Ph.D.
   Nattachet Plengvidhya, M.D

Master degree

1. *Student name:* *Miss Umpa Yasamut*
   Thesis title: *Autophagic Gene Expression Profiling in Dengue Virus-infected HepG2 Cells*
   Date of graduation: *May 20, 2010*
   Advisory committee: *Pa-thai Yenchitsomanus, Ph.D.*
   Thawornchai Limjindaporn, MD., Ph.D.
   Chatchawan Srisawat, M.D., Ph.D.

2. *Student name:* *Mr. Atthapan Morchang*
   Thesis title: *Apoptotic Gene Expression Profiling in Dengue Virus-infected HepG2 Cells*
   Date of graduation: *May 20, 2010*
   Advisory committee: *Thawornchai Limjindaporn, MD., Ph.D..*
   Pa-thai Yenchitsomanus, Ph.D.
   Chatchawan Srisawat, M.D., Ph.D.

3. *Student name:* *Miss Aussara Panya*
   Thesis title: *Human ZHX2 and γ-globin Gene Expression in Erythroleukemic Cell Line*
   Date of graduation: *January 28, 2010*
   Advisory committee: *Chayanon Peerapittayamongkol, M.D., Ph.D.*
   Pa-thai Yenchitsomanus, Ph.D.
   Chatchawan Srisawat, M.D., Ph.D.

4. *Student name:* *Mr. Natapol Duangtum*
Thesis title: *Interaction between Human Kidney Anion Exchanger 1 (kAE1) and Kinesin Family Member 3B (KIF3B) in Human Kidney Cells*
Date of graduation: *April 1, 2009*
Advisory committee: *Thawornchai Limjindaporn, M.D., Ph.D.*
Pa-thai Yenchitsomanus, Ph.D.
Chatchawan Srisawat, M.D.,Ph.D.

5. *Student name:* *Miss Jatuporn Sujjitjoon*
Thesis title: *Functional Study of a Novel Pathogenic Mutation (G554fsX556) of Hepatocyte Nuclear Factor-1Alpha in Thai Patients with Maturity-Onset Diabetes of the Young*
Date of graduation: *April 10, 2008*
Advisory committee: *Suwattanee Kooptiwut, M.D., Ph.D.*
Napatawn Banchuin, M.D., Ph.D.
Pa-thai Yenchitsomanus, Ph.D.
Nattachet Plengvidhya, M.D.

6. *Student name:* *Miss Titikan Chukijrungroat*
Thesis title: *Functional Study of Paired Box 4 (Pax4) R192H Single Nucleotide Polymorphism Identified in Thais with Maturity-Onset Diabetes of the Young (MODY) and Early-onset Type 2 Diabetes Mellitus*
Date of graduation: *April 10, 2008*
Advisory committee: *Suwattanee Kooptiwut, M.D., Ph.D.*
Nattachet Plengvidhya, M.D.
Pa-thai Yenchitsomanus, Ph.D.
Supornpim Chearskul, M.D.

7. *Student name:* *Miss Pairoa Praihirunkit*
Thesis title: *Association Studies of Calgranulin Genes and Renal Stone Formation in Northeastern Thai Population*
Date of graduation: *November 13, 2007*
Advisory committee: *Varaporn Akkarapatumwong, Ph.D.*
Pa-thai Yenchtisomanus, Ph.D.
Wanna Thongnoppakhun, Ph.D.
Surapon Piboonpocanun, Ph.D.

8. *Student name:* *Miss Phuttawadee Phuengcharoen*
Thesis title: *Search for Protein Interacting with Kidney Isoform of Anion Exchanger 1 (kAE1) by GAL4-based Yeast Two-Hybrid*
Date of graduation: *October 22, 2007*
Advisory committee: *Varaporn Akkarapatumwong, Ph.D.*
Pa-thai Yenchtisomanus, Ph.D.
Witoon Tirasophon, Ph.D.
Surapon Piboonpocanun, Ph.D.

5. Current Students

Doctoral degree

1. *Student name:* *Miss Prapaporn Jungtrakoon*
   Thesis title: *Identification of Gene Responsible for Matuality-Onset-Diabetis of the Young (MODY) in Thais by Genome Y Linkage Analysis*
   Advisor: *Pa-thai Yenchtisomanus, Ph.D.*

2. *Student name:* *Miss Oranud Praditsap*
   Thesis title: *Genome-Wide Linkage Study: Identification of Genes Involving in Renal Stone Formation in Northeastern Thai Population*
   Advisor: *Pa-thai Yenchtisomanus, Ph.D.*

3. *Student name:* *Miss Nalin-on Nuiplot*
   Thesis title: *Identification of Protein Interacting with Kidney Isoform of Anion Exchanger 1 (kAE1)*
   Advisor: *Varaporn Akkarapatumwong, Ph.D.*

4. *Student name:* *Miss Sasiprapa Khunchai*
   Thesis title: *The Role of Dengue Virus Nonstructural Protein 5 in Cytokine Production and Inflammatory Response of Dengue Virus Infection*
   Advisor: *Pa-thai Yenchtisomanus, Ph.D.*

5. *Student name:* *Miss Jatuporn Sujjitjoon*
   Thesis title: *Role of Paired Box 4 (PAX4) Gene in the Pathogenesis of Diabetes Mellitus*
   Advisor: *Pa-thai Yenchtisomanus, Ph.D.*

6. *Student name:* *Mr. Natapol Duangtum*
   Thesis title: *Roles of Kinesin in Kidney Anion Exchanger 1 (kAE1) Trafficking*
   Advisor: *Pa-thai Yenchtisomanus, Ph.D.*

7. *Student name:* *Miss Aussara Panya*
   Thesis title: *In entitling*
   Advisor: *Pa-thai Yenchtisomanus, Ph.D.*

8. *Student name:* *Miss Patta Phumesin*
   Thesis title: *In entitling*
   Advisor: *Pa-thai Yenchtisomanus, Ph.D.*

9. *Student name:* *Miss Umpa Yasamut*
   Thesis title: *In entitling*
   Advisory committee: *Thawornchai Limjindaporn, M.D., Ph.D.*

10. *Student name:* *Mr. Atthapan Morchang*
    Thesis title: *In entitling*
    Advisory committee: *Thawornchai Limjindaporn, M.D., Ph.D.*

Master Degree Students:

1. *Student name:* *Miss Thanyaporn Dechtaweewat*
   Thesis title: *The Role of hnRNP C1/C2 and Dengue Virus NS1 Association in Dengue Virus Replication*
   Advisor: *Pa-thai Yenchtisomanus, Ph.D.*

2. *Student name:* *Mrs. Aroonroong Suttitheptumrong*
   Thesis title: *RNA Aptamer against Dengue Virus Capsid Protein*
   Advisor: *Thawornchai Limjindaporn, MD., Ph.D.*

3. *Student name:* *Miss Nichapatr Saokaew*
   Thesis title: *Selection and Production of Human Scfv Specific to Dengue Virus Proteins*
   Advisor: *Pa-thai Yenchtisomanus, Ph.D.*

4. *Student name:* *Miss Rochanawan Sootichote*
   Thesis title: *Role of Toll-Like Receptor 4 in Paclitaxel Resistance of Breast Cancer*
   Advisor: *Chanitra thuwajit, Ph.D.*

5. *Student name:* *Mr. Chaiyadol Tuntasit*
   Thesis title: *The Role of Dengue Envelope Protein in Cellular Apoptosis*
   Advisor: *Thawornchai Limjindaporn, MD., Ph.D.*

6. *Student name:* *Miss Pornnipa Mahawong*
   Thesis title: *The Effect of Estrogen on Oxidative Stress and Endoplasmic Reticulum Stress (ER Stress) in impaired Pancreatic Beta Cell Function with High Glucose*
   Advisor: *Suwattanee Kooptiwut, M.D., Ph.D.*

7. *Student name:* *Miss Keerati Wanchai*
   Thesis title: *Effect of Estrogen on Angitensin II Receptor type 1 Expression in Impaired Pancreatic Beta Cell Function with High Glucose*
   Advisor: *Suwattanee Kooptiwut, M.D., Ph.D.*

8. *Student name:* *Mr. Phongphet Benjaponwattana*
   Thesis title: *Determination of Sequence Variations in Candidate Genes for Renal Stone Formation*
   Advisor: *Varaporn Akkarapatumwong, Ph.D.*

Conference Arrangements

Our TRF-CHE Senior Research Scholar Group together with The Genetic Society of Thailand arranged 3 conferences:

1. ***"Molecular Genetics of Complex and Common Genetic Diseases"*** *on September 29, 2008, at the Royal River Hotel (near the Thonburi Bridge), Bangkok. There were about 204 participants in this conference; 166 registered participants and 38 members of our TRF-CHE Senior Research Scholar Group.*

   The topics of lectures included:

   1. *Molecular genetics of complex and common genetic diseases by Prof. Dr. Pa-thai Yenchisomanus*
   2. *Bioinformatics in human genetics and genomics by Dr. Sissades Tongsima*
   3. *Molecular genetics of diabetes mellitus by Lect. Dr. Nattachet Plengvidhya*
   4. *Molecular genetics of diabetic complications by Dr. Watip Tangjittipokin*
   5. *Molecular genetics of systemic lupus erythrematosus by Assoc. Prof. Dr. Nattiya Hirankarn*
   6. *Molecular genetics of autism by Assoc. Prof. Dr. Pornprot Limprasert*
   7. *Molecular genetics of stroke by Lect. Dr. Manop Pithukpakorn*
   8. *Molecular genetics of cancers (part I) by Prof. Dr. Pornchai O-charoenrat*
   9. *Molecular genetics of cancers (part II) by Assoc. Prof. Dr. Pimpichaya Patmasiriwat*
   10. *Molecular genetics of susceptibility to infectious diseases by Lect. Dr. Prapat Suriyaphol*

2. ***"Human Genomics and Molecular Biology 2009"*** *on December 14, 2009, at the S.D. Avenue Hotel, Bangkok. There were about 207 participants in this conference; 166 registered participants and 41 members of our TRF-CHE Senior Research Scholar Group.*

   The topics of lectures included:

   1. *Human Genomics and Molecular Biology by Prof. Dr. Pa-thai Yenchisomanus*
   2. *Genomic Analysis and Bioinformatics by Lect. Dr. Prapat Suriyapol*
   3. *Genetic and Genomic Analysis in Kidney Stone by Lect. Dr. Nanyawan Rungroj*
   4. *Genetic and Genomic Analysis in Diabetes by Dr. Watip Tangjittipokin*
   5. *Transcription Factor and Promoter Analysis in Human Diseases by Assist. Prof. Dr. Suwattanee Kooptiwut*
   6. *Transcriptomic Analysis and Human Diseases by Asst. Prof. Chatchawan Srisawat*
   7. *Proteomic Analysis in Human Cancers by Dr. Mutita Junking*
   8. *Protein-Protein Interaction Analysis and Human Diseases by Assist. Prof. Dr. Thawornchai Limjindaporn*

3. *"**Human Genomics and Molecular Biology 2010**" on December 13, 2010, at the S.D. Avenue Hotel, Bangkok. There were about 145 participants in this conference; 93 registered participants and 52 members of our TRF-CHE Senior Research Scholar Group.*

The topics of lectures included:

1. *Overview on Research Activities of TRF-CHE Senior Research Scholar Group by Prof. Dr. Pa-thai Yenchisomanus*
2. *Kidney Stone Disease in Northeastern Thai Population by Prof. Dr. Pa-thai Yenchisomanus*
3. *Genetic Association Study of Kidney Stone Disease by Lect. Dr. Nanyawan Rungroj*
4. *Genome-wide Linkage Analysis of Kidney Stone Disease by Miss Oranud Praditsup*
5. *Genetics and Clinical Intervention of Diabetes by Assoc. Prof. Dr. Nattachet Plengvidhya*
9. *Molecular Genetic Study of Diabetes Diabetes by Dr. Watip Tangjittipokin*
6. *Type 2 Diabetes and Adiponectin by Miss Prapaporn Jungtrakoon*
7. *Viral Protein Interaction with Molecular Chaperones to Regulate Infection by Assist. Prof. Dr. Thawornchai Limjindaporn*
8. *Cell Death Gene Expression Profiling: Role of RIPK2 and p38 MAPK in Dengue Virus-Mediated Apoptosis by Assist. Prof. Dr. Thawornchai Limjindaporn*
9. *Nuclear Localization of Dengue Virus Capsid Protein Required for Daxx Interaction and Apoptosis by Lect. Dr. Janjuree Netsawang*
10. *Molecular Virology and Clinical Manifestations of Hepatitis B Virus Infection by Lect. Dr. Watcharasak Chotiyaputta*
11. *Evolution of Therapeutic Antibody by Prof. Dr. Wanpen Chaicumpa*
12. *Heterosubtypic Immunity to Influenza Mediated by Liposome Adjuvanted H5N1 Recombinant Protein Vaccines by Dr. Kanyarat Thueng-In*
13. *Human Transbody: The Novel Paradigm for Fighting Infectious Disease by Dr. Ornnuthchar Poungpair*

Appendix I

Publications in peer-reviewed international journals

Appendix II

Publications in peer-reviewed national journals

Appendix III

Abstracts in international proceedings

Appendix IV

Abstracts of international oral presentations

Appendix V

Abstracts of national oral presentations

Appendix VI

Abstracts of international poster presentations

Appendix VII

Abstracts of national poster presentations

Appendix VIII

Awards

Author Query Sheet

Manuscript Information	
Journal Acronym	TMBC
Volume and issue	27
Author name	Yenchitsomanus
Manuscript No. (if applicable)	459311

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Impaired trafficking and intracellular retention of mutant kidney anion exchanger 1 proteins (G701D and A858D) associated with distal renal tubular acidosis

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Abstract

Novel compound heterozygous mutations, G701D, a recessive mutation, and A858D, a mild dominant mutation, of human *solute carrier family 4*, *anion exchanger*, *member 1* (*SLC4A1*) were identified in two pediatric patients with distal renal tubular acidosis (dRTA). To examine the interaction, trafficking, and cellular localization of the wild-type and two mutant kidney AE1 (kAE1) proteins, we expressed the proteins alone or together in human embryonic kidney (HEK) 293T and Madin-Darby canine kidney (MDCK) epithelial cells. In individual expressions, wild-type kAE1 was localized at the cell surface of HEK 293T and the basolateral membrane of MDCK cells. In contrast, kAE1 G701D was mainly retained intracellularly, while kAE1 A858D was observed intracellularly and at the cell surface. In co-expression experiments, wild-type kAE1 formed heterodimers with kAE1 G701D and kAE1 A858D, and promoted the cell surface expression of the mutant proteins. The co-expressed kAE1 G701D and A858D could also form heterodimers but showed predominant intracellular retention in HEK 293T and MDCK cells. Thus, impaired trafficking of the kAE1 G701D and A858D mutants would lead to a profound decrease in functional kAE1 at the basolateral membrane of α-intercalated cells in the distal nephron of the patients with dRTA.

Keywords: *SLC4A1, anion exchanger 1 (AE1), Band 3, distal renal tubular acidosis (dRTA), membrane protein trafficking*

Introduction

Solute carrier family 4, *anion exchanger*, *member 1* (*SLC4A1*) or *anion exchanger 1* (*AE1*) encodes both erythroid AE1 (eAE1) and kidney AE1 (kAE1), which mediates chloride/bicarbonate (Cl^-/HCO^-_3) exchange in red cells and acid-secreting ∝-intercalated cells of renal collecting duct, respectively. Erythroid AE1 also regulates red cell morphology through interactions with the underlying membrane cytoskeleton [1,2]. Thus, *SLC4A1* mutations can result in morphological abnormalities of red cells, anion transport defects in red cells, and/or impaired acid secretion in the kidney causing distal renal tubular acidosis (dRTA) [3,4]. The *SLC4A1* mutations have been found to be associated with either autosomal dominant (AD) or autosomal recessive (AR) dRTA, determined by mutational sites and trafficking behaviors of the mutant proteins [5,6]. AR dRTA may result from either homozygosity of a single *SLC4A1* mutation or compound heterozygosity of two different *SLC4A1* mutations. Several reported genotypes include: G701D/G701D, SAO/G701D, SAO/delV850, SAO/A858D, delV850/delV850, V488M/V488M, G701D/S773P and SAO/Q759H [7–13]. The *SLC4A1* G701D recessive mutation (band 3 Bangkok I), first described in a Thai kindred [11], was frequently observed in Southeast Asia in both

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homozygote and compound heterozygote with Southeast Asian ovalocytosis (SAO) mutation [5,7,8,12,14]. Heterozygous individual expressing the A858D mutation were symptomless but unable to acidify their urine when challenged with frusemide/fludrocortisone and were classified as incomplete dRTA, although this test is not the 'gold standard' as oral ammonium chloride administration test for dRTA. The mild dominant phenotype for the A858D mutation is in contrast to the complete dRTA induced by other dominant dRTA mutations such as R589H. Recently, we have identified two pediatric patients with AR dRTA caused by novel compound heterozygous *SLC4A1* G701D/A858D mutations in a Thai family [14,15]. To better understand the molecular mechanism of the disease caused by this compound heterozygous *SLC4A1* mutations, we investigated the interaction, trafficking and cellular localization of mutant kidney AE1 (kAE1) proteins (G701D and A858D) expressed alone or together in human embryonic kidney (HEK) 293T and Madin-Darby canine kidney (MDCK) epithelial cells.

Materials and methods

Plasmid constructs and mutagenesis

Plasmid constructs and mutagenesis for experiments in HEK 293T cells. Recombinant plasmids, pcDNA3-kAE1 WT-His, pcDNA3-kAE1 WT-HA, pcDNA3-kAE1 WT-Myc, pcDNA3-kAE1 G701D-His, pcDNA3–kAE1 G701D-HA, and pcDNA3-kAE1 G701D-Myc were constructed as previously described [16]. pcDNA3-kAE1 A858D-His, pcDNA3-kAE1 A858D-HA and pcDNA3 kAE1 A858D-Myc were generated by site-directed mutagenesis.

Plasmid constructs and mutagenesis for experiments in MDCK epithelial cells. Retroviral expression plasmid pFBneo-kAE1HA557 was constructed by shuttling the cDNA encoding the entire human kidney anion exchanger carrying an external hemagglutinin (HA) tag inserted into position 557 [17] into *Xho* I site of the retroviral expression vector pFBneo (Stratagene, La Jolla, CA). The pFBneo-kAE1HA557 mutants were constructed using Stratagene QuickChange site-directed mutagenesis kit and confirmed by automated sequencing (ACGT, Toronto, Canada).

Expression studies in HEK 293T cells

Cell culture and transfection. Cultured HEK 293T cells were transfected with 1 μg of recombinant plasmid DNA per 3.5 cm well either by using Lipofectamine 2000 (Invitrogen, USA) or DEAE-dextran method as previously described [16]. The HEK 293T cells were individually transfected with each of the recombinant constructs, and were also co-transfected with pcDNA-kAE1 WT-His and pcDNA-kAE1 G701D-HA (or pcDNA-kAE1 A858D-HA), pcDNA-kAE1 G701D-His and pcDNA-kAE1 A858D-HA, pcDNA-kAE1 G701D-HA and pcDNA-kAE1 A858D-His.

SDS-PAGE and Western blot analysis. The kAE1 and mutant proteins were analyzed by 10% SDS-PAGE and Western blot analysis as previously described [16]. Briefly, the transfected HEK 293T cells were solubilized with lysis buffer [1mM EDTA, 0.5% (v/v) Igepal (Nonidet P-40 detergent), 150 mM NaCl, 0.2% (v/v) bovine serum albumin, 10 mM Tris-HCl pH 7.5 and protease inhibitor cocktail]. After centrifugation to remove insoluble material, whole cell extracts were subjected to SDS gel electrophoresis and electroblotted to nitrocellulose membranes. The proteins were detected by incubating the membranes with primary antibodies (anti-His, anti-HA or anti-Myc), then with the secondary antibody conjugated to horseradish peroxidate (HRP), and detected with SuperSignal West Pico Chemiluminescent Substrated (Pierce, USA) plus Western Blotting Detection System. The chemiluminescence signal was captured by exposing the membrane to an X-ray film, which was then developed to reveal the immunoreactive bands.

Affinity co-purification. HEK 293T cells were individually transfected with the plasmid constructs or were co-transfected with four different pairs of the constructs described above. Two days after transfection, the cells were washed, lysed, and the lysate incubated with 40 μl of washed Co^{2+} chelate resins (BD Bioscience, USA) at 4°C for 12–16 h as previously described [16]. After incubation, the resin was collected and washed thoroughly. 6×Histidine-tagged-kAE1 proteins that bound to Co^{2+} chelate resins were eluted with 2× SDS-PAGE sample loading buffer containing 2% (v/v) 2-mercaptoethanol and heated at 65°C for 5 min. The samples were subjected to electrophoresis on 10% SDS-PAGE and the co-purified kAE1-HA proteins were detected by Western blot analysis using anti-HA antibody and HRP-conjugated secondary antibody.

Co-immunoprecipitation. The transfected and co-transfected HEK 293T cells were prepared as

mentioned in the previous section and processed as previously described [16]. The non-specific binding proteins in the cell lysate were removed by Protein G-Sepharose beads, recovered by centrifugation. The supernatant were then used for immunoprecipitation by adding anti-6×His antibody and then Protein G-Sepharose beads. The mixture was incubated at 4°C with shaking for 12–16 h and then the beads were collected and washed thoroughly. Protein complexes were eluted with 2× SDS-PAGE sample loading buffer and heated at 65°C for 5 min. The protein complexes were analyzed by 10% SDS-PAGE and Western blot method using anti-HA antibody and HRP conjugated secondary antibody.

Immunofluorescence and confocal microscopy. HEK 293T cells were grown on cover glasses for transfection and co-transfection with the recombinant plasmid constructs. To examine cellular localization of HA-tagged or Myc-tagged kAE1, 48 h after transfection, the transfected HEK 293T cells were processed as previously described [16]. Briefly, the transfected HEK 293T cells were washed with PBS and fixed with 4% paraformaldehyde in PBS. After washing with 100 mM glycine in PBS, the cells were permeabilized with 0.2% Triton X-100. The cells were then incubated with primary antibody followed by secondary antibody conjugated with a fluorescein dye. Localization of the expressed proteins was examined by a confocal microscope (Zeiss LSM510 META, Germany).

Flow cytometry. Myc or HA epitopes were inserted into the third extracellular loop of kAE1, known to have no effect on kAE1 conformation or transport function, to allow detection of the recombinant protein on surface of intact cells. Thus, expression of kAE1-Myc or kAE1-HA on cell surface could be determined by fluorescence staining and flow cytometry as previously described [16]. Briefly, 2 days after transfection, the cells were collected and re-suspended in chilled DMEM containing with 2% fetal bovine serum. Then, the cells were fixed, wash and incubated with mouse anti-Myc or rabbit anti-HA antibody for 1 h. After incubation the cells were washed twice with chilled DMEM containing with 2% fetal bovine serum. Then, goat anti-mouse antibody conjugated with Alexa 488 or donkey anti-rabbit antibody conjugated with Cy3 (Molecular Probes, Eugene, Or, USA) was used to probe mouse anti-Myc or rabbit anti-HA antibody, respectively. The cells were washed and analyzed by using FACSortTM flow cytometer (Becton-Dickinson, USA).

Expression studies in MDCK cells

Cell culture. MDCK cells stably expressing kAE1 proteins were generated as described previously [18]. Briefly, HEK 293T cells were cotransfected with three retroviral plasmids pVpack-GP, pVpack-VSVG (vesicular stomatitis virus glycoprotein), and pFBNeo-kAE1 HA557 using FuGene 6 Transfection reagent (Roche Diagnostics, Indianapolis, IN). The virus-containing supernatant was collected 24–36 h later and added to 30–50% confluent MDCK cells, in the presence of 8 μg/ml of polybrene (Sigma, St. Louis, MO). The infected cells were selected with 1 mg/ml geneticin (G418, Sigma). Polarized MDCK cells were obtained by growing confluent infected cells on Transwell polycarbonate filters (Corning Headquarters, Corning, NY) for 4–5 days. As previously described, the viral infection results in a heterogenous population of kAE1-expressing cells [19].

Western blot analysis. SDS-PAGE and Western blotting were performed as previously described [20]. Briefly, infected MDCK cells were lysed in PBS containing 1% $C_{12}E_8$ detergent and protease inhibitors Leupeptin (1 μM), Aprotinin (1 μM), PMSF (200 μM), and pepstatin A (1 μM). After centrifugation to remove insoluble material, 15 μg of total protein per lane were loaded and resolved on an 8% SDS-PAGE and transferred to a nitrocellulose membrane. The blotted proteins were detected with a mouse anti-HA monoclonal antibody (Covance, Princeton, NJ), followed by an anti-mouse antibody coupled to horseradish peroxidase.

Co-immunoprecipitation. Co-immunoprecipitations were performed as previously described [19]. Briefly, co-infected MDCK cells to express HA- and Myc-tagged proteins were lysed in 0.5 ml of PBS containing 1% $C_{12}E_8$ and centrifuged to remove insoluble materials. An aliquot was saved as total fraction while the remaining supernatant was incubated with 10 μl rabbit anti-Myc antibody followed by 40 μl protein G-Sepharose. After three washes, the protein was eluted with Laemmli buffer, resolved by SDS-PAGE, blotted and detected with a mouse anti-HA antibody followed by an anti-mouse antibody coupled to horseradish peroxidase.

Immunocytochemistry. MDCK cells were grown on glass coverslips, or on semipermeable Transwell polycarbonate filters (Corning Headquarters, Corning, NY) to confluency for 4–5 days. Cells were fixed using 3.8% formaldehyde for 15 min, then washed

once with 100 mM glycine, permeabilized with 0.2% Triton X-100 for 15 min and blocked for 30 min with 1% BSA. A 1/1,000 mouse anti-HA antibody (Covance) and 1/500 rabbit anti-Myc antibody (Stressgen Biotechnologies, San Diego, CA) in 1% BSA were added to the sample for 30 min. After several washes, 1/1,000 of Alexa 488-conjugated anti-mouse antibody (Molecular Probes, Eu- gene, OR) or Cy3-conjugated anti-rabbit antibody (Jackson Immunoresearch Laboratories, West Grove, PA) was added for 30 min before observation using a Zeiss laser confocal microscope LSM 510. For co-expression experiments, the co-infected cells were fixed and permeabilized using the same protocol as above described. The samples were then incubated with 1/1,000 mouse anti-HA antibody (Covance, Princeton, NJ) and 1/500 rabbit polyclonal anti-Myc antibody (Santa Cruz Biotechnologies, Santa Cruz, CA), followed, after several washes, by 1/1,000 of Alexa 488-conjugated anti-mouse antibody (Molecular Probes, Eugene, OR) and Cy3-conjugated anti-rabbit antibody (Jackson Immunoresearch Laboratories). Samples were examined using a Zeiss laser confocal microscope LSM 510.

Results

We have recently identified two pediatric patients in a Thai family with dRTA caused by novel compound heterozygous *SLC4A1* G701D/A858D mutations [14]. Here, we have conducted *in vitro* studies to examine the molecular defect associated with this compound heterozygous condition. HEK 293T and MDCK cells were transfected with recombinant plasmids expressing tagged versions (HA, Myc, His) of wild-type kAE1, kAE1 G701D or kAE1 A858D, or co-transfected with all pairs of plasmids. Protein expression was examined by Western blotting; protein dimerization by co-purification and co-immunoprecipitation; and subcellular localization by immunofluorescence staining and confocal microscopy. Additionally, cell surface expression of wild-type and mutant kAE1 tagged with either Myc or HA epitope was analyzed by flow cytometry.

HEK 293T cells

Expression and interaction of wild-type and mutant kAE1 proteins in HEK 293T cells. HEK 293T cells transfected with the recombinant plasmids expressed His-, Myc- or HA-tagged kAE1 WT, kAE1 G701D, or kAE1 A858D produced similar levels of immunoreactive proteins with the estimated molecular weight (MW) of ~96 kDa (Figure 1A). Thus, the dRTA mutants are not targeted for rapid degradation in transfected HEK cells.

The interactions between kAE1 WT and kAE1 G701D or kAE1 A858D, and between kAE1 G701D and kAE1 A858D co-expressed in HEK 293T cells were examined by affinity co-purification and co-immunoprecipitation. His-tagged kAE1 WT or mutants were purified using Co^{2+} resins and interacting HA-tagged proteins were detected by immunoblot using anti-HA antibody (Figure 1B). The purified fraction from control HEK 293T cells expressing only kAE1 WT-HA or kAE1 WT-His did not show any kAE1 WT bands on the HA blot as expected (Figure 1B, lanes 1 and 2). The purified fraction from HEK 293T cells co-expressing kAE1 WT-His and kAE1 WT-HA showed heterodimer formation between the two tagged kAE1 proteins (Figure 1B, lane 3). When kAE1 WT-His was co-expressed with kAE1 G701D-HA or kAE1 A858D-HA, it could form heterodimers with the dRTA mutants (Figure 1B, lanes 4 and 5).

To mimic the compound heterozygous kAE1 G701D and kAE1 A858D mutations, the cells were co-transfected to express kAE1 G701D-His and kAE1 A858D-HA or kAE1 A858D-His and kAE1 G701D-HA. Co-expression of the two dRTA mutants showed that they could interact with each other (Figure 1B, lanes 6 and 7). The pull-down experiments show that kAE1 WT is able to form heterodimers with the dRTA mutants, but also that the dRTA mutants can form heterodimers.

Co-immunoprecipitation experiments showed the same results when kAE1 proteins were immunoprecipitated with anti-His antibody and associated HA-tagged kAE1 proteins were detected by immunoblotting with anti-HA antibody (Figure 1C). The co-purification and co-immunoprecipitation results indicate that the two dRTA mutations do not seriously affect the protein folding and interactions responsible for forming kAE1 dimers.

Sub cellular localization of wild-type and mutant kAE1 dRTA proteins in HEK 293T cells. The subcellular localization of wild-type and mutant kAE1 proteins in HEK 293T cells were examined by using immunofluorescence and confocal microscopy. Transfected HEK 293T cells expressing either tagged kAE1 WT-Myc or kAE1 WT-HA showed predominant expression at the cell surface (Figure 2A) as previously described [16,21,22]. The kAE1 G701D was mainly localized intracellularly with little or no cell surface localization (Figure 2A) as previously reported [18,19]. kAE1 A858D-Myc or kAE1 A858D-HA

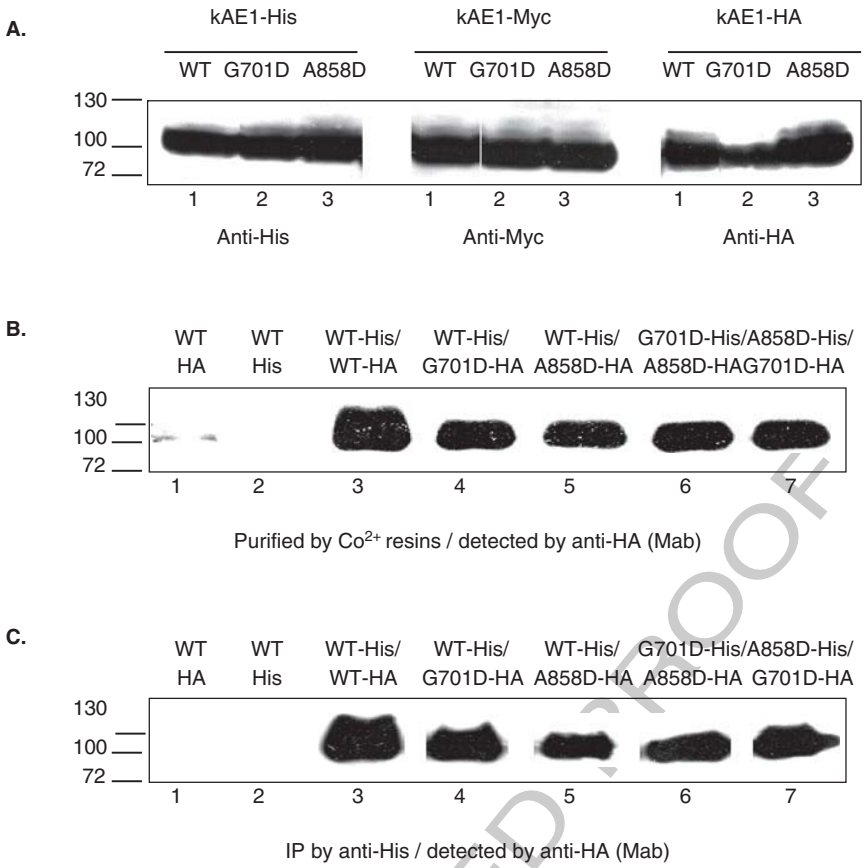

Figure 1. (A) Western blot analysis of wild-type and mutant kAE1 proteins, tagged with 6xHis or Myc or HA, expressed in HEK 293T cells, (B) affinity co-purification, and (C) co-immunoprecipitation of wide-type and mutant kAE1 proteins in HEK 293T cells. HEK 293T cells transfected or co-transfected with the plasmid constructs expressing kAE1-His and kAE1-HA were lysed and 6xHis-tagged oligomers were purified by Co^{2+} resin or immunoprecipitated (IP) by mouse anti-His antibody followed by Protein G-sepharose. The bound protein were eluted by 2× Laemmli buffer and detected by Western blot analysis using rabbit anti-HA antibody. Lanes 1–2 are individually expressed kAE1 WT-HA and kAE1 WT-His, respectively. Lanes 3–5 are kAE1 WT-His co-expressed with kAE1 WT-HA, kAE1 G701D-HA, and kAE1 A858D, respectively. Lane 6 is kAE1 G701D-His co-expressed with kAE1 A858D-HA. Lane 7 is kE1 A858D-His co-expressed with kAE1 G701D-HA. The results show that kAE1 WT-His could interact with either kAE1 WT-HA, kAE1 G701D-HA, or kAE1 A858D-HA. In addition, kAE1 G701D-His could interact with kAE1 A858D-HA, and kAE1 A858D-His could interact with kAE1 G701D-HA.

was observed both at the cell surface and intracellularly (Figure 2A). These results indicate that kAE1 WT can traffic efficiently to the cell surface in transfected HEK 293T cells. The kAE1 A858D mutant can also traffic to the cell surface, but not as efficiently as the wild-type kAE1. In contrast, the kAE1 G701D mutant fails to traffic to the cell surface.

Co-expression and localization of wild-type and mutant kAE1 proteins in HEK 293T cells. We next determined the effect of the dRTA mutants on the wild-type kAE1 trafficking and localization and *vice versa*, and the effect of the two dRTA mutants on each other using co-expression studies. Co-expression of kAE1 WT-Myc with kAE1 WT-HA showed cell surface expression and co-localization of both kAE1 proteins as expected (Figure 2B). Co-expression of kAE1 WT-Myc with kAE1 G701D-HA or kAE1 WT-Myc with kAE1 A858D-HA showed cell surface expression and co-localization of wild-type and the mutant kAE1 proteins (Figure 2B). There was a strong co-localization of kAE1 WT with the kAE1 A858D mutant at the cell surface with little intracellular staining of the kAE1 A858D mutant. While the kAE1 A858D mutant can traffic to the cell surface when expressed alone, its trafficking is facilitated when co-expressed with kAE1 WT. There was also co-localization of kAE1 WT with the kAE1 G701D mutant at the cell surface, indicating that kAE1 WT could rescue the cell surface trafficking of the kAE1 G701D mutant, In contrast to the kAE1 A858D mutant, the kAE1 G701D mutant retained a

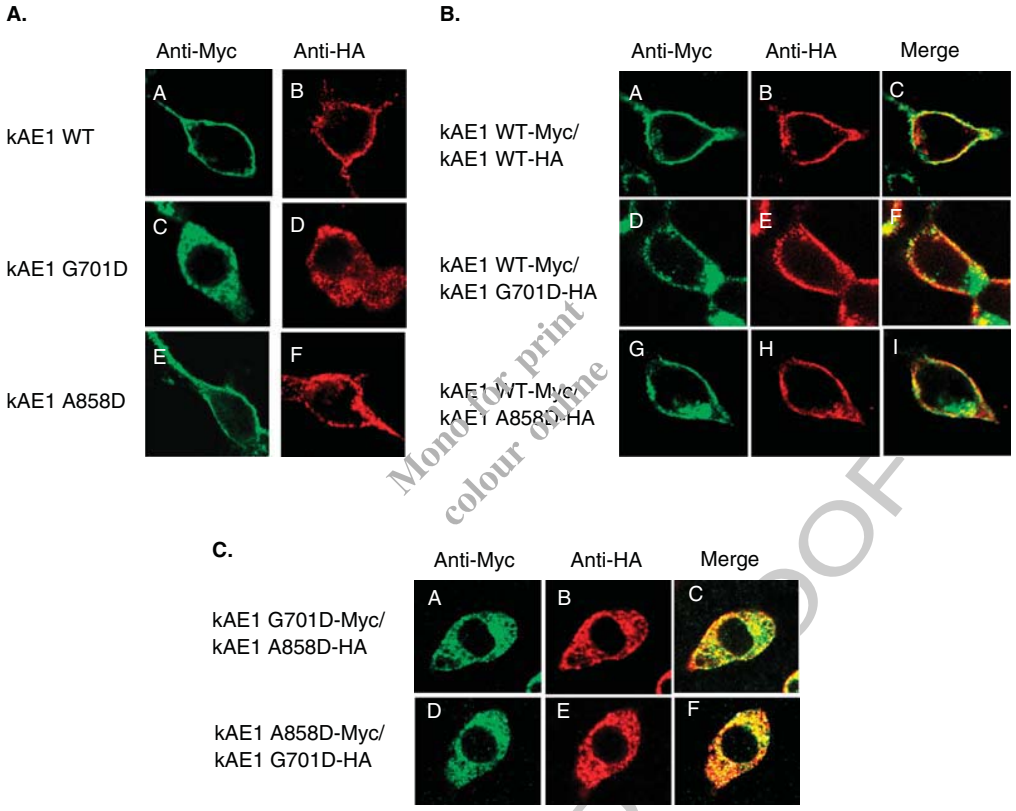

Figure 2. (A) Immunofluorescence staining of kAE1 WT, kAE1 G701D, and kAE1 A858D, tagged with Myc or HA, expressed in HEK 293T cells, (B) immunofluorescence staining of kAE1 WT-Myc co-expressed with either kAE1 WT-HA, kAE1 G701D-HA, or kAE1 A858D-HA, and (C) immunofluorescence staining of kAE1 G701D-Myc co-expressed with kAE1 A858D-HA, and kAE1 A858D-Myc co-expressed with kAE1 G701D-HA in HEK 293T cells. Transiently transfected or co-transfected HEK 293T cells expressing kAE1-Myc or kAE1-HA were fixed and permeabilized and then stained with anti-Myc or anti-HA antibody followed by anti-mouse IgG-Alexa 488 antibodies (green) or anti-rabbit IgG-Cy3 (red). Yellow color indicates co-localized proteins. Immunofluorescence images were captured by using Zeiss LSM510 META confocal microscopy. (This figure is reproduced in colour in *Molecular Membrane Biology* online.)

predominant intracellular staining even when co-expressed with kAE1 WT. These results indicate that kAE1 WT facilitates the trafficking of kAE1 A858D and kAE1 G701D to the cell surface, but the rescue of kAE1 G701D is less complete. This rescue effect likely occurs through the formation of heterodimers in the endoplasmic reticulum. The heterodimers that contain a wild-type subunit can exit the ER more efficiently and traffic to the cell surface than the mutant homodimers.

To mimic the compound heterozygous *SLC4A1* G701D and A858D mutations found in the patients, kAE1 G701D-Myc and kAE1 A858D-HA or A858D-myc and kAE1 G701D-HA were co-expressed in HEK 293T cells. The two dRTA mutants were largely expressed intracellularly with poor localization at the plasma membrane (Figure 2C). Unlike wild-type kAE1, the kAE1 A858D mutant that could traffic to the cell surface when expressed alone, but was unable to rescue kAE1 G701D trafficking to the cell surface. In this case the kAE1 G701D had a dominant effect on the kAE1 A858D mutant, retaining it intracellularly. These results indicate that the wild-type kAE1 could rescue both mutant dRTA kAE1 proteins from intracellular retention to the plasma membrane but that kAE1 G701D and kAE1 A858D fail to rescue each other to the cell surface.

Analysis of individual expression and co-expression of kAE1-Myc and kAE1-HA tagged protein by flow cytometry in HEK 293T cells. Myc or HA epitopes were inserted in the third extracellular loop at the position 557 of kAE1 to allow immuno-detection of intact cells expressing the protein at the cell surface. The HEK 293T cells that individually expressed kAE1 WT-Myc, kAE1 G701D-Myc or kAE1 A858D-Myc had mean fluorescence intensities of 34.57 ± 2.24, 1.14 ± 0.35 or

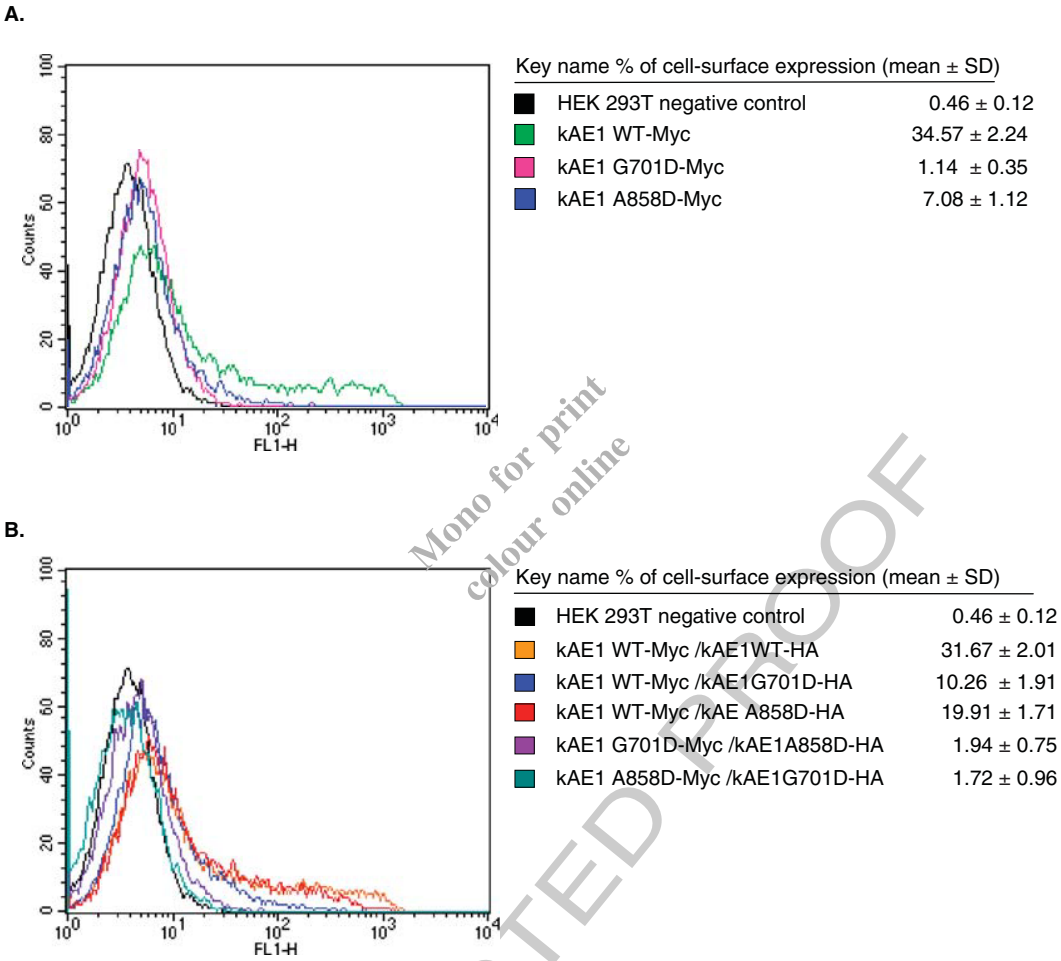

Figure 3. Cell-surface expression of Myc557-tagged kAE1 in transfected HEK 293T cells measured by flow cytometry. The transfected cells were incubated with mouse anti-Myc antibody followed by anti-mouse immunoglobulin G antibody conjugated with Alexa 488. Fluorescence intensity was detected by flow cytometry with kAE1-positive cells showing heterogeneous expression above background levels. (A) HEK 293T expressing cells kAE1 WT-Myc (green), kAE1 G701D-Myc (pink), and kAE1 A858D-Myc (blue). (B) HEK 293T cells expressing kAE1 WT-Myc and kAE1 WT-HA (orange), kAE1 WT-Myc and kAE1 G701D-HA (blue), kAE1 WT-Myc and kAE1 A858D-HA (red), kAE1 G701D-HA and kAE1 A858D-HA (purple), and kAE1 A858D-Myc and kAE1 G701D-HA (greenish blue). The experiments were conducted in triplicates and percentages of cell-surface expression of Myc557-tagged kAE1 (mean ± SD) were shown. (This figure is reproduced in colour in *Molecular Membrane Biology* online.)

7.08 ± 1.12, respectively (Figure 3A). This confirms the low level of cell surface expression of the dRTA mutants, especially the G701D mutant, relative to wild-type kAE1.

HEK 293T cells co-expressing kAE1 WT-Myc with either kAE1 G701D-HA (10.26 ± 1.91) or kAE1 A858D-HA (19.91 ± 1.71) showed higher levels of cell surface expression of the mutants than when expressed alone. This indicates that kAE1 WT is able to rescue the cell surface expression of both dRTA mutants. The level of cell surface expression was still less than that of kAE1 WT-HA (31.67 ± 2.01) (Figure 3B), indicating that the rescue was not complete, especially in the case of the kAE1 G701D. The transfected cells co-expressing both kAE1 G701D-Myc and kAE1 A858D-HA or A858D-myc and kAE1 G701D-HA presented very low signal levels (1.94 ± 0.75 and 1.72 ± 0.96) when compared to when the mutants were co-expressed with kAE1 WT (Figure 3B). These results indicate that kAE1 A858D has a more severe trafficking impairment when co-expressed with kAE1 G701D than when expressed alone. Furthermore, the kAE1 A858D mutant could not rescue kAE1 G701D trafficking to the cell surface. In contrast, kAE1 WT could rescue both kAE1 G701D and kAE1 A858D mutant proteins to the cell surface with more efficient rescue of the kAE1 A858D mutant.

MDCK cells

Expression and heterodimerization of wild-type and mutant kAE1 proteins in MDCK cells. To determine the expression of kAE1 G701D and kAE1 A858D mutant proteins tagged with HA in MDCK epithelial cells, immunoblot analyses of whole cell extracts using anti-HA antibody (Figure 4A) or anti-kNt-AE1 antibody (Figure 4B) were performed. The immunoblot in Figure 4A shows the HA-tagged proteins, while that in Figure 4B shows the full complement of expressed kAE1 proteins using an anti-kNt-kAE1 antibody. The immunoblot results show that the wild-type and mutant kAE1 proteins could be expressed and detected in MDCK cells with the wild-type protein showing higher expression levels relative the dRTA mutants. The wild-type kAE1 protein ran as two closely-spaced bands (Figure 4A and B, lanes 1), a major upper band and a minor lower band. Enzymatic deglycosylation experiments (not shown) confirmed previous results [19] that the upper band contained complex oligosaccharide that had exited the ER, while the lower band contained high mannose oligosaccharide. The kAE1 G701D contained about equal amounts of the two glyco-forms, indicating impaired exit from the ER. The kAE1 A858D contained a higher proportion of complex oligosaccharide than the kAE1 G701D mutant, suggesting a less severe impairment in ER exit.

To mimic the heterozygous state kAE1 WT-Myc was co-expressed with kAE1 G701D-HA or kAE1 A858D-HA in MDCK cells. To mimic the compound heterozygous state found in the patients with dRTA the kAE1 G701D-HA was co-expressed with kAE1 A858D-Myc (Figure 4C and D). Figure 4C shows a control experiment that HA-tagged protein immunoprecipitated using the anti-HA antibody could be detected by the anti-Nt-kAE1 antibody. Figure 4D shows that two dRTA kAE1 mutants could interact with the wild-type kAE1, and importantly also with each other. The kAE1 WT-Myc immunoprecipitated using the anti-Myc antibody was associated with the kAE1 G701D-HA and A858D-HA as detected by anti-HA antibody (Figure 4D, lanes 4 and 5). The kAE1 G701D-HA was co-immunoprecipitated with the kAE1 A858D-Myc (Figure 4D, lane 6) and in reverse (Figure 4D, lane 7), showing that the two mutants could interact with each other in MDCK cells. Interestingly, the immunoblots (Figure 4D) showed only the upper complex band. Thus, kAE1 WT can interact with the kAE1 G701D and A858D mutants and exit the ER. The lack of detection of high mannose forms in the co-immunoprecipitation experiments (Figure 4D) that are present in the control immunoprecipitation experiment (Figure 4C) indicates that the ER-retained forms are likely unstable and degraded. This is consistent with the lower levels of expression observed for the dRTA mutants relative to the wild-type protein when expressed in MDCK cells.

Examination of cellular localization of dRTA kAE1 mutants in MDCK cells. The wild-type and dRTA mutant proteins or the two mutants, carrying a HA or Myc epitope to discriminate the different proteins, were expressed alone or together and the relative location of the tagged proteins were determined by immunofluorescence and confocal microscopy in both non-polarized and polarized MDCK cells. We were particularly interested in the effect of co-expression of different forms of kAE1 on their mutual localization. The wild-type protein when expressed alone was predominantly found at the cell surface of the non-polarized MDCK cells (Figure 5A left panel) and at the basolateral membrane after polarization (Figure 5A right panel and Figure 6). In contrast, the kAE1 G701D was predominantly found intracellularly in non-polarized and polarization MDCK cells. The kAE1 A858D also showed expression at the cell surface and basolateral membrane, however there was also considerable intracellular localization (Figure 5A).

When the kAE1 WT was co-expressed with the kAE1 G701D in the same cells, both proteins were detected at the cell surface (Figure 5B), however with strong intracellular staining for kAE1 G701D. When the kAE1 WT was co-expressed with the kAE1 A858D (Figure 5C), both proteins were localized to the cell surface, with some intracellular staining for the kAE1 A858D mutant (Figure 5C). These results suggest that the co-expressed wild-type kAE1 could partially rescue both mutant kAE1 proteins from intracellular retention to the plasma membrane. There was however a pool of mutant protein that was still localized intracelluarly even when co-expressed with the wild-type kAE1, with no evidence of a major pool of intracellular wild-type kAE1.

The results of co-expressed kAE1 G701D and kAE1 A858D showed that kAE1 G701D was co-localized with kAE1 A858D intracellularly but also at the cell surface in non-polarized and polarized MDCK cells (Figure 5D). The kAE1 A858D could partially rescue the kAE1 G701D to cell surface, however there was still strong intracellular staining observed for the kAE1 G701D even in the presence of the kAE1 A858D mutant.

To confirm cell surface expression, Figure 6 shows localization of the kAE1 protein and E-cadherin, a basolateral marker. The kAE1 WT strongly

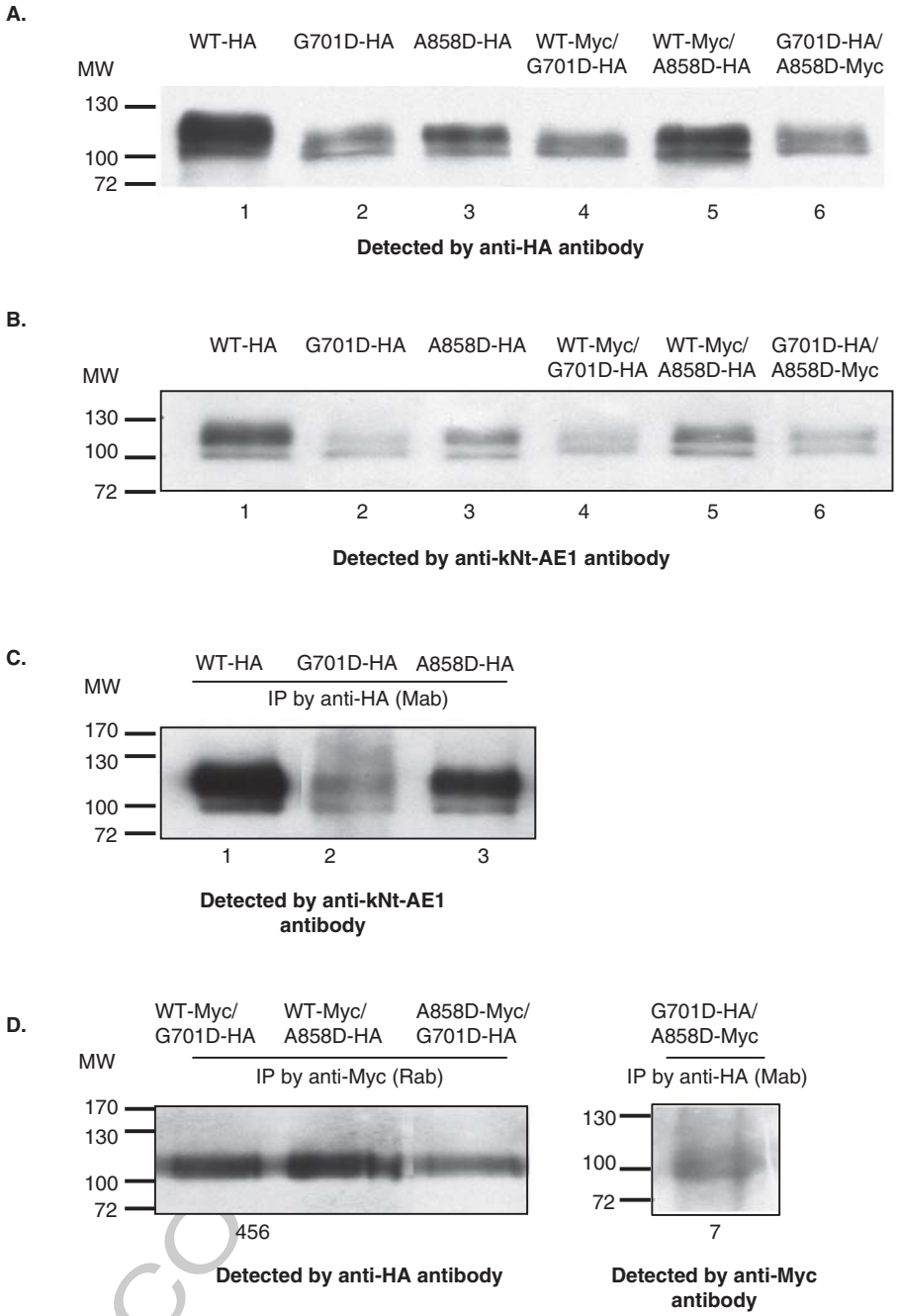

Figure 4. Western blot analysis of total cell extracts of HA557- or Myc557-kAE1 WT, G701D, and A858D (A), and co-immunoprecipitation studies (B and C). MDCK cells were infected or co-infected for 12 days and lysed by 1% $C_{12}E_8$ buffer. The cell extracts were loaded onto 8% SDS-PAGE and transferred to nitrocellulose membrane. For co-immunoprecipitation, the cell extract was incubated with mouse anti-HA or rabbit anti-Myc antibodies followed by Protein G-Sepharose. The bound protein were eluted by 2× Laemmli buffer and detected by Western blot. (A) The total expressed kAE1 protein was detected by mouse anti-HA followed by anti-mouse IgG-HRP antibodies. (B) The total expressed kAE1 protein was detected by rabbit anti-Knt-AE1 antibody. (C) HA557-kAE1 WT (lane 1), G701D (lane 2) and A858D (lane 3) were co-immunoprecipitated (Co-IP) by mouse anti-HA antibody and detected by rabbit anti-kNt-AE1 antibody followed anti-rabbit IgG-HRP conjugated antibody. D: Co-IP of Myc557-kAE1 WT with kAE1 WT-HA (lane 1), Myc557-kAE1 WT with kAE1 G701D-HA (lanes 2 and 4), kAE1 WT-Myc with kAE1 A858D-HA (lanes 3 and 5), kAE1 G701D-HA with kAE1 A858D-Myc (lanes 6 and 7). The samples of lanes 4–6 were co-immunoprecipitated by rabbit anti-Myc, detected by mouse anti-HA and followed by anti mouse IgG-HRP antibody. The sample of lane 7 was co-IP by mouse anti-HA, detected by rabbit anti-myc and followed by anti rabbit IgG-HRP antibody.

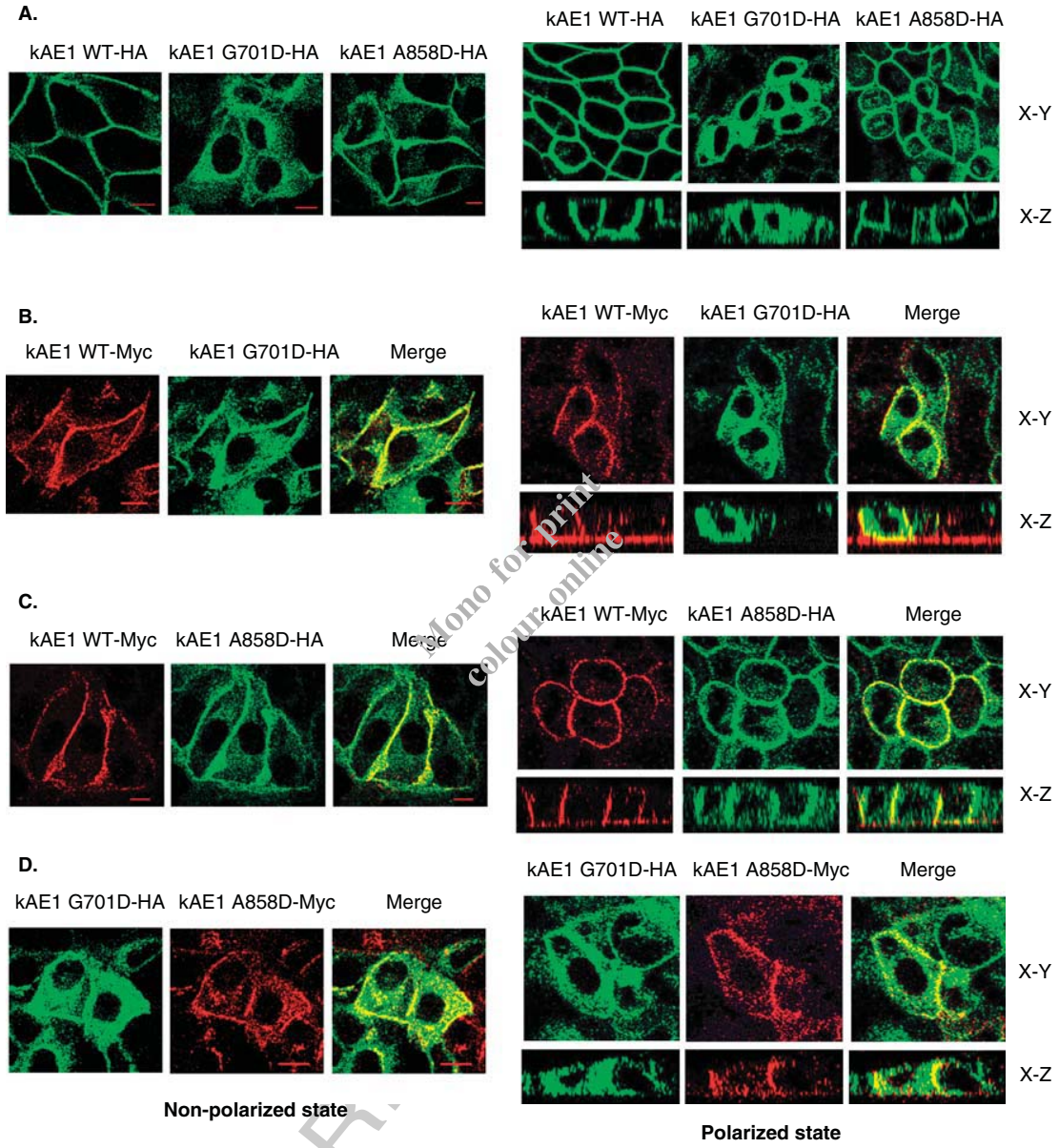

Figure 5. (A) Immunofluorescence staining of kAE1 WT-HA557, kAE1 G701D-HA557 and kAE1 A858D-HA557, and (B) co-immunofluorescence staining of kAE1 WT-Myc557 and kAE1 G701D-HA557, (C) kAE1 WT-Myc557 and kAE1 A858D-HA557, and (D) kAE1 G701D-HA557 and kAE1 A858D-Myc557 in MDCK cells. MDCK cells were fixed, permeabilized and incubated with mouse anti-HA followed by anti-mouse IgG-Alexa488 antibodies (green) or rabbit anti-myc and mouse anti-HA followed by anti-rabbit IgG-cy3 (red). Yellow color indicated co-localization of the proteins. The images were observed by Zeiss LSM510 confocal microscope. Bar represents 10 μm. X–Y shows the top view of middle section and X–Z shows the side view of the polarized cells. (This figure is reproduced in colour in *Molecular Membrane Biology* online.)

co-localized with E-cadherin as did the kAE1 A858D, which also showed some intracellular staining. In contrast, kAE1 G701D was very strongly localized intracellularly and there was very little co-localization with E-cadherin at the basolateral membrane. When the kAE1 G701D was co-expressed with the kAE1 A858D; however, some co-localization of the kAE1 G701D mutant with E-cadhrein was observed. Thus, the kAE1 A858D could only poorly facilitate trafficking of the kAE1 G701D to the basolateral membrane.

Discussion

Novel compound heterozygous *SLC4A1* G701D/A858D mutations causing dRTA have recently

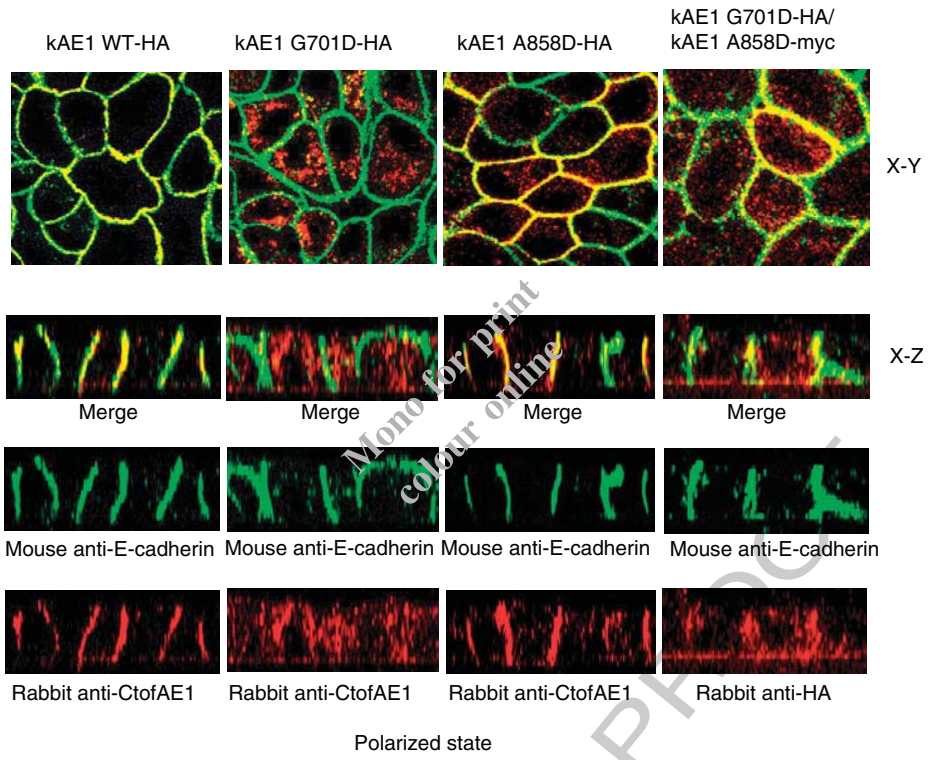

Figure 6. Co-immunofluorescence staining of individually expressed kAE1 WT-HA557, kAE1 G701D-HA557, or kAE1 A858D-HA557, and co-expressed kAE1 G701D-HA557 and kAE1 A858D-Myc557 in MDCK cells. Viral infected MDCK cells were grown on semipermeabilized filter (polarized state) for 6 days until polarized. Then cells were fixed, permeabilized and incubated with mouse anti-E-cadherin (epithelia basolateral marker) followed by anti mouse-Alexa488 (green), rabbit anti-Ct of AE1, or rabbit anti-HA (for co-expression only to detect kAE1 G701D) followed by anti rabbit IgG-Cy3 (red) antibodies. Yellow color indicates co-localization of the kAE1 proteins with E-cadherin at the basolateral membrane. The cells were observed by using Zeiss LSM510 confocal microscope. X–Y shows the top view of the middle section and X–Z shows the side view of the polarized cells. (This figure is reproduced in colour in *Molecular Membrane Biology* online.)

been identified in two pediatric patients from a Thai family [14]. The interactions between wild-type kAE1 and the two mutant kAE1 (G701D and A858D) associated with dRTA and between each other were examined by co-immunoprecipitation and affinity co-purification. The wild-type kAE1 protein could form heterodimers with kAE1 G701D and kAE1 A858D proteins, while the two mutant proteins could form heterodimers with each other. This indicates that, these two mutations do not seriously affect the regions of kAE1 involved in the formation of dimers.

When expressed alone, kAE1 WT was found at the cell surface of HEK 293T and non-polarized MDCK cells and the basolateral membrane of polarized MDCK cells indicating efficient trafficking of kAE1 in the cell lines, particularly polarized MDCK cells. The kAE1 A858D mutant was also found at the cell surface, with some intracellular localization, suggesting a mild trafficking defect. In contrast, the kAE1 G701D mutant was very poorly expressed at the cell surface and predominantly retained intracellularly as noted previously [16,21].

In this paper, we have examined the cellular localization of kAE1 in the heterozygous and compound heterozygous G701D/A858D conditions by co-expression of wild-type kAE1 with either kAE1 G701D or A858D, and by co-expression of both mutant proteins to mimic the circumstance in α-intercalated kidney cells of the patients. The results showed that wild-type kAE1 was able to rescue trafficking kAE1 A858D and partially rescue kAE1 G701D to the cell surface. In contrast, the kAE1 A858D could only poorly rescue the kAE1 G701D, which maintained a predominant intracellular localization in non-polarized MDCK and polarized MDCK cells. Heterozygous patients expressing the wild-type protein and the dRTA mutants are able to express the proteins at the cell surface due to rescue by the wild-type protein. Co-expression of kAE1 G701D and kAE1 A858D however would result in impaired anion transport activity in the α-intercalated cells of the distal nephron of the patients with the compound heterozygous G701D/A858D due to their poor cell surface expression.

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Rapid detection of *solute carrier family* 4, *member 1* (*SLC4A1*) mutations and polymorphisms by high-resolution melting analysis

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HRM analysis

ABSTRACT

Objective: The objective of this study is to develop and evaluate a high-resolution melting (HRM) method for detection of *SLC4A1* mutations and polymorphisms.

Design and methods: The HRM method was optimized for detection of 18 known *SLC4A1* variants. It was then used for analysis of 16 blind DNA samples highly enriched with two common mutations, Southeast Asian ovalocytosis (SAO) and band 3 Bangkok 1 (G701D), to compare the results with that of the conventional procedures.

Results: The HRM method was able to detect all 18 *SLC4A1* variants. In the samples in which homozygous wild-type and homozygous variant could not be distinguished by difference plots, they were spiked with a sample carrying known homozygous genotype, resulting in their clear differentiation. The HRM method had 100% efficiency for detection of mutations in the blind DNA samples, when compared with that of the conventional techniques.

Conclusions: The developed HRM method is efficient and reproducible for detection of *SLC4A1* mutations and polymorphisms.

Introduction

The *solute carrier family 4, member1* (*SLC4A1*) or *anion exchanger 1* (*AE1*) gene is located on chromosome 17q21–q22, encoding both erythroid AE1 (eAE1) and kidney AE1 (kAE1) isoforms using different promoters and alternative splicing mechanism [1]. eAE1 is the most abundant transmembrane protein of the red blood cell (RBC) where it is involved in the electroneutral exchange of chloride/bicarbonate (Cl^-/HCO^-_3) and cytoskeleton anchorage [2]. kAE1 is expressed at the basolateral membrane of renal acid-secreting alpha-intercalated cells of the kidney collecting duct, which also mediates chloride/bicarbonate exchange [3]. Defects of *SLC4A1* may cause morphological changes of RBC, including spherocytosis and ovalocytosis, or distal renal tubular acidosis (dRTA)—a kidney disease characterized by a urinary acid-secreting defect resulting in hyperchloremic metabolic acidosis.

A number of missenses, frameshifts, and deletional mutations of *SLC4A1* were reported to result in both autosomal dominant (AD) [4–7] and autosomal recessive (AR) dRTA [8]. Additionally, combined defects of ovalocytosis and dRTA were originally reported in Thai patients by our group [9] to result from compound heterozygous Southeast Asian ovalocytosis (SAO) and band 3 Bangkok 1 mutations. SAO is caused by an in-frame 27-nucleotide deletion in exon 11 of *SLC4A1* giving rise to an absence of 9 amino acids at the positions 400–408 and band 3 Bangkok 1 by a missense mutation resulting in a glycine (G) to aspartic acid (D) substitution at the position 701 of band 3 protein (G701D).

Several methods have been developed to detect *SLC4A1* variations, including polymerase chain reaction-single stranded conformation polymorphism (PCR-SSCP), PCR and restriction fragment length polymorphism (PCR-RFLP), allele specific amplification (ASA), and direct DNA sequencing [9]. These methods are time-consuming open-tube assays that require additional post-PCR processing such as restriction enzyme digestion, electrophoresis, or sequencing. The process in transferring PCR products for further analyses creates a high risk of contamination.

High-resolution melting (HRM) analysis is a simple closed-tube assay that can scan or detect sequence variations by examining

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melting curves of amplicons [10]. Applications of HRM for detection genetic variations in *BRCA1*, *BRCA2* [11], *FGFR3* [12] and *CFTR* [13] have been reported. The HRM assay requires PCR reaction with a saturating double-stranded DNA-binding dye, an instrument for high-resolution melting (melting equipment), and software for converting and generating the difference plot. The melting curve of the amplicon is monitored by collecting fluorescent intensity of double-stranded DNA, dropped by denaturation while the temperature is raised. DNA samples with heterozygous sequence variations form heteroduplexes that are readily identified by analysis of the shape and width of melting curve. Melting curve can be converted by two different methods, the derivative plot (−dF/dT vs. temperature) for finding the melting temperature (Tm) of amplicon and the difference plot for scanning sequence variations. The difference plot method provides clearer distinction of sequence variations than the derivative plot one [10]. In this study, we have developed and optimized the HRM method for detection of *SLC4A1* mutations and polymorphisms and evaluated the efficiency of this method for mutation analysis in a collection of DNA samples highly enriched with SAO and G701D mutations.

Material and methods

Subjects' DNA samples

The ethical approval and informed consent were obtained before the collection of blood samples from the patients with dRTA and their family members, which were conducted in our previous studies [9,14–16]. The patients with dRTA were diagnosed by clinical and laboratory investigations including the presence of hyperchloremic metabolic acidosis or positive short acid loading test (ALT) showing inability of the patient kidneys to acidify urine to pH below 5.5 after the acid (ammonium chloride) load. Genomic DNA samples were extracted from white blood cells using a standard phenol–chloroform protocol. Concentrations of DNA samples were determined by spectrophotometric method and diluted to 25 ng/μL for screening by PCR-SSCP and direct DNA sequencing. Subsequent detection of *SLC4A1* variants was performed by PCR and electrophoresis, and PCR-RFLP [9], which are herein called the conventional methods.

Thirty-four DNA samples containing known *SLC4A1* mutations and polymorphisms were used for optimization of the amplification and HRM analysis using LightCycler 480 II (Roche Diagnotics, Germany) machine. Then, a second group of 16 DNA samples enriched with two common *SLC4A1* mutations (SAO and G701D) as detected by the conventional methods was used for evaluation of reproducibility of the HRM method.

In addition, 28 and 40 DNA samples containing the SAO and G701D mutations, respectively, were taken from our collection for testing sensitivity, and two groups (48 and 49) of DNA samples containing homozygous wild-type sequences at each respective position were selected for testing specificity.

Primers and PCR conditions

The sequence of *SLC4A1* was obtained from GenBank (accession number GI:171460929) for designing PCR primers (Table 1). These primer-pairs generated amplicons with the sizes between 114 and

Table 1
SLC4A1 variants used for analyses in this study.

Name	Type of variant	rs no.	Location	Position	Nucleotide change	Primer sequences (5′→ 3′)	Annealing temp (°C)	PCR product (bp)
M31T	SNP	–	Exon3	Codon 31	ATG>ACG	5′-CTTCGTTGGGGGTGGAAGAG-3′ 5′-GTGGAGAAGGGGAGAGGACAAG-3′	65	235
K1+11	SNP	rs999716	Intron3	IVS3+580	G>A	5′-CAGTTTGGGACAAGGGCGTG-3′ 5′-TGATGAAGTGAAGGGACCTCTCC-3′	67	491
IVS3+701	SNP	rs2074106	Intron3	IVS3+701	C>A	5′-TGGGAGGAGAGAAGGGAGTCTG-3′ 5′-TGATGAAGTGAAGGGACCTCTCC-3′	65	122
D38A	SNP	rs5034	Exon4	Codon 38	GAC>GCC	5′-GTCTCTGAGGCTCACAGTGGATG-3′ 5′-GGTAGTCTGTGGCTGTTGC-3′	65	116
K56E	SNP	rs5036	Exon4	Codon 56	GAG>AAG	5′-TACCACACCACATCACACC-3′ 5′-ATCCCCTTGCTCCTCTCTTCC-3′	65	114
E72D	SNP	rs13306788	Exon5	Codon 72	GAG>GAT	5′-TGAGCACCCACTATGCCCTG-3′ 5′-CCAGGCCCCATTCTCCCCCA-3′	63	175
IVS5+27	SNP	rs2074107	intron5	IVS5+27	C>T	5′-GCTGAGATGGATGGAGGCGG-3′ 5′-CAGCACCCCACAACAATCCTC-3′	65	191
F266F	SNP	–	Exon9	Codon 266	TTT>TTC	5′-CGGTGCTGGGCTTCGTGAGG-3′ 5′-TCAGCCACCATGCAGGTCC-3′	60	226
SAO	Mutation[a]	–	Exon11	Codon 400-408	Δ 27 bp	5′-CCTCACCTCCTCCAGCTACTCC-3′ 5′-CAGAAGTTGGGGCTGAGACAGAG-3′	62	318
S438S	SNP	rs13306781	Exon12	Codon 438	TCG>TCA	5′-AATGATCTCCTGACCTTGATCC-3′ 5′-GTCAGTGGGGCAAGGACAG-3′	58	217
C479W	Mutation[b]	–	Exon13	Codon 479	TGC>TGG	5′-CTGTCATGTCCCCCGCAC-3′ 5′-CACCACCAACACCACCAGCA-3′	58	172
R589C	Mutation[a]	–	Exon14	Codon 589	CGC>TGC	5′-CAGAATGCCTTGGTTTTCTGC-3′ 5′-GACAGGCGAGGAGGGTATG-3′	60	224
R602H	Mutation[b]	–	Exon15	Codon 602	CGT>CAT	5′-AAGGACGGAGGTGGGGAGTGACTG-3′ 5′-GGAAATGAGGACCTGGGGGGTATC-3′	70	201
G701D	Mutation[b]	–	Exon17	Codon 701	GGC>GAC	5′-TACCCCTCACCTTCCCCTAC-3′ 5′-GGCATGGGTGACGGAACGCA-3′	70	173
IVS17+19	SNP	rs2285643	intron17	IVS17+19	G>A	5′-GTAGTAGGCATGGGTGGGGT-3′ 5′-GGGGCAGGAGGATGGTGAAG-3′	65	230
IVS17-48	SNP	rs13306780	intron17	IVS17-48	T>G	5′-ATATGGTGCCTGTGTTTTATTCCC-3′ 5′-ACTTGGGTGGCTTGAACAGA-3′	65	189
S773P	Mutation[b]	–	Exon18	Codon 773	TCC>CCC	5′-AACCTGGGCTGAGAGTGTGC-3′ 5′-GGGCAGCCAGAAAAGGGTCC-3′	65	210
A858D	Mutation[c]	–	Exon19	Codon 858	GCC>GAC	5′-GGTACAGGACCCTTTTCTGG-3′ 5′-GCCTGCCCTAGTTCTGAGAC-3′	60	334

[a] Autosomal dominant (AD) dRTA.
[b] Autosomal recessive (AR) dRTA.
[c] Mild autosomal dominant dRTA.

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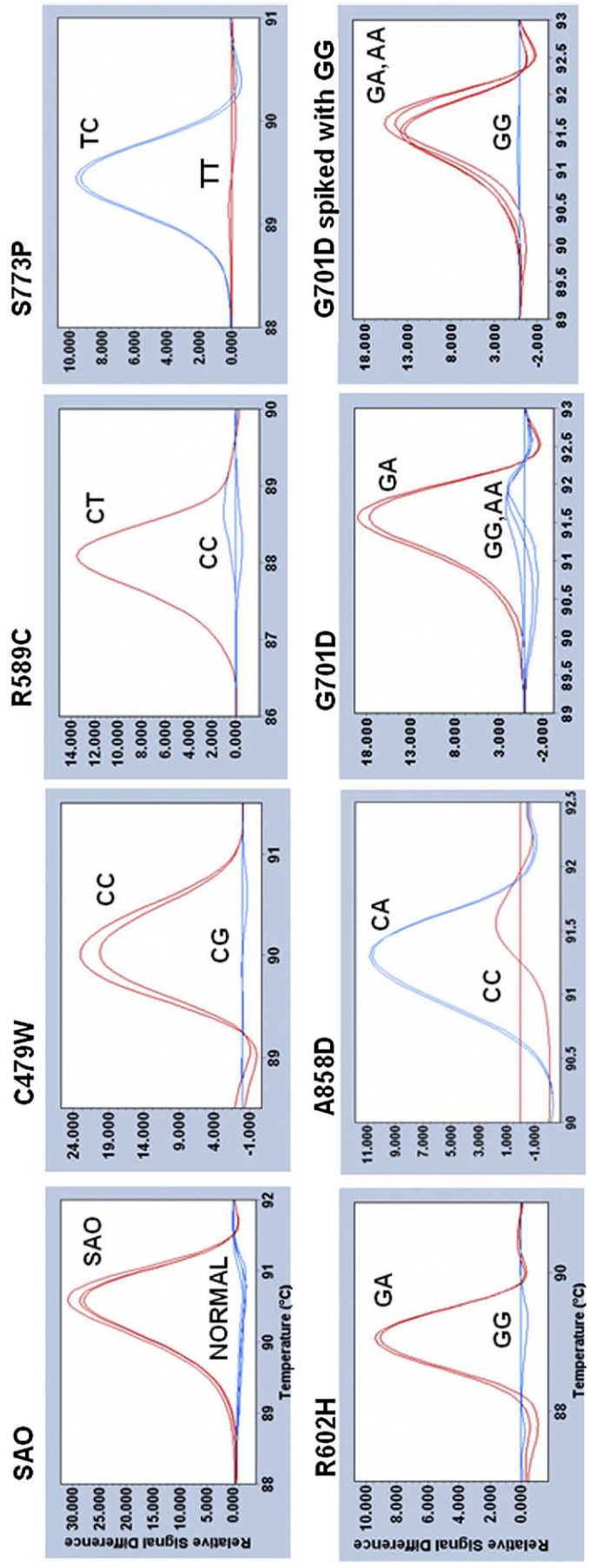

Fig. 1. Detection of seven *SLC4A1* mutations by HRM analysis. The difference plots of melting profiles of the amplicons from wild-types and heterozygous SAO, C479W, R589C, S773P, R602H, A858D and G701D mutations could clearly be distinguished. The difference plots of melting profiles of the amplicons from homozygous G701D (AA) and homozygous wild-type (GG) clustered together but they were differentiated after spiking with a DNA sample carrying homozygous wild-type (GG) before the analysis.

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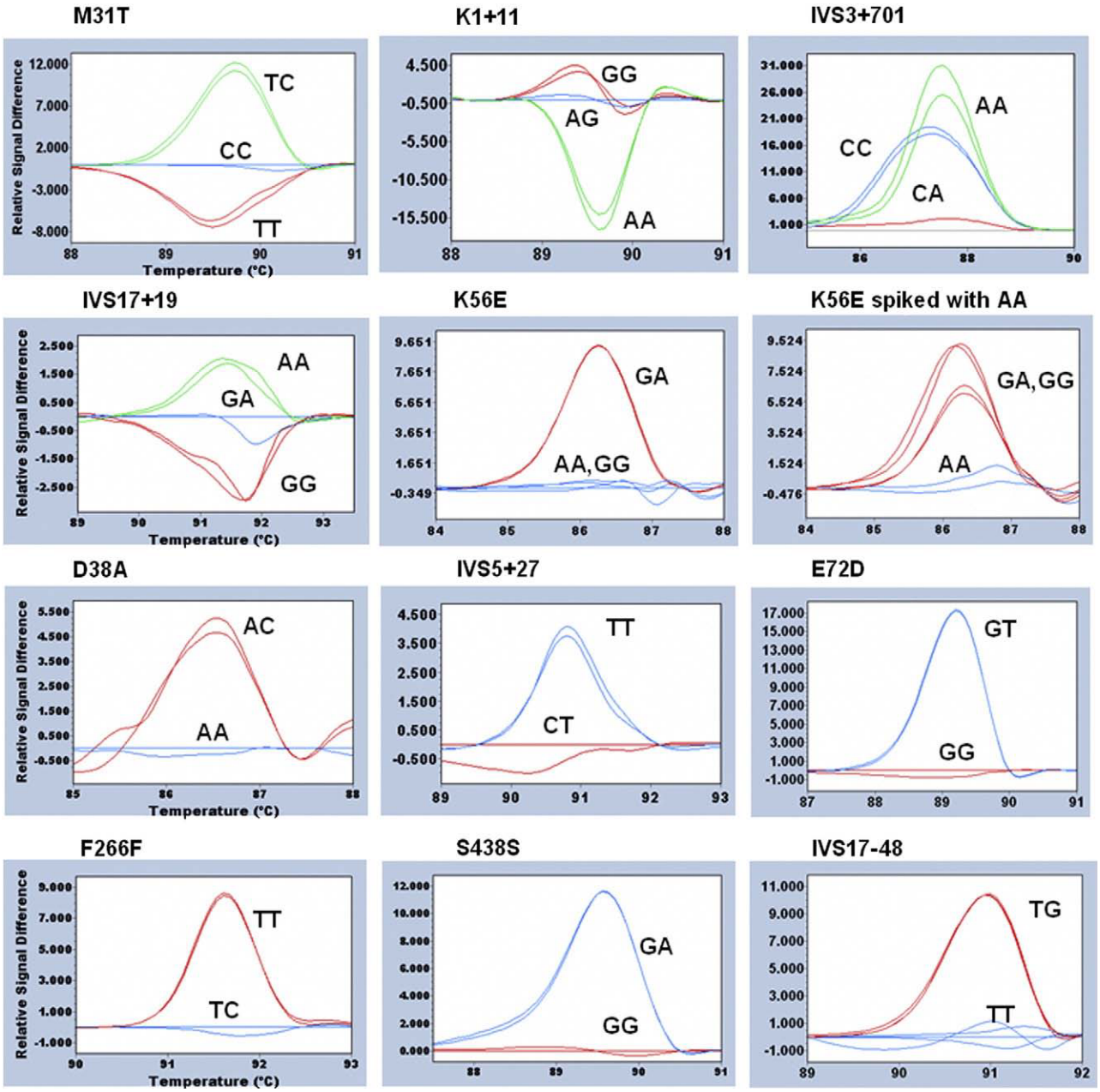

Fig. 2. Detection of eleven single nucleotide polymorphisms (SNPs) of *SLC4A1* by HRM analysis. The difference plots of melting profiles of amplicons from homozygous wild-types could be distinguished from that of heterozygous and homozygous M31T, K1+11, IVS3+701, and IVS17+19. The difference plots of melting profiles of amplicons from AA and GG genotypes of K56E were not distinguishable from each other but they could be differentiated by spiking with a DNA sample with AA genotype before the analysis. Six SNPs including D38A, IVS5+27, E72D, F266F, S438S and IVS17-48 were found only in heterozygous conditions; the difference plots of melting profiles of their amplicons were distinguishable from those of the wild-types.

491 base-pairs (bp). All PCR reactions were optimized by performing reactions in 20 μL volumes containing 125 ng genomic DNA in 1× Immolase buffer, 0.5 μM of each primer, 0.2 mM dNTP, 1.5 mM $MgCl_2$ and 0.25 unit of DNA polymerase (Immolase, Bioline, USA). PCR was carried out in a GeneAmp PCR System 9700 (PE Applied Biosystems, USA) Thermal Cycler with an initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation (95 °C for 20 s), annealing (58–70 °C for 20 s), and extension (72 °C for 25 s). The PCR products were used for analysis of *SLC4A1* variations by the conventional methods.

Restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) analysis of PCR products was conducted for the variant that created or abolished a restriction site. The reaction was made up in a 15 μL volume containing 10 μL of PCR product, 1× of reaction buffer, 0.15 μL BSA (100 μg/mL), and 5 U of restriction enzyme. The reaction was incubated overnight at 37 °C. The digested PCR fragments were separated on a 3% agarose gel by electrophoresis, stained with ethidium bromide, and visualized under ultraviolet light. The electrophoretic patterns of digested DNA fragments were recorded by a gel documentation.

HRM analysis

PCR and HRM analysis for each *SLC4A1* variant were performed in a single run on a LightCycler 480 II machine (Roche Diagnotics, Germany). The PCR reaction was modified from that of the

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conventional method by addition with 1× Resolight dye (Roche Diagnotics, Germany) and preparation in 96-well plate. The PCR condition included an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 20 s, 65 °C for 20 s, and 72 °C for 25 s. Before the high-resolution melting step, the product was heated to 96 °C for 1 min and then cooled to 40 °C. Melting curves were obtained by increasing the temperature to 96 °C with 25 acquisitions of continuous florescence detection. The melting curves were normalized, temperature-shifted and converted to difference plots by Gene Scan software.

In some cases, when the melting curves and difference plots of amplicons from the samples with homozygous wild-type and homozygous variants were unable to be differentiated, they were further analyzed by spiking the DNA sample carrying homozygous wild-type into the tested samples to allow the detection of heteroduplex DNA molecules and to differentiate between the two conditions; while the homozygous wild-type did not generate any heteroduplex DNA molecule, the homozygous variants did.

Results

SLC4A1 variations and detections

The PCR-SSCP method was initially conducted for screening *SLC4A1* variants (data not shown). The PCR products with mobility shifts of single stranded DNA were further analyzed by DNA sequencing and their results were compared with the published sequence of *SLC4A1* (accession number GI:171460929). This revealed 18 sites of sequence variations in the studied DNA samples; 7 were mutations and 11 were single nucleotide polymorphisms (SNPs). An in-frame deletion of 27 bp (codons 400–408) was found in exon 11 whereas base substitutions in exons 13, 14, 15, 17, 18 and 19 resulted in amino acid changes; otherwise, SNPs were observed in exons 3, 4, 5, 9, and 12, and in introns 3, 5, and 17. Several conventional methods including PCR-SSCP, PCR and electrophoresis, PCR-RFLP, and allele specific amplification (ASA) were designed for detection of these variants, depending on the property of sequence changes.

Optimization of HRM analysis

The amplification and HRM analysis for each *SLC4A1* variant were optimized in a single run using LightCycler 480 II machine. The primer-pairs generated amplicons with the sizes between 114 and 491 bp (Table 1). The amplicons with fluorescent dye were then melted to generate the melting curves and difference plots. The difference plots showed relative melting curves with reference to a chosen genotype (base-line). The genotypes of DNA samples used for optimization of HRM analysis were known from the analyses by the conventional methods. Not all three genotypes were found for each

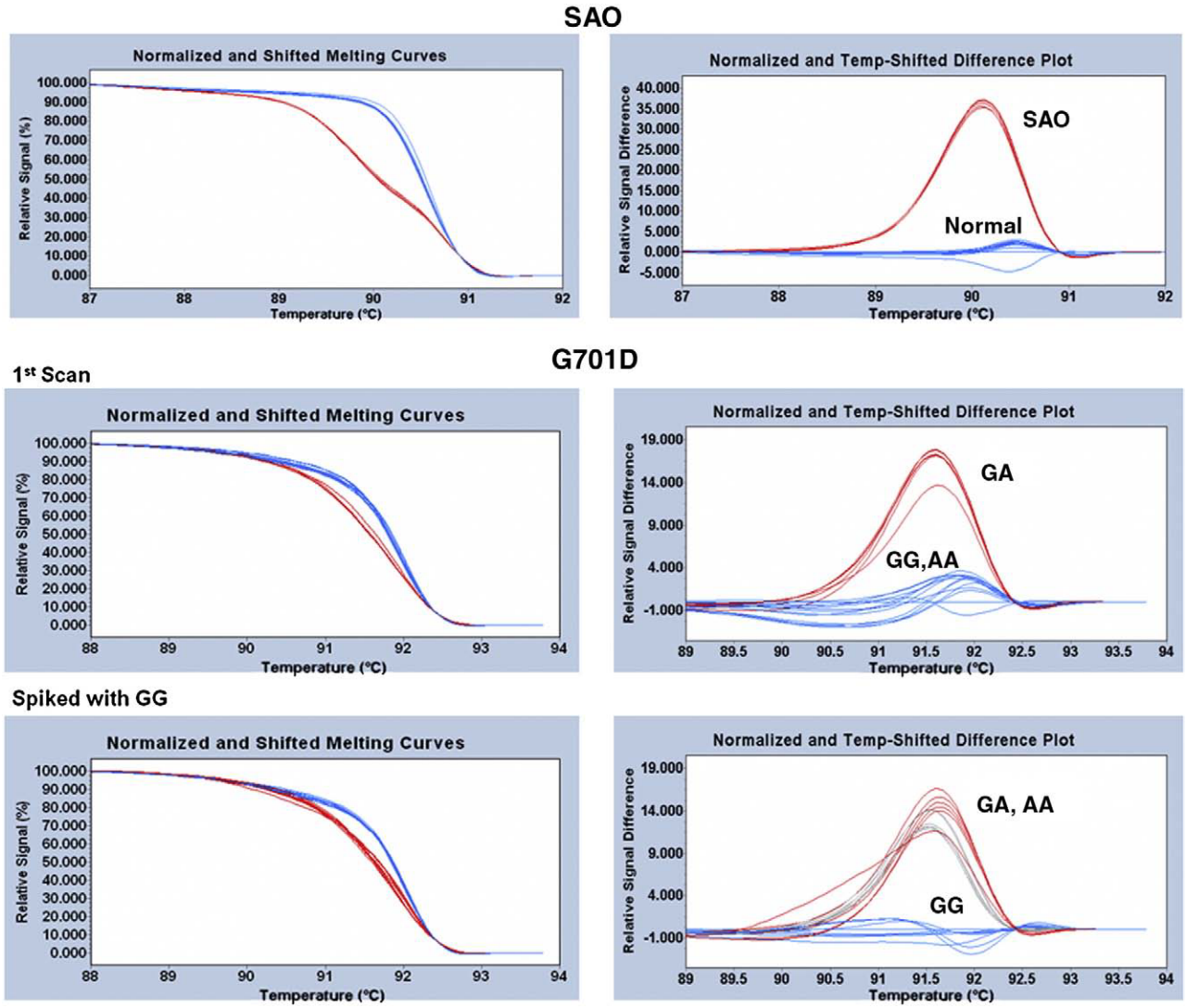

Fig. 3. Melting curves and difference plots of the HRM analysis for genotyping of SAO and G701D mutations in 16 blinded samples.

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Table 2
Genotyping of blinded DNA samples in SAO and G701D mutation by HRM analysis.

Samples	Genotype of exon 11	Exon 17			Result
		1st scan	2nd spike with G/G	Genotype	
S1	SAO/N	G/G or A/A	G/G	G/G	SAO/N
S2	SAO/N	G/A	G/A	G/A	SAO/G701D
S3	N/N	G/G or A/A	G/G	G/G	N/N
S4	N/N	G/G or A/A	G/A	A/A	G701D/G701D
S5	N/N	G/A	G/A	G/A	N/G701D
S6	N/N	G/G or A/A	G/A	A/A	G701D/G701D
S7	N/N	G/G or A/A	G/G	G/G	N/N
S8	N/N	G/A	G/A	G/A	N/G701D
S9	SAO/N	G/G or A/A	G/G	G/G	SAO/N
S10	N/N	G/G or A/A	G/G	G/G	N/N
S11	N/N	G/G or A/A	G/A	A/A	G701D/G701D
S12	N/N	G/G or A/A	G/A	A/A	G701D/G701D
S13	N/N	G/G or A/A	G/G	G/G	N/N
S14	N/N	G/A	G/A	G/A	N/G701D
S15	SAO/N	G/A	G/A	G/A	SAO/G701D
S16	N/N	G/G or A/A	G/A	A/A	G701D/G701D

SLC4A1 variant. Six of seven *SLC4A1* mutations (SAO, C479W, R589C, S773P, R602H, and A858D) were found as heterozygous genotypes. The difference plots of these heterozygous mutants could clearly be distinguished from that of homozygous wild-type (Fig. 1). The G701D mutation was found as both heterozygous and homozygous genotypes. The difference plots of heterozygous G701D could be distinguished from that of homozygous wild-type but the difference plot of homozygous G701D was clustered with that of homozygous wild-type (Fig. 1). The homozygous G701D was further analyzed by spiking with a DNA sample carrying known homozygous wild-type before the PCR and HRM analysis. This allowed generation of heteroduplex DNA molecules, which made as successful a detection as that for the heterozygous G701D.

Four *SLC4A1* SNPs (M31T, K1+11, IVS3+701 and IVS17+19) had the distinguishable difference plots of all three genotypes (Fig. 2). The AA and GG genotypes of K56E were not initially distinguishable from each other (Fig. 2) but they were after spiking with a DNA sample with the AA genotype. Six *SLC4A1* SNPs (D38A, IVS5+27, E72D, F266F, S438S and IVS17-48) that contained two genotypes showed distinguishable difference plots for each genotype (Fig. 2).

Analysis of SAO and G701D mutations by the HRM method

To evaluate reproducibility of the HRM method for analysis of SAO and G701D mutations, 16 DNA samples highly enriched with these two mutations (SAO and G701D) were blindly taken from our collection. These samples were subjected to the HRM analysis. The melting profiles and the difference plots of SAO and G701D mutations are illustrated in Fig. 3. The difference plots of 16 DNA samples showed that 4 samples had heterozygous SAO mutation while 12 samples had no SAO mutation. The first scan of G701D mutation revealed that 5 samples contained heterozygous G701D mutation but 11 samples had either homozygous wild-type or homozygous G701D. These samples were further analyzed by spiking with a sample carrying homozygous wild-type which could distinguish five samples with homozygous G701D from six samples with homozygous wild-type (Fig. 3, Table 2). The conventional methods for detection of *SLC4A1* mutations, PCR for SAO (27 bp deletion) and PCR-*Hpa*II digestion for G701D were also performed in order to compare the results with that of the HRM analysis (Fig. 4). The results of the HRM analysis and that of the conventional methods were completely compatible.

To evaluate sensitivity of the HRM method for detection of SAO and G701D mutations, we conducted the melting analyses for 28 and 40 samples with these two mutations, respectively, in order to determine the results with true positivity and false negativity. To evaluate its specificity, we performed the same analysis for 48 and 49 control samples (without mutations at the two corresponding positions, respectively) in order to determine the results with true negativity and false positivity, as described by Norambuena et al. [17]. The samples for sensitivity and specificity experiments were placed alongside to each other in the same run using 384-well format (i.e., 28 samples with SAO and 48 control samples were placed in wells A1 to D4, and 40 samples with G701D and 49 control samples were placed in wells A1 to D17 in a different experiment). Altogether, we found that all 28 samples with SAO and all 40 samples with G701D were positive, without false negativity (Supplementary Table 1). Thus, sensitivity of the HRM method for detection of both SAO and G701D mutations is 100%. Two of 48 control samples (for the SAO mutation) showed the results with false positivity; the specificity of the HRM method for detection at this position is 95.83%. The amplicons of two samples with the false-positive results were analyzed by DNA sequencing, which were found to also carry a known SNP (rs5015); otherwise, the specificity should reach 100%. One of 49 control sample (for the G701D mutation) showed the result with false positivity once in the duplicate assays; the specificity at this position is thus 98.98%. Furthermore, we tested well-to-well variations of the LightCycler 480 II machine in the HRM analysis for detection of SAO and G701D mutations. Slight shifts of melting curves were observed in the HRM analysis using both 96-well and 384-well formats but almost all of these did not cause any change in reading the results. However, greater variations of the results were noticed in one of the two replicate assays in one each of 46 wells tested (using 384-well format) for analysis of the two mutations that could lead to errors in reading the results (Supplementary Fig. 1).

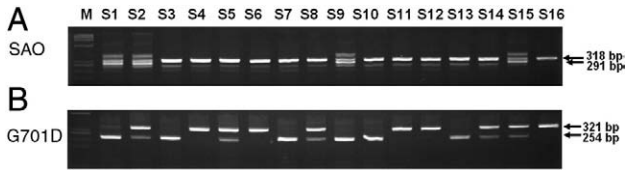

Fig. 4. Genotyping of SAO and G701D mutations by agarose gel electrophoresis. (A) The SAO mutation was analyzed by amplification of exon 11 of *SLC4A1* gene. Lane 1 is standard DNA markers, lanes 2–16 are tested DNA samples; normal samples produced a fragment of 318 bp, and samples with heterozygous SAO mutation generated two fragments of 318 and 291 bp as well as two bands of heteroduplex DNA. (B) The G701D mutation was analyzed by amplification of exon 17 of *SLC4A1* gene, followed by *Hpa*II digestion. Lane 1 is standard DNA markers, lanes 2–16 are tested DNA samples; normal samples showed a digested fragment of 254 bp but no observable fragment of 67 bp, samples with homozygous G701D mutation resulted in an undigested fragment of 321 bp, and samples with heterozygous G701D mutation contained both 254 and 321 bp fragments.

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Discussion

High-resolution melting (HRM) analysis of amplicon by using double-stranded DNA dye was introduced by Wittwer et al. [10]. The advantages of the HRM method for analysis of genetic variants are that amplification and detection steps can be conducted in the same reaction tube (or well), post-PCR processing is not needed, only standard PCR reagents and a saturating DNA dye are required for the analysis, and the process is rapid and has high through-put. In addition, the HRM method can identify heterozygous single-base changes in PCR products with a sensitivity >95% and specificity >99% [18]. Thus, this single close-tube method has recently become popular for detection and scanning of mutations in many genes [11–13,19–27].

In the present study, we have developed and evaluated the PCR and HRM method for detection of *SLC4A1* mutations and polymorphisms. The detection of *SLC4A1* variants was successful by matching the difference plot patterns of amplicon melting with that of known *SLC4A1* variants that had been confirmed by DNA sequencing. The results showed that all known 18 *SLC4A1* variants could be detected by the HRM analysis. It was also shown in the experiment that the deletional mutation attributable to the absence of 27 bp in *SLC4A1* exon 11 causing SAO could be detected as well as the mutations resulted from single-base substitutions or SNPs. The difference between melting temperature (Tm) of heterozygous genotype and that of homozygous genotype is approximately 1.0 °C or lower [10], depending on nature of base change and length of amplicon, but it has readily been identified by difference plot in the HRM analysis [10]. In some cases, however, the melting curves and difference plots in the HRM analysis of the samples with either homozygous wild-type or homozygous variant could not be differentiated. The second HRM analysis after spiking the tested samples with the one that was known to carry the homozygous wild-type genotype was required to allow the detection of heteroduplex DNA molecules. Using this approach, the detection of all homozygous *SLC4A1* variants was achievable.

We also evaluated the reproducibility of the HRM method for detection of SAO and G701D mutations by blind experiments of DNA samples enriched with these two mutations—the compound heterozygous condition of SAO and G701D mutations or homozygous G701D mutation are common causes of AR dRTA in pediatric patients in Thailand and other Southeast Asian countries. The results of the HRM analysis for these two mutations in the blind studies of DNA samples were completely compatible with those which were analyzed by the conventional methods including PCR or PCR-RFLP and gel electrophoresis method. Furthermore, the sensitivities of the HRM method for detection of SAO and G701D mutations were found to attain at the levels of 100% and their specificities were 95.83% and 98.98%, respectively. The sensitivities and specificities might be reduced by the effect of well-to-well variation if the tested samples were spread out within the block of the LightCycler machine. From the results of our study, we found that the HRM analysis is a rapid and efficient single-close-tube method for detection of *SLC4A1* mutations and polymorphisms. It should be a method of choice for screening carriers with *SLC4A1* mutations causing AR dRTA in the affected families and for using in prenatal diagnosis of AD or AR dRTA resulted from known *SLC4A1* mutations in the affected families.

For unknown *SLC4A1* mutations in unrelated patients with dRTA, the HRM method is able to be used as an initial screening prior to DNA sequencing. Heterozygous unknown *SLC4A1* mutations should easily be detected as their heteroduplex molecules are produced. To detect homozygous unknown *SLC4A1* mutations, it will also require spiking the tested samples with a known homozygous sample. In some particular situation where two mutations lie in the same amplicon, the HRM result, if positive, will only be an indicator for a definite identification by DNA sequencing. For the mutations that occur in a CG-rich region, they may not be detected by the HRM method but should be analyzed by the direct DNA sequencing technique.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.clinbiochem.2009.12.010.

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Band 3 Edmonton I, a novel mutant of the anion exchanger 1 causing spherocytosis and distal renal tubular acidosis

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Running title: Mis-trafficking of a new AE1 mutant

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ABSTRACT

Distal renal tubular acidosis (dRTA)[2] and hereditary spherocytosis (HS) are two diseases that can be caused by mutations in the gene encoding the anion exchanger 1 (AE1, Band 3). dRTA is characterized by defective urinary acidification, leading to metabolic acidosis, renal stones and failure to thrive. HS results in anemia, which may require regular blood transfusions and splenectomy. Mutations in the gene encoding AE1 rarely cause both HS and dRTA. Here, we describe a novel AE1 mutation, Band 3 Edmonton I, which causes dominant HS and recessive dRTA. The patient is a compound heterozygote with the new mutation, C479W and the previously described mutation, G701D. Red blood cells from the patient presented a reduced amount of AE1. Expression in a kidney cell line showed that kAE1 C479W is retained intracellularly. As kAE1 is a dimer, we performed co-expression studies and found that in kidney cells, kAE1 C479W and G701D proteins traffic independently from each other despite their ability to form heterodimers. Therefore, the patient carries one kAE1 mutant that is retained in the Golgi (G701D) and another kAE1 mutant (C479W) located in the endoplasmic reticulum of kidney cells, and is thus likely unable to reabsorb bicarbonate into the blood. We conclude that the C479W mutant is a novel trafficking mutant of AE1, which causes HS due to a decreased cell surface AE1 protein and results in dRTA due to its intracellular retention in kidney.

Keywords

Anion exchanger 1, membrane proteins, kidney, red blood cells, hereditary disease, trafficking.

[1] These authors contributed equally to this work

[2]**Abbreviation list**: AE1, anion exchanger 1; C479W, Cys479 Trp; dRTA, distal renal tubular acidosis; Endo-H, endoglycosidase H; G701D, Gly701 Asp; GPA, glycophorin A; HA, hemagglutinin; HRP, horseradish peroxidase; HS, hereditary spherocytosis; ICC, intercalated cells; MDCK, Madin-Darby canaine kidney cells; PNGase-F, peptide-N-glycosidase F; SAO, Southeast Asia ovalocytosis; TM, transmembrane domain; WT, wild-type.

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INTRODUCTION

The human anion exchanger 1 (AE1), encoded by the *SLC4A1* gene, is a dimeric or tetrameric membrane glycoprotein which exchanges bicarbonate for chloride in red blood cells (RBC) and in type A intercalated cells (ICCs) in kidneys (kAE1) [1]. It plays a central role for optimization of respiration by participating in CO_2 removal and in the maintenance of acid-base balance in kidneys [2].

Naturally occurring mutations in the *SLC4A1* gene can lead to hereditary spherocytosis (HS), a disease that can cause severe anemia [3], or distal renal tubular acidosis (dRTA) [4], characterized by nephrocalcinosis, metabolic acidosis and failure to thrive. dRTA mutations can be either dominantly or recessively inherited. Patients with a different mutation in each allele are compound heterozygotes. Dominantly inherited dRTA mutants alter the normal trafficking of kAE1 WT in kidney cells, while recessively inherited mutants can have their trafficking facilitated by the co-expression with kAE1 WT [5-8].

Mutations that cause both HS and dRTA are extremely rare. RBC-specific proteins such as glycophorin A (GPA) may act as chaperones to improve eAE1 trafficking to the RBC surface, and thus might correct targeting defects caused by dRTA mutations in RBC. However, these proteins are absent from ICCs, thus patients develop dRTA. Patients with HS and dRTA are either homozygous or compound heterozygous. There are only two case reports of patients homozygous for AE1 mutations that cause both dRTA and HS. Band 3 Coimbra (V488M) was found in a newborn with severe HS, who also developed dRTA [9]. The patients' RBC did not contain detectable AE1 [10], and when expressed in the kidney epithelial cell line Madin-Darby canine kidney (MDCK), the AE1 mutant was retained intracellularly [11]. Band 3 Courcouronnes, was described in a homozygous patient carrying the S667F substitution [12], resulting in HS and incomplete dRTA. This mutant was retained intracellularly when expressed in MDCK cells, but was partially found at the cell surface when co-expressed with GPA in *Xenopus* oocytes. Compound heterozygote AE1 gene mutations have previously been reported in Asia. These patients who usually have dRTA but no HS carry a Southeast Asia Ovalocytosis (SAO) allele (that causes a condition where RBC are abnormally rigid) and another mutation [13]. Recently, a compound heterozygous patient carrying the E522K and G701D mutations was described with both HS and dRTA [14]. In kidney epithelial cells, the E522K/G701D heterodimers were retained intracellularly, likely causing complete dRTA in this patient.

We report another compound heterozygote patient carrying the G701D mutation and a novel C479W mutation on the other allele. The patient displayed severe HS and complete dRTA as an infant. In this paper, we describe the characterization of the molecular and cellular defects associated with the combination of these two mutations.

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EXPERIMENTAL

Case report

The patient is a 19 years female with Caucasian Scandinavian origin. The patient was diagnosed with severe HS early after birth requiring splenectomy at age 3 with improvement of her hematological condition. Failure to thrive and nephrocalcinosis detected at age 2.5 led to diagnosis of complete dRTA, which improved after prescription of daily oral sodium bicarbonate and potassium chloride. The patient did not present auditory deficits or cognitive impairment. The father has mild HS but no renal symptoms, and the mother is healthy. No other family member has dRTA. Protocols involved in this project have been reviewed and approved by the Health Research Ethics Board (Biomedical panel) at the University of Alberta.

Analysis of *SLC4A1* mutations

Genomic DNA was isolated from blood samples drawn from the patient, her parents and two siblings. *SLC4A1* was analyzed by polymerase chain reaction and a standard dye-terminator cycle sequencing method. The segregation of mutations in the family was examined by the PCR and restriction fragment length polymorphism or derived cleaved amplified polymorphism methods. Analysis indicated that the patient carries nucleotide substitutions in exons 13 (TGC>TGG) and 17 (GGC>GAC), resulting in amino acid changes C479W (band 3 Edmonton I) and G701D (band 3 Bangkok I), respectively (Figure 1 B). The Memphis I polymorphism (K56E), which was previously found associated with the G701D mutation in Southeast Asia [15], is not present in this family, suggesting an independent G701D mutation. To our knowledge, this case is the first reported instance of G701D in a non-Asian family.

Analysis of red blood cell membrane

40 µg of total erythrocyte membrane proteins per lane were loaded on 8 % SDS-PAGE gel followed by Coomassie blue staining. For western blots, 10 µg of total proteins per lane were loaded on 8 % SDS-PAGE gel. After migration, proteins were transferred on a nitrocellulose membrane, blocked with 3 % skimmed milk, incubated with a rabbit antibody detecting the last 15 residues of AE1 (a kind gift from Dr. Reithmeier, University of Toronto) followed by a goat anti-rabbit antibody coupled to horseradish peroxidase (HRP) (Jackson Immunoresearch, West Grove, PA, USA). Relative quantification of the band intensity of proteins was performed using the ImageJ software.

Construction of AE1 mutants

Human kidney (kAE1) cDNA, containing the sequence encoding a HA epitope in position 557 of the protein, and the kAE1 G701D cDNA cloned into the viral vector pFB-Neo (Stratagene, La Jolla, CA, USA) were previously described [5]. The kAE1-HA557 wild-type (WT) construct was submitted to site-directed mutagenesis using QuikChange site-directed mutagenesis kit (Stratagene). Using the QuikChange site-directed mutagenesis kit, we also introduced the C479W mutation in kAE1 devoid of HA epitope, subcloned in pcDNA3 vector (Invitrogen, Carlsbad, CA, USA) [16]. The presence of mutations was verified by automated DNA sequencing.

Cell culture, transfections and viral infections

Viral infections were performed as previously described [5]. Briefly, HEK 293 cells were transfected with p-VPack-GP, p-VPack-VSV-G and pFB Neo kAE1-HA557 WT or mutant plasmids using FuGENE 6 (Roche Applied Science, Indianapolis, IN, USA). Cell culture supernatant containing infectious viral particles were added to dividing MDCK cells complemented with 8 µg / ml of polybrene. After 24 hours incubation, cells expressing AE1 were selected with 1 mg / ml geneticin. Despite the constant presence of geneticin in the cell culture medium, the level of kAE1 protein expression in MDCK cells progressively decreases within 2 to 3 weeks. Consequently, all our

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experiments are performed within 3 weeks post-infection on a heterogeneous population of MDCK cells that have not been cloned.

Western-blots and enzymatic deglycosylation

MDCK cells expressing kAE1-HA557 WT or mutants were lysed in PBS containing 1 % Triton X-100 and protease inhibitors (Sigma Aldrich, St Louis, MO, USA), and 1 / 10 of cell lysate supernatant was either left untreated or digested using 1,000 units of endoglycosidase H (endo H, New England Biolabs (NEB), Mississauga, ON, Canada), or 500 units of peptide-N-glycosidase F (PNGase-F, NEB) at 37°C for 1 hour. Samples were then loaded on 8 % SDS-PAGE gel, proteins detected with mouse anti-HA antibody (Covance, Emeryville, CA, USA) followed by a goat anti-mouse antibody coupled to HRP.

Expression and functional assay in *Xenopus* oocytes

Erythroid AE1 (eAE1) WT cloned in pSP65 plasmid was used to substitute cysteine 479 by a tryptophan by PCR with the QuikChange site-directed mutagenesis kit (Stratagene). One positive clone was entirely sequenced before further use. 10 ng of eAE1 WT or C479W mutant and 2.5 ng of GPA were co-injected and oocytes were kept at 19°C in modified Barth saline (composition in mM: NaCl: 85; KCl: 1; $NaHCO_3$: 2.4; $MgSO_4$: 0.82; $Ca(NO_3)_2$: 0.33; $CaCl_2$: 0.41; HEPES: 10; NaOH: 4.5; pH 7.4; 10 U/ml penicillin and 10µg/ml streptomycin). Measurements of intracellular pH (pHi) variations were done by incubation of *Xenopus* oocytes in the following medium (mM): NaCl 63.4; KCl 1; HCO_3^- 24; $MgSO_4$ 0.82; $Ca(NO_3)_2$ 0.33; $CaCl_2$ 0.41; HEPES/NaOH 5, pH 7.35; CO_2 5%, O_2 95% until an equilibrium is reached. Then, oocytes were bathed in a medium without Cl^- (NaGluconate 63.4 mM; KGluconate 1 mM; HCO_3^- 24 mM; $MgSO_4$ 0.82 mM; $Ca(NO_3)_2$ 0.74 mM HEPES/NaOH 5 mM pH 7.35, CO_2 5%, O_2 95%). Traces are representative of intracellular pHi recordings from different oocytes expressing eAE1 WT or C479W mutant co-expressed with GPA (hGPA-BSXG plasmid was a kind gift of Dr. Ashley Toye, University of Bristol) to ensure maximal expression of the mutant.

Cell surface biotinylation

MDCK cells expressing kAE1-HA557 WT or mutant were incubated with 0.5 mg / ml of EZ-link NHS-SS-Biotin (MJS Biolynx, Brockville, ON, Canada) for 15 minutes at 4°C. Cells were then washed with PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.35) containing 0.3 % BSA, lysed and after centrifugation, an aliquot (50 µl) was saved for Western-blotting. UltraLink Immobilized monomeric avidin (Thermo Scientific, Rockford, IL, USA) was added to the cell lysates at 4°C for 1 hour. Eluted proteins were loaded on 8 % SDS-PAGE gels and proteins were detected using the mouse anti-HA antibody (Covance).

Immunocytochemistry

MDCK cells expressing kAE1-HA557 WT or mutants were grown on glass coverslips or on semi-permeable Transwell polycarbonate filters (Corning Inc., Corning, NY, USA), fixed, permeabilized or kept intact and blocked with 1 % BSA. Cells were then incubated with mouse (Covance) or rat anti-HA (Roche, Basel, Switzerland) antibody, rabbit anti-giantin antibody (Covance), rabbit anti-calnexin antibody (kind gift from Dr. David Williams, University of Toronto), or rat anti-E-cadherin antibody (Sigma). Secondary antibodies were goat anti-rabbit antibody coupled to Alexa 488 (Molecular Probes, Carlsbad, CA, USA), donkey anti-rat antibody coupled to Alexa 488 (Molecular Probes), donkey anti-rat antibody coupled to Cy5 (Jackson Immunoresearch), goat anti-mouse coupled to Cy3 (Jackson Immunoresearch) or to Alexa 488 (Molecular Probes). HEK 293 or MDCK cells transiently transfected with kAE1 C479W (with no HA epitope) were fixed, permeabilized and blocked with 1 % BSA. Cells were then incubated with either mouse Bric155 antibody (IBGRL, Bristol, England) or a rabbit antibody detecting the last 15 residues of AE1 (gift from Dr. Reithmeier), followed by anti-mouse or anti-rabbit antibodies coupled to Cy3 (Jackson Immunoresearch). Samples were examined using an Olympus IX81 microscope equipped with a Nipkow spinning-disk optimized by Quorum Technologies (Guelph, ON, Canada).

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Oocytes used for immunofluorescence detection of AE1 were from the same batches as the ones used for pHi measurements. Oocytes were fixed 20 minutes in 3% paraformaldehyde Phosphate Buffer 100 mM pH 7.4 (PBS), then rinced in PBS and incubated overnight in PBS 15% sucrose at 4 °C. Four oocytes for each experimental condition were frozen simultaneously in tissue freezing medium (Jung, Leica Microsystems Nussloch, Heidelberg, Germany) by immersion in isopentane at -180°C and cryosectioned (7 µm, Leica CM3050).
Cryosections were placed on silane coated glass slides (Electron Microscopy Sciences, Hatfield, PA, USA) and heated for 1 hour at 50°C. Samples were then incubated in PBS 1% Bovine Serum Albumin (BSA) before addition of antibodies against AE1 N-terminal domain (anti-CDB3 1/100), at room temperature for 1 hour. Following washing with PBS 1% BSA, a 1/1000 dilution of fluorescein isothiocyanate-labeled anti-rabbit antibody was added for 1 hour at room temperature. The samples were washed in PBS and mounted. Immunofluorescence was carried out at room temperature and samples were viewed using an Axioplan2 Zeiss microscope.

Immunoprecipitations

MDCK cells expressing kAE1-HA557 WT or mutants were lysed in PBS containing 1 % Triton X-100 and protease inhibitors. Aliquots of the cell lysates were saved as total fraction, the remaining cell lysates were incubated with rabbit anti-myc antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), followed by Protein G-sepharose. The bound AE1 proteins were eluted with Laemmli buffer before detection by western blot using a mouse anti-HA antibody (Covance).

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RESULTS

The red blood cells from the patient carry reduced amount of plasma membrane AE1

Sequencing of the *SLC4A1* gene indicated that the patient is compound heterozygote with the previously described G701D substitution (Band 3 Bangkok I) [15] that she inherited from her healthy mother, and a novel C479W mutation, designated as *'Band 3 Edmonton I'*. As the father who is heterozygous for the C479W novel mutation has HS only (Figure 1 A), this new mutation is dominantly inherited for HS. Since the C479W mutation only causes dRTA when combined with the recessive dRTA mutation G701D [15], we conclude that the novel C479W mutation is recessive for dRTA. HS typically occurs when α- or β-spectrin, ankyrin, protein 4.2 or AE1 proteins are defective and reduced in RBC [17]. To measure the amount of plasma membrane proteins in the patient's RBC, we performed an SDS-PAGE followed by staining using Coomassie blue on ghost membranes (Figure 2 A). Spectrin, protein 4.1 and 4.2 amounts do not display obvious reduction (Figure 2 A). However, AE1 was decreased to about 65 % and 80 % of normal amount in the patient's and father's RBC, respectively (Figure 2 B). The intensity of two lower molecular weight bands (approx. 70 kDa and 35 kDa as indicated by arrowheads) that may correspond to AE1 degradation fragments was also reduced in the patient's RBC. The amount of ankyrin is also slightly decreased. The decreased amount of AE1 in patient and father's RBC is likely the reason for appearance of HS symptoms.

The kAE1 C479W mutant is not functional at the surface of Xenopus oocytes

To test whether the new C479W mutation affects AE1's function, we expressed and tested the presence of functional eAE1 WT and C479W at the cell surface of *Xenopus* oocytes by immunostaining (Figure 3 A). Glycophorin A is a protein acting like a chaperone that facilitates the surface trafficking of eAE1 in RBC [13]. In absence or presence of glycophorin A, the eAE1 C479W mutant protein was detected at the cell surface, in contrast with oocytes where no cRNA was injected or in absence of primary antibody. We next compared the functional activity of eAE1 WT or C479W mutant expressed at the cell surface of *Xenopus* oocytes by measuring variations of the intracellular pH over time, in presence or absence of extracellular chloride. If the AE1 protein is functional, switching the chloride-containing extracellular solution to one containing gluconate will result in intracellular alkalinization caused by the exchange of intracellular chloride with extracellular bicarbonate [18]. Figure 3 B shows that in contrast with eAE1 WT, there was no alkalinization in oocytes expressing eAE1 C479W, indicating that this novel mutant is inactive in *Xenopus* oocytes.

In MDCK cells, the kAE1 C479W mutant does not carry complex glycosylation

To evaluate the effect of the new C479W mutation on kAE1 biosynthesis in kidney cells, we introduced the C479W mutation in the cDNA encoding human kidney AE1 (kAE1 C479W) and expressed it in MDCK cells that do not express endogenous AE1 [19, 20]. This construct as well as kAE1 WT contains an extracellular hemaglutinin (HA) epitope in position 557, a modification that does not affect folding or trafficking of AE1 [5, 20, 21]. kAE1-HA557 WT (later on referred to as kAE1 WT) is targeted to the basolateral membrane of polarized MDCK cells [5, 20], a similar location to that in type A-intercalated cells. Immunoblots from MDCK cells indicated that kAE1 WT migrates as two major bands (Figure 4 A). kAE1 WT carries a single N-glycosylation site in position 642. Figure 4 B shows that the upper band is endoglycosidase H (endo H)-resistant (lane H) but peptide-N-glycosidase F (PNGase-F)-sensitive (lane F), indicating that in MDCK cells, kAE1 WT was processed to complex oligosaccharide and had moved from the ER to the medial Golgi. Processing of kAE1 protein was efficient in MDCK cells as 73 % of kAE1 WT carried complex oligosaccharide. The lower

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molecular weight band, endo-H and PNGase F sensitive, corresponds to kAE1 WT in the ER carrying high mannose oligosaccharide [5].
kAE1 G701D also carries complex oligosaccharide but kAE1 G701D's processing in the medial Golgi is less efficient than in MDCK cells expressing kAE1 WT as seen by the slight predominance of the core-glycosylated fraction (53 % of high mannose glycosylated kAE1 G701D) [5]. In contrast, the kAE1 C479W mutant runs as a low-molecular weight band corresponding to high mannose-glycosylated kAE1 protein. These experiments suggest that the intracellular trafficking of the kAE1 C479W mutant from the ER is impaired in MDCK cells.

The kAE1 C479W mutant does not traffic to the cell surface in kidney cells

We determined the level of cell surface expression of kAE1 WT, G701D and C479W proteins by cell surface biotinylation (Figure 5 A), and immunostaining on MDCK cells (Figure 5 B and C). In contrast with kAE1 WT, neither the kAE1 C479W nor the G701D mutant were biotinylated and thus neither were detectable at the cell surface (figure 5 A). The absence of biotinylated GAPDH indicated that no biotinylation reagent had leaked inside cells during the experiment. As kAE1 G701D or C479W did not seem to reach the plasma membrane, we determined their respective intracellular location by immunostaining in MDCK cells (Figure 5 B and C). kAE1 WT was predominantly located at the plasma membrane and displayed minimal co-localization with the ER-marker calnexin (Figure 5 B). In polarized MDCK cells, kAE1 WT co-localized with the basolateral marker E-cadherin (Figure 5 C). The kAE1 G701D mutant showed partial co-localization with calnexin but was predominantly retained in the Golgi, as seen by co-localization with giantin [5]. However, the kAE1 C479W mutant co-localized with calnexin, indicating that this new mutant is retained in the ER. When expressed in HEK 293 or MDCK cells, the kAE1 C479W mutant protein devoid of HA epitope was also retained intracellularly (data not shown), indicating that the HA epitope did not cause the intracellular retention of kAE1 C479W mutant. Neither of the mutants co-localized with the basolateral membrane marker E-cadherin in polarized MDCK cells. Interestingly, the kAE1 C479W mutant was not present at the cell surface of kidney MDCK cells but eAE1 C479W mutant trafficked to the plasma membrane of *Xenopus* oocytes. This suggests that the cellular environment plays a role in trafficking of the C479W mutant, as was previously observed for other dRTA mutants such as eAE1 or kAE1 R589H, S613F [5, 18, 20] or kAE1 R901X (Walton) [5, 18-20, 22]. These results indicate that trafficking of the novel kAE1 C479W mutant is altered in kidney cells.

The kAE1 C479W mutant can physically interact with the kAE1 G701D mutant in MDCK cells

AE1 forms dimers [23] and individuals carrying normal and mutant AE1 or two different AE1 mutants may express AE1 heterodimers in their kidney cells. To determine the molecular mechanisms resulting in dRTA in the patient's kidney, we co-expressed kAE1 G701D and C479W in MDCK cells, as well as kAE1 WT with either kAE1 G701D or kAE1 C479W to mimic the situation in the parent's kidney cells. To distinguish kAE1 mutant proteins from kAE1 WT, we introduced different epitopes in position 557: kAE1 G701D or kAE1 WT carried a myc epitope while kAE1 C479W protein carried an HA epitope. We determined whether kAE1 WT-myc or G701D-myc protein could physically interact with kAE1 C479W-HA mutant in MDCK cells using co-immunoprecipitation. Figure 6 A shows that kAE1 C479W mutant can physically interact with kAE1 WT or with kAE1 G701D mutant.

When co-expressed, the kAE1 C479W and G701D mutants do not overlap

We then examined the respective location of kAE1 C479W and G701D within MDCK cells by immunostaining (Figure 6 B). When co-expressed in polarized MDCK cells, we observed that the kAE1 G701D and C479W mutants remained located in the Golgi and in the ER, respectively. Neither

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protein was localized to the cell surface. Despite their ability to physically interact, no obvious change of individual mutants' cellular location was detectable in cells co-expressing both proteins (compare Figure 4 B and 6 B). This suggests that the kAE1 G701D and C479W mutants do not substantially overlap at sites where either protein is present in high concentrations in kidney cells, and the patient likely does not have functional kAE1 protein at the basolateral membrane of intercalated cells.

We next wondered whether the co-expression of kAE1 WT and kAE1 G701D or C479W mutant could restore cell surface trafficking of the mutant in MDCK cells. As the patient's father and mother do not display kidney symptoms, we anticipate that sufficient amount of functional kAE1 is present at the basolateral membrane of type-A intercalated cells in these individuals. We thus co-expressed kAE1 WT and C479W or G701D mutant in MDCK cells, and we investigated whether the co-expression of kAE1 WT could restore the mutant's trafficking to the cell surface by immunofluorescence (Figure 6 C). We used the extracellular HA epitope to determine if co-expressing kAE1 WT-myc could restore trafficking of kAE1 C479W-HA or kAE1 G701D-HA mutants to the plasma membrane of MDCK cells: blue staining corresponds to kAE1 mutant whose trafficking to the cell surface has been restored by the presence of kAE1 WT stained in red. Although kAE1 WT-myc could partially restore trafficking of kAE1 G701D-HA in MDCK cells, there was no plasma membrane kAE1 C479W-HA mutant in MDCK cells co-expressing kAE1 WT-myc (Figure 6 C), indicating that kAE1 WT cannot restore the cell surface trafficking of the kAE1 C479W mutant.

DISCUSSION

We report the case of a new patient with both HS and complete dRTA, who is compound heterozygote for two *SLC4A1* mutations – the previously described G701D mutation and a novel C479W mutation (Figure 1 C). Our data indicate that the patient's RBC display a 35 % reduction of plasma membrane AE1 (Figure 2), which likely results in decreased anion transport function in RBC and in destabilizing interactions between cytoskeleton and plasma membrane [17].

The G701D mutation was previously described in compound heterozygous dRTA patients from South East Asia, in combination with either SAO or other dRTA mutations [13, 15, 24, 25]. Our patient is the second compound heterozygous individual with both HS and dRTA, as G701D was recently found in a compound heterozygous Taiwanese patient in combination with the novel E522K mutation [14]. Interestingly, our patient has a Caucasian origin and is therefore the first one reported with the G701D mutation in a non-Asian background. When expressed in *Xenopus* oocytes, the G701D mutant was retained intracellularly unless co-expressed with GPA [13, 15]. These results indicate that GPA may act like a chaperone on the trafficking of AE1 in RBC [26]. In contrast, our results indicate that in absence or presence of GPA, the C479W mutant is targeted to the surface of *Xenopus* oocytes (Figure 3). However, given that the patient's RBC display approximately a 35 % reduction of plasma membrane AE1 (Figure 2), we hypothesize either that the cell surface location of the mutants in *Xenopus* oocytes does not reflect trafficking of the mutant proteins in RBC, or that recovery of the mutant protein's trafficking by GPA is incomplete in RBC. Further investigations are required to fully understand the molecular basis for HS in this patient.

The patient also developed dRTA and nephrocalcinosis at an early age. To investigate the molecular mechanisms for development of dRTA, we expressed the kAE1 WT, G701D or C479W proteins in MDCK cells. As previously observed [5, 7], the kAE1 G701D mutant was predominantly located in the Golgi in MDCK cells (Figure 5 B and C). In MDCK cells, we determined that the kAE1 C479W mutant did not carry complex oligosaccharides (figure 4), did not traffic to the plasma membrane (figure 5 A) and predominantly co-localized with the ER marker calnexin (figure 5 C and D). The intracellular retention of kAE1 G701D and C479W in MDCK cells indicate that these two mutant proteins have a trafficking defect in kidney cells. As kAE1 protein is a dimer, we next mimicked the

situation found in the kidneys of the mother and father's patient by co-expressing kAE1 WT with either kAE1 G701D or C479W in MDCK cells. We found that kAE1 WT could rescue the trafficking of kAE1 G701D to the cell surface as was previously observed (Figure 6 C) [5, 7, 27]. However, no cell surface kAE1 C479W protein was detectable when co-expressed with kAE1 WT in MDCK cells. This result is in contrast with previous findings on other recessive dRTA mutants whose trafficking to the plasma membrane was restored by co-expression with kAE1 WT [5, 7], including the recently described case of a novel compound heterozygous patient carrying the E522K and G701D mutations. Further, despite their ability to interact with each other (Figure 6 A), the two kAE1 G701D and C479W mutants were both retained intracellularly and did not substantially overlap when co-expressed in polarized MDCK cells (Figure 6 B). These results suggest that kAE1 G701D / C479W heterodimers may represent a minority of kAE1 dimers in cells co-expressing both proteins or may be prematurely degraded. Together, these data suggest that the patient, who is compound heterozygous for the G701D and C479W mutations, does not have functional kAE1 at the basolateral membrane of intercalated cells, and thus developed dRTA.

The highly conserved cysteine 479 is one of the three cysteine residues located in the transmembrane domain of AE1 (Figure 1 B) [28]. AE1 in which all five cysteine residues were replaced by serine was functional, suggesting that a cysteine residue in position 479 is not absolutely required for the protein's function [29]. However, it is possible that substituting a cysteine into a bulky tryptophan residue at the membrane interface alters the structure of the region around transmembrane domain 3 (TM3) and forces TM3 to adopt a conformation unfavorable for the protein's function. Indeed, tryptophan residues display a striking preference for extracellular boundary of TM in membrane proteins [30, 31]. Alternatively, the C479W substitution may stabilize TM3 and reduce its conformational stability for substrate transport as cysteine 479 was proposed to be part of the pore-lining helix [29]. Consistently, our results indicate that in *Xenopus* oocytes, the C479W mutant located at the cell surface does not alkalinize the oocyte after an acid load, in contrast with AE1 WT, suggesting that the kAE1 C479W mutant is misfolded. As for other protein-misfolding-diseases such as nephrogenic diabetes insipidus [32], we hypothesize that protein synthesis quality control systems recognize and retain the C479W mutant intracellularly in kidney cells, which results in dRTA.

Cysteine 479 is located eleven amino acids away from the arginine 490, which dominantly causes HS when substituted with cysteine (Band 3 Bicetre I) [33]. In COS-7 and the erythroleukemia cell line K562, eAE1 R490C mutant does not traffic to the plasma membrane and is retained in the ER. Furthermore, the eAE1 R490C has a dominant-negative effect on the trafficking of the eAE1 WT protein [34].

In conclusion, the clinical recognition that this patient was different from thus far described patients with dRTA and HS led to a multidisciplinary collaborative investigation. Our results suggest that the new C479W mutation causes major trafficking defects in kAE1 protein in both RBC and kidney cells.

AUTHOR CONTRIBUTION

Carmen Chu and Naomi Woods contributed equally to this work, and performed the majority of the experiments under the supervision of Emmanuelle Cordat. Helene Guizouarn, Franck Borgese and Bernard Pellissier performed *Xenopus* oocytes experiments, Nunghathai Sawasdee sequenced the patient and family's DNA under the supervision of Pa-thai Yenchitsomanus, Manjula Gowrishankar is the clinician who is treating the patient.

ACKNOWLEDGMENTS

The authors thank the patient and family for their invaluable contribution to this study. We are also grateful to Sandra Ungarian for excellent technical assistance and to Drs. Reinhart Reithmeier and Ashley Toye for providing constructs and antibodies. Part of this work was presented at the annual ASN meeting 2008.

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FIGURE LEGENDS

Figure 1: The patient has a red blood cell defect and is compound heterozygote with a novel mutation on the SLC4A1 gene.

(**A**) Family pedigree of the patient. Arrow locates the patient with HS and dRTA. (**B**) Sequencing results obtained from blood samples of the patient and patient's father and mother. Exons 13 and 17 were amplified by PCR and subjected to direct sequencing. The patient showed nucleotide substitutions in exons 13 (TGC>TGG) and 17 (GGC>GAC), resulting in amino acid changes C479W (band 3 Edmonton I) and G701D (band 3 Bangkok I), respectively. The exon 13 (TGC>TGG) mutation was also observed in the patient's father while the exon 17 (GGC>GAC) mutation was detected in the patient's mother. (**C**) Topology model of the anion exchanger 1, showing the location of the novel mutation C479W and of the previously described G701D mutation. The Coimbra mutation (V488M) is also displayed. The "Y" letter on the fourth extracellular loop corresponds to the single N-glycosylation site on arginine 642.

Figure 2: The red blood cells from the patient carry reduced amount of plasma membrane AE1

(**A**) Ghosts were prepared and 40 μg of total proteins were loaded on 8 % SDS-PAGE gel before staining with Coomassie Blue. (**B**) 10 μg of total proteins from ghosts were loaded on 8 % SDS-PAGE gel and after transfer on a nitrocellulose membrane, AE1 was detected using a rabbit antibody detecting the 15 carboxyl-terminal residues of the protein, followed by an anti-rabbit antibody coupled to horseradish peroxidase (HRP). The table below the blot shows quantification of the amount of AE1 in red blood cell membranes. The quantification was performed using ImageJ software. Results are expressed in percent of AE1 in control red blood cell membranes.

Figure 3: The C479W mutant is inactive at the surface of Xenopus oocytes

(**A**) Cryosections of non-injected *Xenopus* oocytes or *Xenopus* oocytes expressing eAE1 WT or C479W that were incubated with a mouse monoclonal anti-AE1 N terminus (anti-CDB3) antibody (gift from Dr. Philip Low, Purdue University) followed by fluorescein isothiocyanate labeled anti-rabbit antibody. Samples were observed using an Axioplan 2 Zeiss microscope. (A) oocyte expressing eAE1 WT without primary antibody, (B) non-injected oocyte, (C) oocyte expressing eAE1 WT, (D) oocyte expressing eAE1 C479W, (E) oocyte co-expressing eAE1 C479W and GPA. Arrowheads in (C), (D) and (E) show staining at the plasma membrane. (**B**) Measurement of intracellular pH in Xenopus oocytes expressing eAE1 WT (left) or C479W (right) proteins and GPA, as outlined in the Material and Methods section.

Figure 4: In MDCK cells, the C479W mutant does not carry complex glycosylation

(**A**) Cell lysates from MDCK cells expressing kAE1 WT, G701D or C479W proteins were loaded on a 8 % SDS-PAGE gel (20 μg of total proteins per lane). After transfer on a nitrocellulose membrane, AE1 was detected using a mouse anti-HA antibody followed by anti-mouse antibody coupled to HRP. The table below the blot indicates the amount of AE1 protein carrying complex oligosaccharide as calculated using ImageJ software from 3 independent experiments. (**B**) Cell lysates from MDCK cells expressing kAE1 WT, G701D or C479W were either left untreated (lane C), incubated with endoglycosidase H (lane H) or peptide-N-glycanase F (lane F) for 1 hour at 37°C, prior to separation on a 8% SDS-PAGE gel. Proteins were detected using mouse anti-HA antibody followed by anti-mouse antibody coupled to HRP. Open circles correspond to protein carrying high mannose oligosaccharides, closed circle corresponds to AE1 carrying complex oligosaccharides, and arrowheads indicate non-glycosylated AE1 proteins.

Figure 5: The C479W AE1 mutant does not traffic to the cell surface in MDCK cells

(**A**) Intact, non-polarized MDCK cells expressing kAE1 WT, G701D or C479W proteins were incubated with the EZ-Link biotinylation reagent before quenching with PBS containing 0.3 % BSA.

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Cells were then lysed and samples were incubated with streptavidin beads. Bound proteins were eluted with 2X sample loading buffer. Fractions from total cell lysate (lane T) or 10 times concentrated bound fraction (lane B) were then loaded on an 8% SDS PAGE gel. Proteins were transferred on nitrocellulose membrane and AE1 was detected by western blot using mouse anti-HA and anti-mouse antibody coupled to HRP. The lack of cytoplasmic GAPDH biotinylation was also assessed using a mouse anti-GAPDH antibody. This experiment is representative of three independent experiments.
(**B**) MDCK cells expressing kAE1 WT, G701D or C479W proteins were fixed, permeabilized and blocked before incubation with rabbit anti-calnexin antibody or rabbit anti-giantin antibody and mouse anti-HA antibody. After washes, cells were incubated with anti-rabbit-Alexa 488 (green) and anti-mouse-Cy3 (red) antibodies.
(**C**) MDCK cells expressing kAE1 WT, G701D or C479W proteins were grown until polarization on semi-permeable polycarbonate filters, then fixed, permeabilized and incubated with rat anti-E-cadherin and mouse anti-HA antibodies. Following washes, anti-rat-Cy3 (red) and anti-mouse-Alexa 488 (green) antibodies were added to the samples, slides were mounted prior to observation using an Olympus IX81 spinning-disc confocal microscope. Yellow staining corresponds to overlap between red and green labeling. x-z corresponds to side view of the cells, x-y shows middle section of the cells. Bar represents 10 μm.

Figure 6: The C479W mutant does not overlap with the G701D mutant and co-expression with kAE1 WT does not rescue its trafficking to the cell surface

(**A**) Non-polarized MDCK cells co-expressing kAE1 WT-myc and kAE1 WT-HA, kAE1 WT-myc and kAE1 C479W-HA or kAE1 G701D-myc and kAE1 C479W-HA were lysed with 1 % triton X-100, an aliquot of the lysate was saved as Total fraction (T) and the remaining extracts were incubated with rabbit anti-myc antibody (IP) followed by protein G-Sepharose. Fraction from the total cell lysate or 15 times concentrated bound proteins (B) were eluted from the resin using Laemmli buffer and the presence of HA-tagged mutant proteins was detected by Western Blot using a mouse anti-HA antibody (IB) followed by an anti-mouse antibody coupled to HRP. This blot is representative of 5 independent experiments.
(**B**) Polarized MDCK cells co-expressing kAE1 WT-myc and kAE1 WT-HA or kAE1 G701D-myc and kAE1 C479W-HA were grown on semi-permeable polycarbonate filters, fixed, permeabilized and incubated with mouse anti-myc (Cell Signalling Technologies) and rat anti-HA (Roche) antibodies followed by donkey anti-rat antibody coupled to the red fluorophore Cy3 and goat anti-mouse coupled to the green fluorophore Alexa 488. Samples were examined using an Olympus IX81 spinning-disc confocal microscope. Bar represents 10 μm. x-y shows top view of the cells, x-z corresponds to side view of the cells.
(**C**) Intact non-polarized MDCK cells co-expressing kAE1 WT-myc and kAE1 WT-HA, kAE1 WT-myc and kAE1 G701D-HA or kAE1 C479W-HA were incubated with rat anti-HA antibody followed by goat anti-rat antibody coupled to Cy5 (blue). After permeabilization, cells were incubated with rat anti-HA and mouse anti-myc antibodies followed by donkey anti-mouse antibody coupled to the red fluorophore Cy3 and donkey anti-rat antibody coupled to Alexa 488 (green). Yellow colour corresponds to overlap between red and green, white colour corresponds to overlap between red, green and blue colors. Samples were observed using an Olympus IX81 spinning-disc confocal microscope. Bar corresponds to 10 μm.

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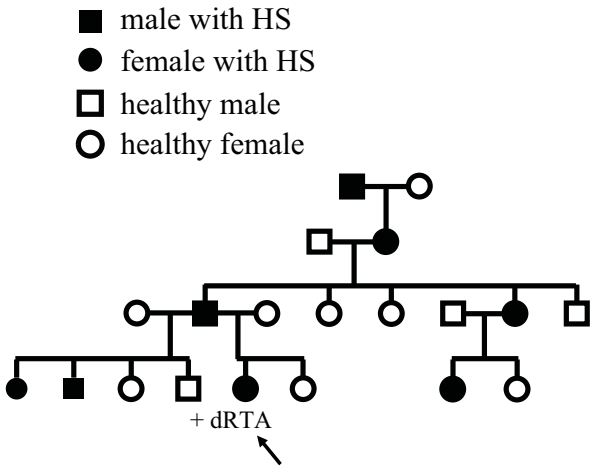
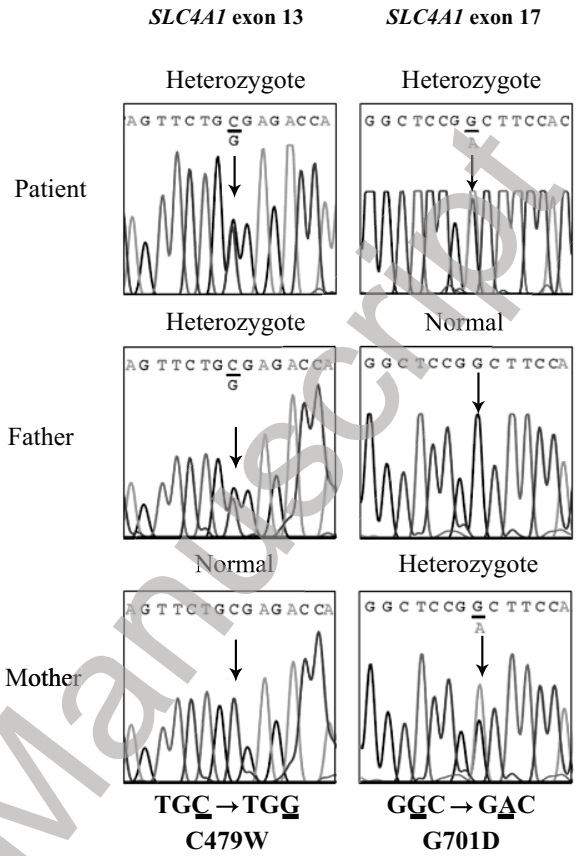
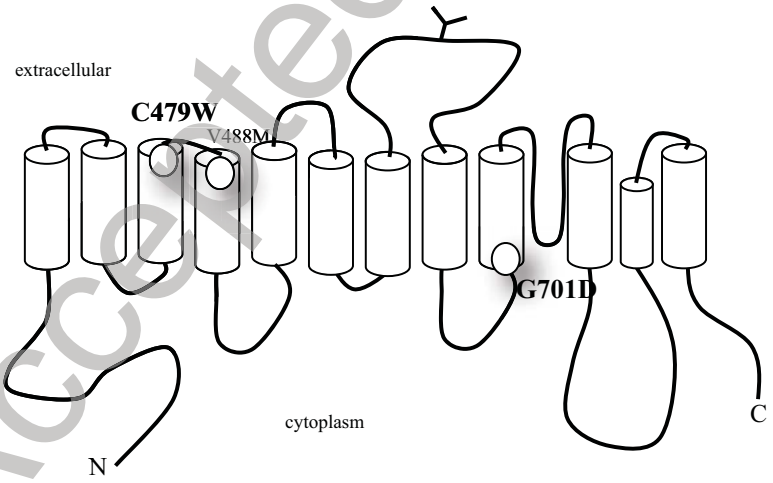
A. Family pedigree



B. Sequencing results from the patient and family



C. Topology model of the anion exchanger 1 protein



Chu et al. Figure 1

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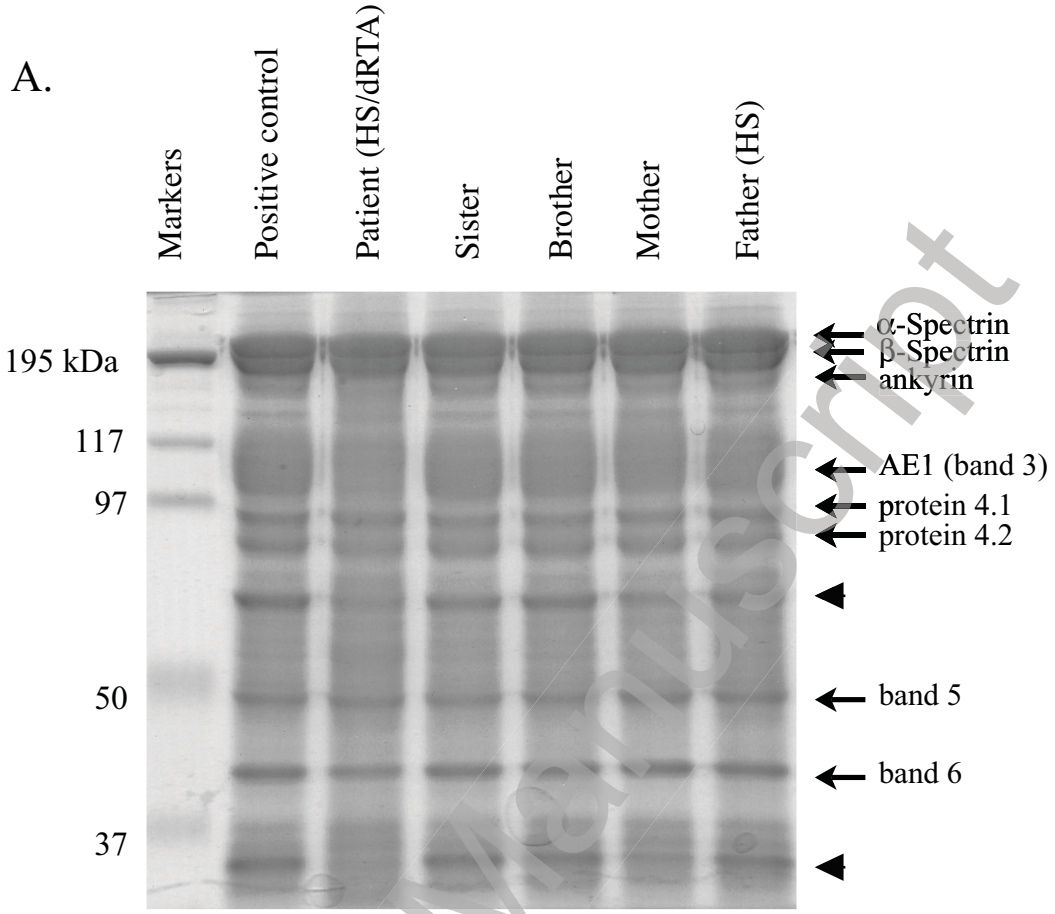

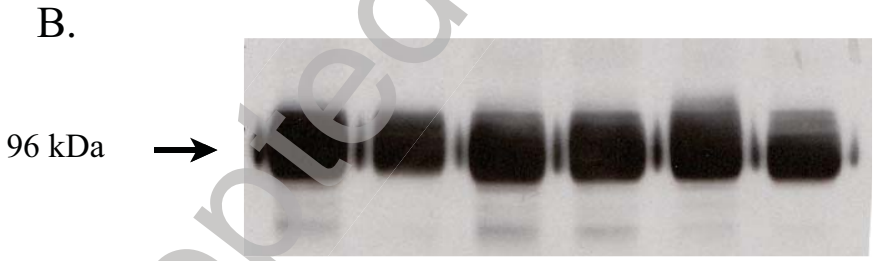

% of AE1	100	64	94	95	95	81
± SD (n = 3)	0	6	4	8	5	8

Chu et al. Figure 2

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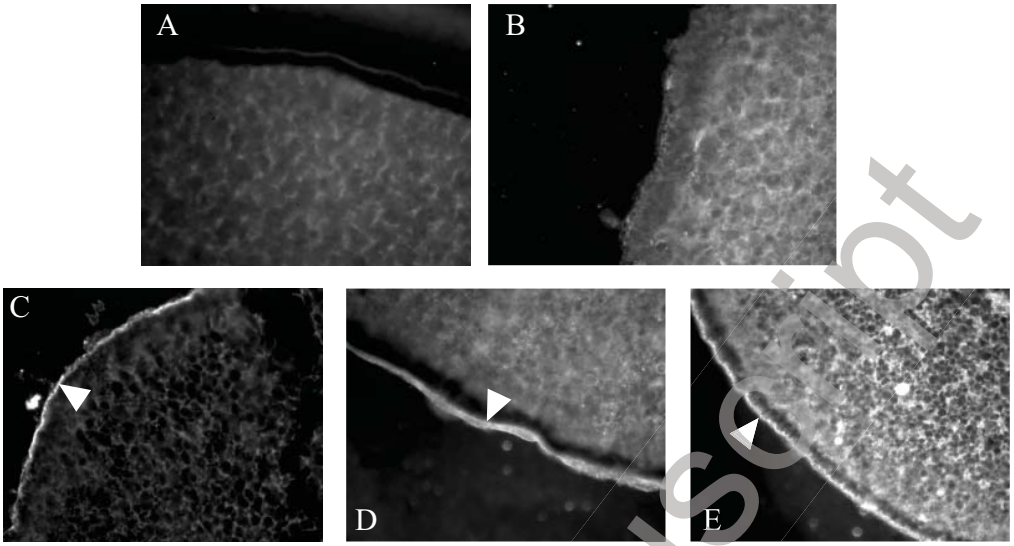
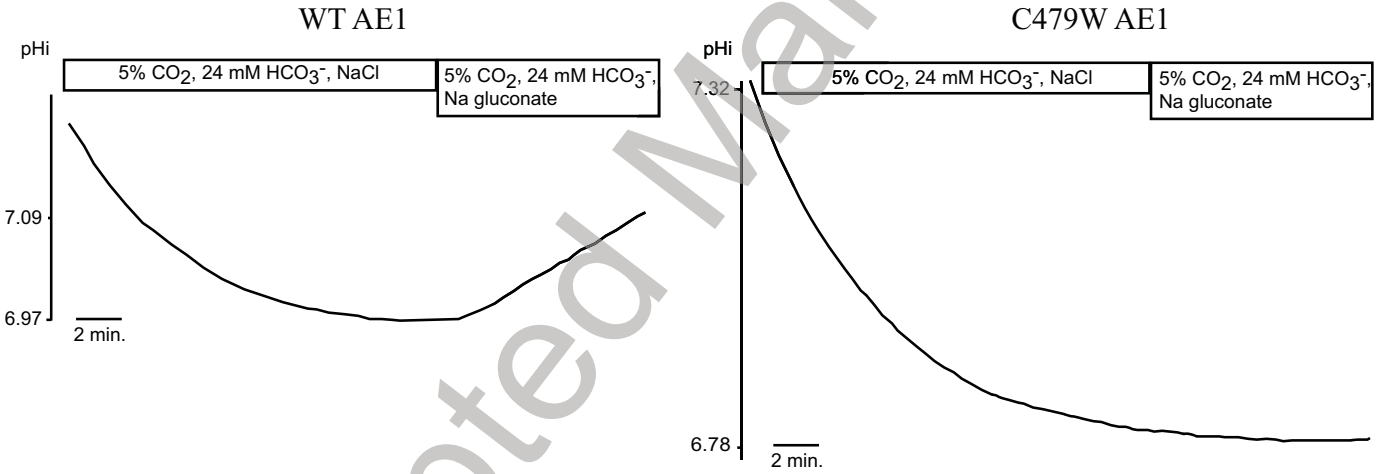
A.



B.



Chu et al. Figure 3

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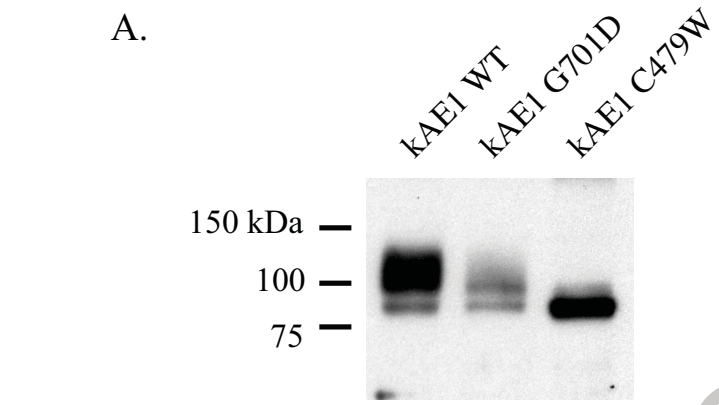
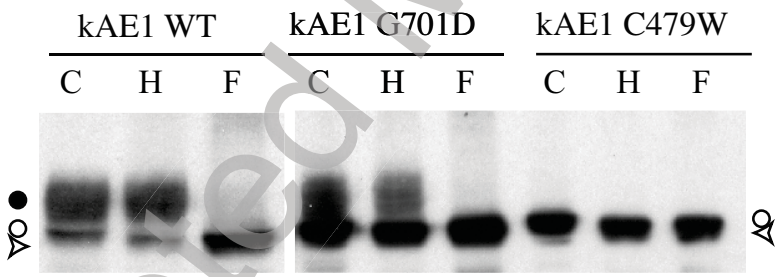
A.



% of complex N-glycan	73	47	8
SD (n = 3)	4	5	6

B.



Chu et al. Figure 4

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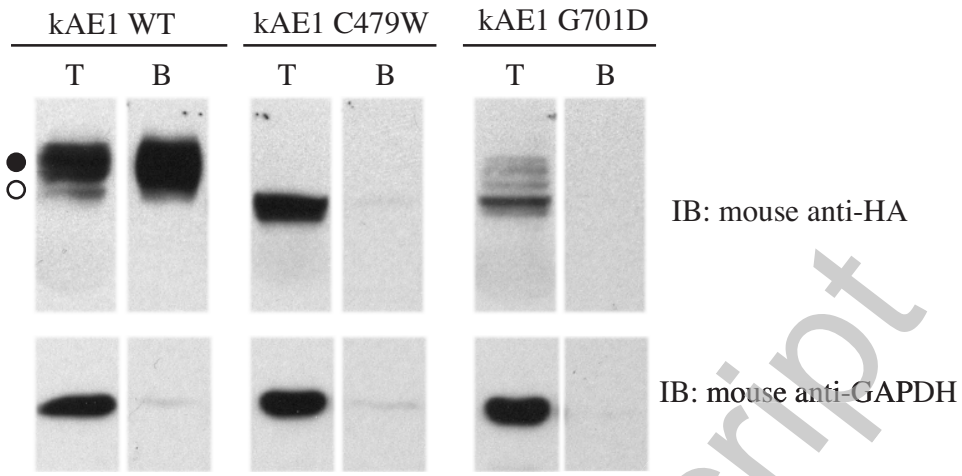
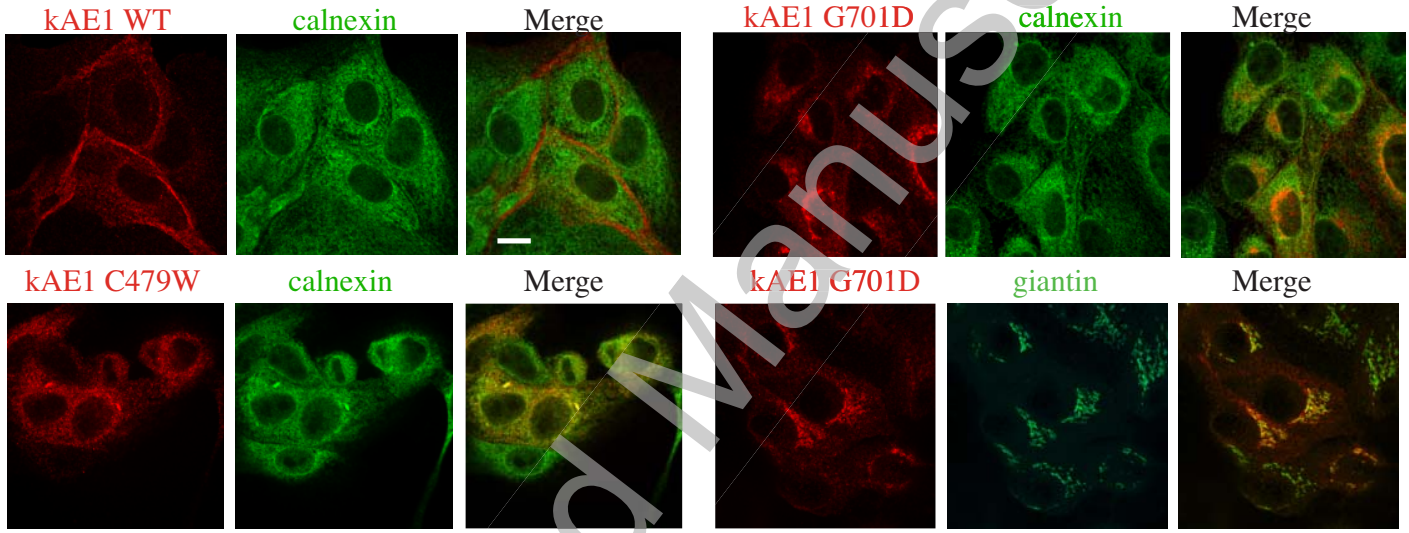
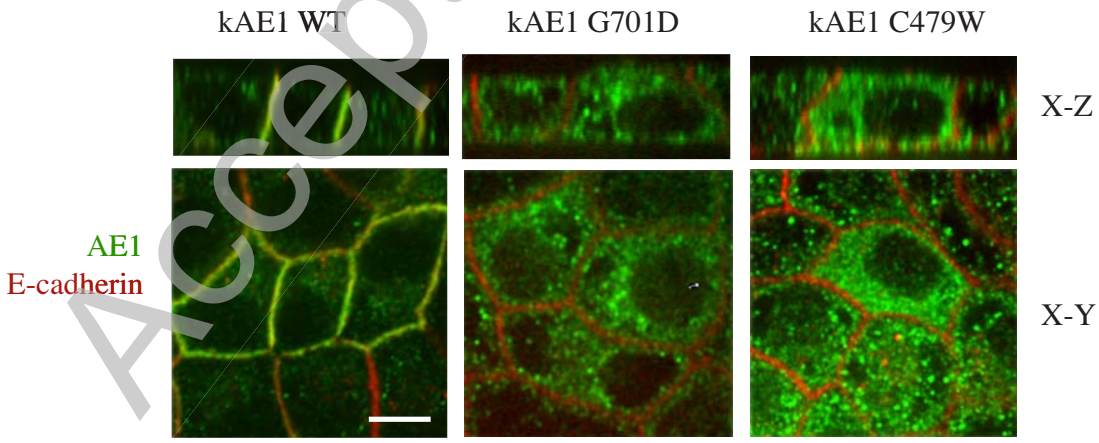
A.



B.



C.



Chu et al. Figure 5

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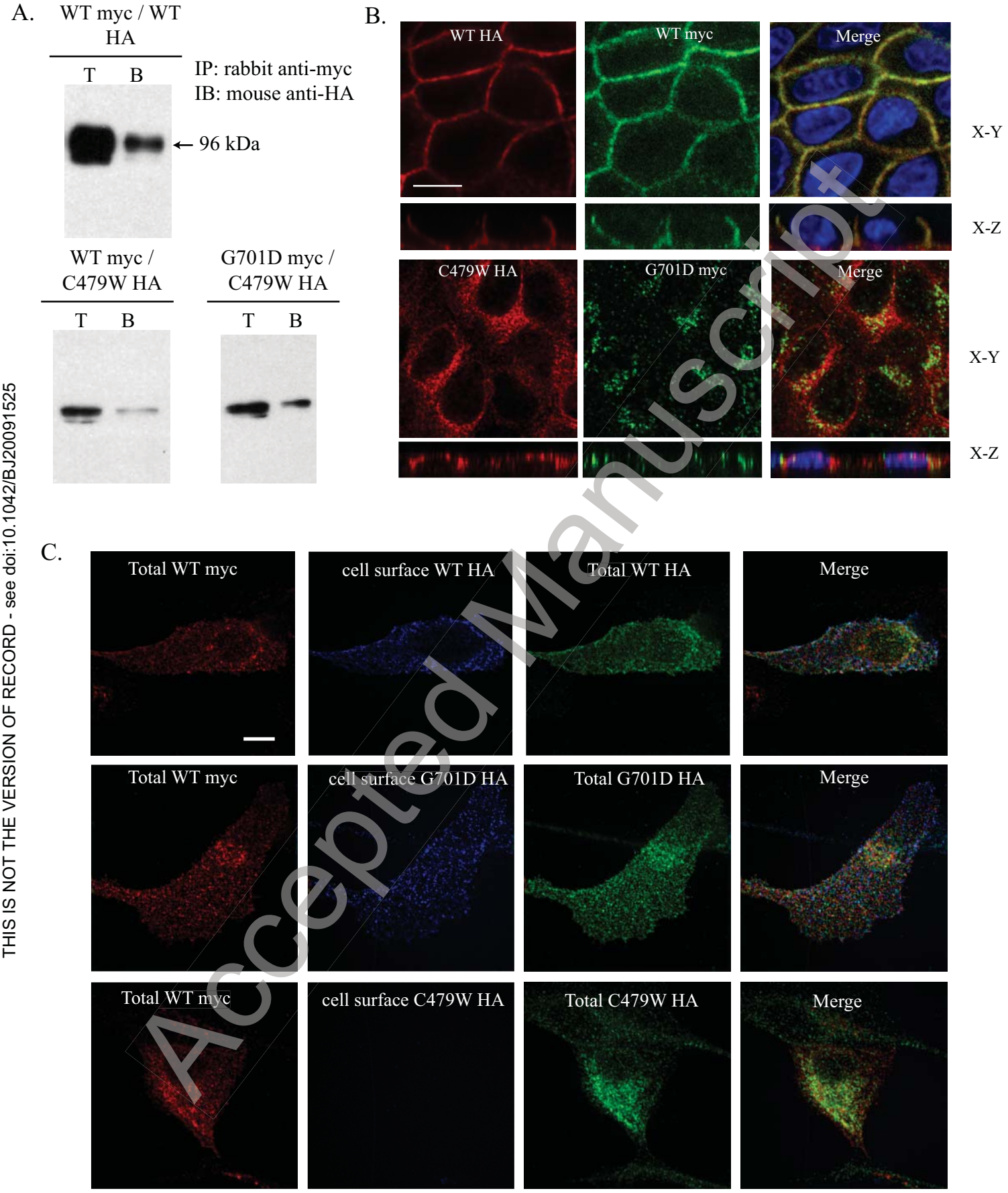

Chu et al. Figure 6

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Nuclear localization of dengue virus capsid protein is required for DAXX interaction and apoptosis

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ABSTRACT

Dengue virus capsid protein (DENVC) localizes to both the cytoplasm and nucleus of dengue virus-infected cells. DENV C contains three nuclear localization signals (NLS), ^{6}KKAR^{9}, ^{73}KKSK^{76}, and the bipartite signal ^{85}RKeigrmlnilnRRRR^{100}. Stable HepG2 cells constitutively expressing DENV C, DENV C (Δ85–100) and DENV C (Δ73–100) were constructed to clarify whether nuclear translocation of DENV C affected apoptosis in liver cell line. While the wild-type DENV C could translocate into the nuclei of HepG2 cells, the mutant DENV Cs were restricted to the cytoplasm. The loss of nuclear localization of both mutant DENV Cs resulted in the disruption of their interactions with the apoptotic protein Daxx. Interestingly, upon treatment with anti-Fas antibody, the HepG2 cells expressing the wild-type DENV C showed significantly more apoptosis compared with the HepG2 cells expressing either mutant DENV C. To identify the amino acids required for DAXX interaction and apoptosis, substitution mutations either (K73A/K74A) or (R85A/K86A) were introduced into the C-terminal region of DENV C, and tested whether these mutations affected its interaction with Daxx and apoptosis. The results demonstrate that ^{73}KK and ^{85}RK of DENV C are important for its nuclear localization, interaction with DAXX and induction of apoptosis. This work is the first to demonstrate that nuclear localization of DENV C is required for DAXX interaction and apoptosis.

1. Introduction

Dengue virus (DENV) infection is one of the most important mosquito-borne viral diseases and is endemic in several countries (Halstead, 2007). Clinical severity of the disease ranges from a predominantly febrile disease, dengue fever (DF), to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which usually occurs in cases with subsequent infection with a different serotype of dengue virus (Halstead, 1988). The patients with DHF generally present with hemorrhagic tendencies, plasma leakage, thrombocytopenia, and hemoconcentration. Liver injury with an elevation of aminotransferases, reactive hepatitis and hepatic failure is frequently observed in the patients with DHF/DSS (Khongphatthanayothin et al., 2005).

DENV belongs to the *Flaviviridae* family. The viral particles contain a single positive-stranded RNA genome, encoding a single precursor polypeptide. Host and viral proteases cleave this polypeptide into three structural proteins (capsid, membrane, and envelope) and seven nonstructural proteins (DENV NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Chambers et al., 1990). Dengue virus capsid protein (DENVC) is a small, highly positively charged, 12-kDa protein that is required for the maturation of viral particles and assembly of the nucleocapsid (Kuhn et al., 2002). DENV C is predicted to be largely alpha-helical structure (Jones et al., 2003). The secondary structure of recombinant DENV C is composed of 4 alpha helices (α1 to α4) and is predominately dimeric (Jones et al., 2003). The domain responsible for homotypic interaction was mapped to residues 37–72 by co-immunoprecipitation (Wang et al., 2004). DENV C localizes in both the cytoplasm and nucleus of dengue virus-infected cells (Bulich and Aaskov, 1992; Tadano et al., 1989; Wang et al., 2002). DENV C contains three nuclear localization signals (NLS), ^{6}KKAR^{9}, ^{73}KKSK^{76}, and the bipartite signal ^{85}RKeigrmlnilnRRRR^{100}. Each has been implicated in the nuclear localization of DENV C (Sangiambut et al., 2008; Wang et al., 2002). The substitution mutations in DENV C (K73A, K74A) or DENV C (R85A, K86A) resulted in an elimination of nuclear local-

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ization in porcine kidney (PS) cells. However, nuclear localization is not required for viral growth (Sangiambut et al., 2008). Thus, the role of DENV C in the nucleus of dengue virus-infected cells is still uncertain.

Our group demonstrated that DENV C physically interacts with Daxx and sensitizes hepatic cells to Fas-mediated apoptosis (Limjindaporn et al., 2007). Daxx is a 740 amino acid protein that contains two amino-terminal amphipathic helices (PAH1, PAH2), a coiled–coiled domain (CC), an acidic domain (D/E), and a carboxyl-terminal serine/proline/threonine rich domain (S/P/T). This protein can be found in the cytoplasm but the majority of it is located in the nucleus. In the cytoplasm, Daxx interacts with Fas at the plasma membrane (Yang et al., 1997). Stimulation of Fas causes translocation of Daxx from the nucleus to the cytoplasm, where it interacts with ASK1 and promotes JNK activation (Chang et al., 1998). However, Daxx has recently been shown to be a predominantly nuclear protein that does not translocate to the cytoplasm in response to cell stress (Lindsay et al., 2009). Therefore, the functional role of cytoplasmic Daxx in response to stimuli remains controversial. In contrast, it is relatively clear that Daxx acts as a transcription repressor in the nucleus. Daxx localizes to the PML nuclear bodies (PML-Nbs), where it interacts with the tumor suppressor PML (Ishov et al., 1999; Zhong et al., 2000). In addition, Daxx interacts with ETS1 and represses transcription activation of ETS1 target genes including anti-apoptotic Bcl2 (R. Li et al., 2000). Daxx also interacts with p53 and potentiates p53-dependent apoptosis (Gostissa et al., 2004). To further investigate the role of DENV C in the nucleus, we created HepG2 cells constitutively expressing either wild-type DENV C or mutant DENV C lacking nuclear-localization signals and assessed their roles in nuclear translocation, protein interaction, and induction of apoptosis. In the present study, we demonstrate that, in the absence of nuclear localization signals, DENV C could neither translocate into the nucleus nor interact with Daxx to induce apoptosis.

2. Materials and methods

2.1. Generation of stable HepG2 cells constitutively expressing DENV C, DENV C (Δ85–100) and DENV C (Δ73–100)

PCR amplification and cloning of DENV C into plasmid pcDNA3.1/His C (Invitrogen) was reported previously (Limjindaporn et al., 2007; Sriburi et al., 2001). DENV C (Δ85–100) and DENV C (Δ73–100) were amplified from the prior clone (Limjindaporn et al., 2007) using the forward primer DENV C *Bam*HI forward (5′-GTA GGA TCC ATG AAT GAC CAA CGG AAA AAG-3′) and the reverse primers DENV C (Δ85–100) *Xho*I reverse (5′-GCA CTC GAG CTA GAA CCC TCT CAA AAC-3′), and DENV C (Δ73–100) *Xho*I reverse (5′-GCA CTC GAG CTA AAT TGT TCC CCA TCT-3′) which resulted in C-terminal truncations of the capsid coding region and removes the NLS. The PCR reactions were carried out in a GeneAmp PCR System 9700 (Applied Biosystems) with the following thermal conditions: 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s, with extension at 72 °C for 2 min, and one cycle of final extension at 72 °C for 10 min. The PCR products were cloned into the plasmid pcDNA3.1/His C (Invitrogen). The integrity of positive clones was verified by DNA sequencing. HepG2 cells were transfected with these plasmids using Lipofectamine 2000 (Invitrogen). Twenty-four hours post-transfection, stable cell colonies were selected in DMEM medium containing 1 mg/ml G418 (Calbiochem) for a month. The isolated G418-resistant colonies were maintained in DMEM medium containing 0.5 mg/ml G418 and examined for the expression of DENV C by Western blot analysis as previously described.

2.2. Generation of HepG2 cells transiently expressing DENV C, DENV C (K73A, K74A), and DENV C (R85A, K86A)

Plasmid pcDNA3.1/His C-DENV C (K73A/K74A) and DENV C (R85A/K86A), were generated using pcDNA3.1/His C-DENV C as a template. Site-directed mutagenesis was performed by inverse PCR using overlapping primers, for DENV C (K73A/K74A) primers forward K73A/K74A (5′-GAG ATG GGG AAC AAT TGC AGC ATC CAA AGC TAT TAA TG-3′) and reverse K73A/K74A (5′-CAT TAA TAG CTT TGG ATG CTG CAA TTG TTC CCC ATC TC-3′), for DENV C (R85A/K86A) primers forward R85A/K86A (5′-GTT TTG AGA GGG TTC GCG GCA GAG ATT GGA AGG ATG-3′) and reverse R85A/K86A (5′-CAT CCT TCC AAT CTC TGC CGC GAA CCC TCT CAA AAC-3′). Thermal cycling was started at 95 °C for 30 s, followed by 18 cycles of 95 °C for 30 s, 55 °C for 1 min and 68 °C for 14 min. The PCR product was digested with *Dpn*I to eliminate methylated parental DNA templates and transformed into competent *E. coli* strain DH5α. The correct mutant clones were verified by DNA sequencing (Fig. 2A).

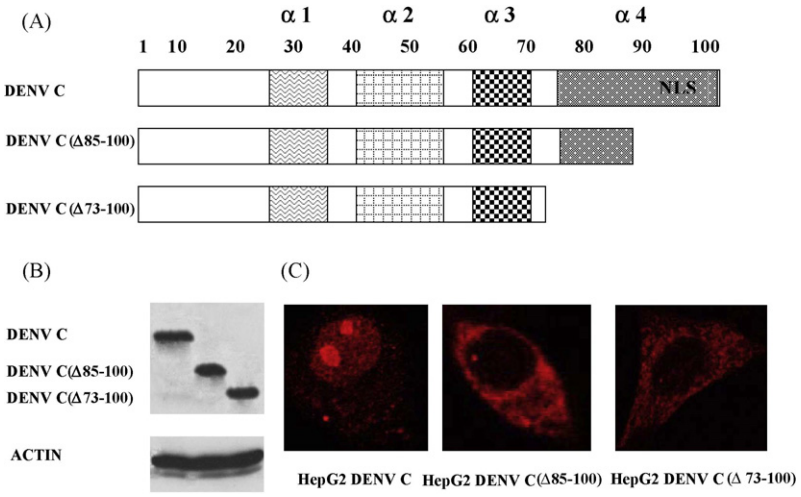

Fig. 1. Generation of HepG2 cells constitutively expressing either wild-type DENV C or mutant DENV C with deletions of nuclear-localization signals, DENV C (Δ85–100) and DENV C (Δ73–100). (A) DENV C is composed of 4 alpha helices (α1 to α4), and contains three NLS at positions 6–9, 73–76 and 85–100, respectively. (B) The expression of DENV C, DENV C (Δ85–100) and DENV C (Δ73–100) in HepG2 cells was verified by Western blot analysis using mouse antibody to DENV C. (C) HepG2 cells constitutively expressing either wild-type DENV C or mutant DENV C were fixed and immunostained with mouse anti-DENV C and Cy3-conjugated rabbit anti-mouse Ig antibody as secondary antibody at RT for 1 hr. Fluorescent images were captured with a confocal microscope (Model LSM 510, Carl Zeiss).

The transient transfection of plasmid pcDNA3.1/His C-DENV C, pcDNA3.1/His C-DENV C (K73A/K74A) or pcDNA3.1/His C-DENV C (R85A/K86A) into HepG2 cells was performed by using FuGene HD (Roche-applied science, Germany). Up to 1×10^6 of HepG2 cells were seeded into a 6-well plate prior transfection. Four micrograms of plasmid DNA, which were pre-incubated with 12 μl of FuGene HD reagent in serum-free media, Optimem (Gibco-BRL, USA), was added to the HepG2 cells in Optimeme containing 3% FBS. Forty-eight hours post-transfection, HepG2 cells were harvested and assayed their expression in HepG2 cells by Western blot analysis using anti-DENV C monoclonal antibody (D2-C1) (Puttikhunt et al., 2009).

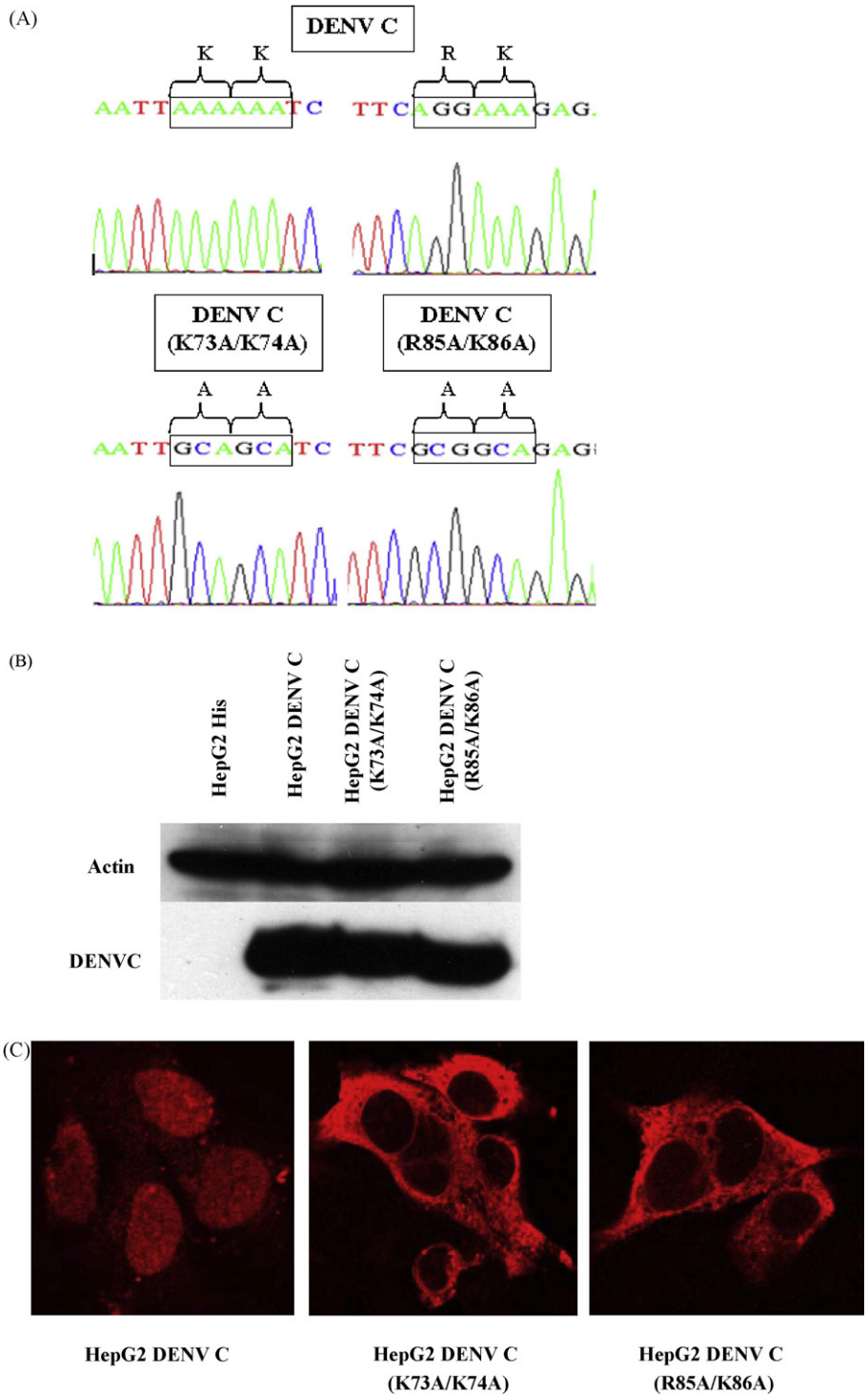

Fig. 2. Generation of HepG2 cells transiently expressing DENV C, DENV C (K73A, K74A), and DENV C (R85A, K86A). (A) Site-directed mutagenesis was employed to generate DENV C (K73A/K74A) and DENV C (R85A/K86A) in pcDNA3.1/His C and subjected to DNA sequencing. (B) The expression of DENV C, DENV C (K73A/K74A) and DENV C (R85A/K86A) in HepG2 cells was verified by Western blot analysis using mouse antibody to DENV C. (C) HepG2 cells transiently expressing DENV C, DENV C (K73A, K74A), and DENV C (R85A, K86A) were fixed and immunostained with mouse anti-DENV C and Cy3-conjugated rabbit anti-mouse Ig antibody as secondary antibody at RT for 1 hr. Fluorescent images were captured with a confocal microscope (Model LSM 510, Carl Zeiss).

2.3. Co-immunoprecipitation

Interaction of DENV C with Daxx was examined by co-immunoprecipitation of transiently or stably transfected cell. Stable HepG2 transformants were grown in DMEM containing 0.5 mg/ml G418 (Gibco-BRL, USA) supplemented with 10% heat inactivated fetal bovine serum (Hyclone, USA), 1 mM sodium pyruvate (Sigma, USA), 1 mM non-essential amino acids (Gibco-BRL, USA), and 1.2% penicillin G–streptomycin at 37 °C in 5% CO_2. Up to 3×10^6 HepG2 transformant cells were lysed with RIPA buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 20 mM Tris–HCl; pH 7.4, 5 mM EDTA. Five micrograms of purified mouse anti-DENV C monoclonal antibody (D2-C1) (Puttikhunt et al., 2009) or 5 μg of rabbit anti-Daxx polyclonal antibody (M-122) from Santa Cruz Biotechnology or 5 μg of isotype-matched control antibody were added to lysates. The mixture was incubated at 4 °C overnight with protein G Sepharose beads (Amersham Pharmacia Biosciences) and washed with washing buffer. The bound proteins were eluted by boiling in SDS sample buffer, subjected to SDS-PAGE and transferred to nylon membranes (Whatman GmbH, Dassel, Germany). The membranes were incubated either with D2-C1 or with M-122, followed by probing either with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse Ig antibody or with HRP-conjugated swine anti-rabbit Ig antibody, respectively. The

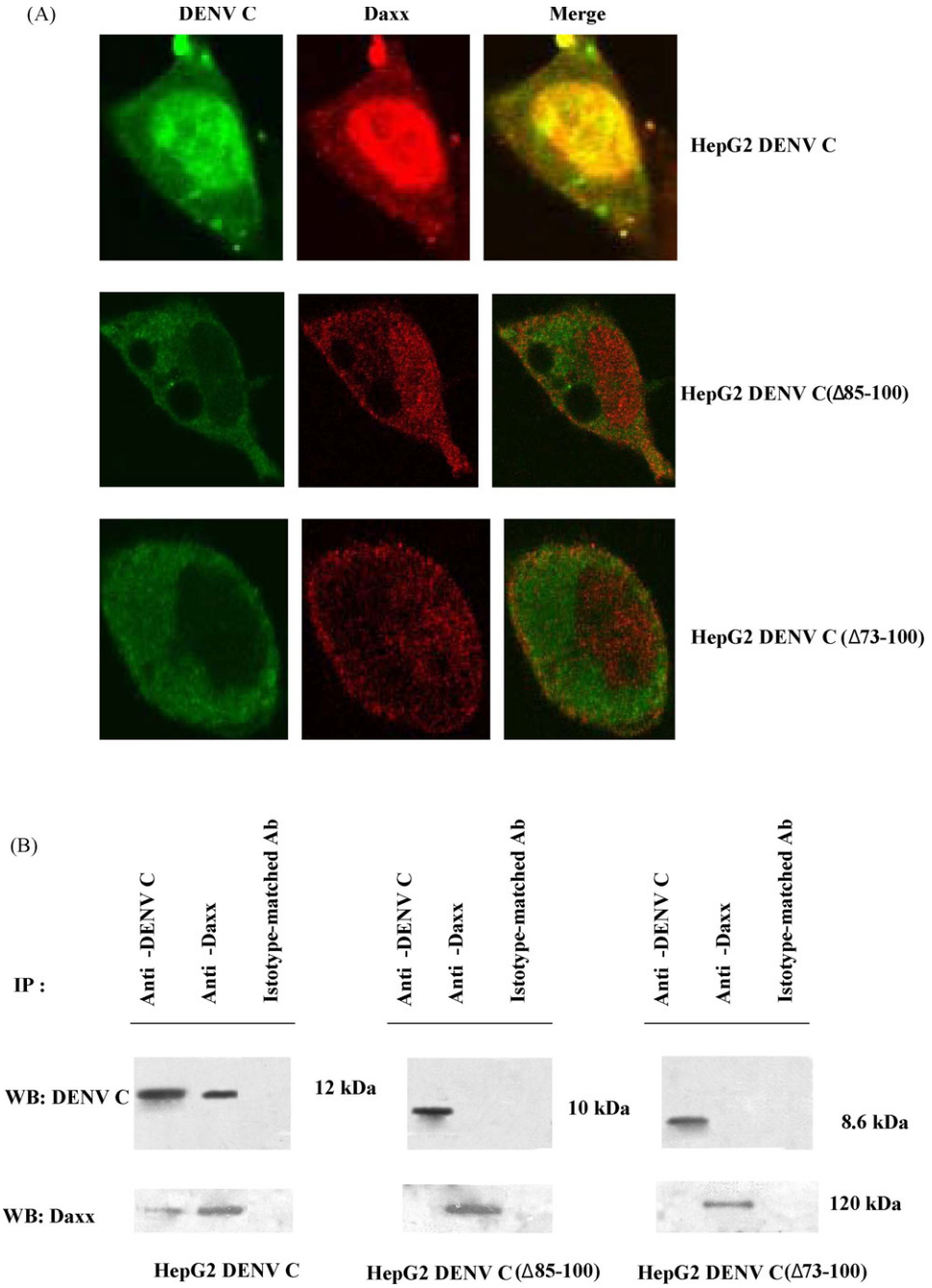

Fig. 3. Interaction of DENV C and Daxx was not observed in HepG2 cells expressing DENV C (Δ85–100), DENV C (Δ73–100) (A) HepG2 cells constitutively expressing either wild-type DENV C or mutant DENV C were fixed and immunostained with mouse anti-DENV C (green) and rabbit anti-Daxx antibody (red). The merged image (yellow) demonstrated co-localization between DENV C and Daxx. (B) The cell lysates from HepG2 cells constitutively expressing either wild-type DENV C or mutant DENV C were immunoprecipitated with either mouse anti-DENV C or rabbit anti-Daxx antibody or isotype-matched control antibodies. The complexes were detected either with mouse anti-DENV C or rabbit anti-Daxx antibody. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

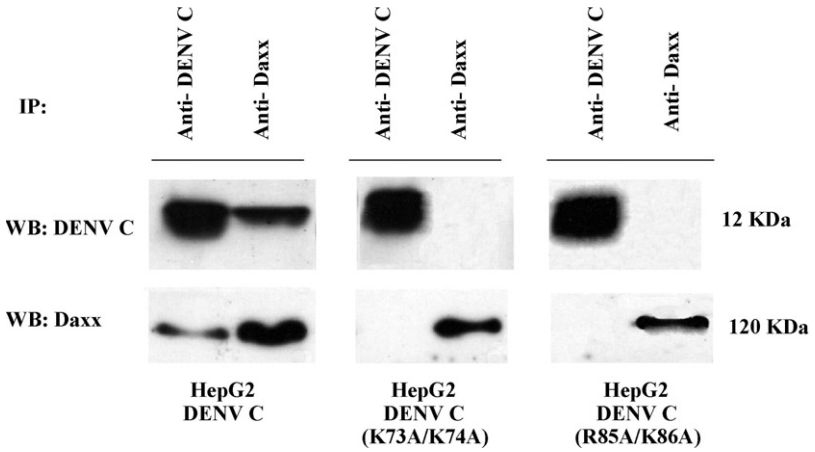

Fig. 4. Co-immnoprecipitation of DENV C and Daxx was not observed in HepG2 cells expressing DENV C (K73A, K74A), or DENV C (R85A, K86A). The cell lysates from HepG2 cells transiently expressing wild-type DENV C or DENV C (K73A, K74A) or DENV C (R85A, K86A), were immunoprecipitated by either mouse anti-DENV C or rabbit anti-Daxx antibody. The complexes were detected either with mouse anti-DENV C or rabbit anti-Daxx antibody.

protein bands were detected using ECL reagents (Amersham Pharmacia Biosciences).

2.4. Co-localization

Stable HepG2 transformants were grown on cover slips as previously described (Limjindaporn et al., 2007), washed, fixed with 4% formaldehyde in PBS for 20 min, and permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. After washing three times with 0.1% Triton X-100 in PBS, cover slips were incubated with both D2-C1 and M-122 for 1 hr, washed and incubated with both Alexa 488-conjugated rabbit anti-mouse Ig antibody (Molecular Probes) and Cy3-conjugated donkey anti-rabbit Ig antibody (Jackson Immunoresearch Laboratories) as secondary antibodies at room temperature for an hour. Fluorescent images were captured with a confocal microscope (model LSM 510, Carl Zeiss).

For localization of DENV C (K73A/K74A) or DENV C (R85A/K86A), the transient transfection of plasmid pcDNA3.1/His C-DENV C, pcDNA3.1/His C-DENV C (K73A/K74A) or pcDNA3.1/His C-DENV C (R85A/K86A) into HepG2 cells was performed by using FuGene HD (Roche-Applied Science, Germany). The transformants were washed, fixed with 4% formaldehyde in PBS for 20 min, and permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. After washing three times with 0.1% Triton X-100 in PBS, cover slips were incubated with D2-C1 for 1 h. After washing, the cover slips were incubated with Cy3-conjugated goat anti-mouse Ig antibody (Molecular Probes) as secondary antibody at room temperature for an hour. Fluorescent images were captured with a confocal microscope (Model LSM 510, Carl Zeiss).

2.5. Apoptosis assays

Stable HepG2 transformants were treated with 0.5 μg/ml of anti-Fas mAb (Sigma) and 1 μg/ml cycloheximide (CHX) for 24 hr in culture medium containing 2% FBS. HepG2 DENV C, HepG2 DENV C (Δ85–100), HepG2 DENV C (Δ73–100) and HepG2 cells containing the empty vector pcDNA3.1/His C, HepG2His, were examined for the presence of apoptotic cells by harvesting both detached and adherent cells, staining for cell surface phosphatidyl serine and assaying caspase-3 activation. For Annexin V/PI staining, samples were suspended in AnnexinV buffer and incubated on ice with FITC-conjugated AnnexinV (BD Biosciences) for 15 min. Immediately prior to analysis, propidium iodide was added and samples were analyzed by flow cytometry (Becton Dickinson). In the assessment of caspase-3 activation, the cells were lysed with RIPA buffer and subjected to Western blot analysis as noted above. The blots were incubated with goat anti-caspase-3 antibody (sc-22139) from Santa Cruz Biotechnology, followed by a HRP-conjugated rabbit anti-goat Ig antibody. Protein bands were detected with ECL reagents.

Analysis of cells transiently tranfected with plasmid pcDNA3.1/His C-DENV C, pcDNA3.1/His C-DENV C (K73A/K74A) or pcDNA3.1/His C-DENV C (R85A/K86A) was performed in a similar manner. Twenty-four hours post-transfection, transfected cells were triggered with 0.5 μg/ml anti-Fas mAb (Sigma) and 1 μg/ml cycloheximide (CHX) containing 2% FBS culture medium for 1 day. Subsequently, both detached and adherent cells were harvested for determining apoptotic cell death. Results were obtained from three independent experiments.

3. Results

3.1. Generation of HepG2 cells expressing DENV C, DENV C (Δ85–100), DENV C (Δ73–100), DENV C (K73A, K74A), and DENV C (R85A, K86A)

Two of the DENV C nuclear-localization signals are located at positions 73–76 and 85–100, respectively (Fig. 1A) (Wang et al., 2002); therefore, we constructed stable HepG2 cells constitutively expressing DENV C, DENV C (Δ85–100) and DENV C (Δ73–100) to clarify whether nuclear translocation of DENV C affected apoptosis in a liver derived cell line. The expression of DENV C, DENV C (Δ85–100) and DENV C (Δ73–100) was verified by Western blot analysis (Fig. 1B). Whilst DENV C was present in the nuclei of HepG2 cells constitutively expressing DENV C, HepG2 cells constitutively expressing DENV C (Δ85–100) and DENV C (Δ73–100) had no detectable DENV C in the nucleus confirming the functional role of these sequences in nuclear localization (Fig. 1C).

Double alanine-substitution mutations in DENV C (K73A, K74A) or DENV C (R85A, K86A) resulted in an elimination of nuclear localization in PS cells (Sangiambut et al., 2008). To evaluate the effect of these mutations on localization in HepG2 cells site-directed mutagenesis was used to generate DENV C (K73A/K74A) and DENV C (R85A/K86A) mutations which were sub-cloned into pcDNA3.1/His C and transiently expressing into HepG2 cells. Introduction of the DENV C (K73A/K74A) or DENV C (R85A/K86A) mutations was verified by DNA sequencing (Fig. 2A) and their expression in HepG2 cells was confirmed by Western blot analysis using anti-DENV C (Fig. 2B). Whilst DENV C was present in the nuclei of HepG2 cells expressing DENV C, HepG2 cells expressing DENV C (K73A, K74A) or DENV C (R85A, K86A) had no detectable DENV C in the nucleus (Fig. 2C).

3.2. Loss of nuclear localization of DENV C contributed to loss of interaction between DENV C and Daxx

Our prior studies demonstrated that DENV C physically interacts with Daxx, but could not distinguish whether this interaction was limited to nuclear versus cytoplasmic forms of these proteins or if interaction occurred in both cell compartments. Co-localization of DENV C and Daxx was clearly evident in stable HepG2 cells constitutively expressing DENV C (Fig. 3A). Anti-DENV C antibody co-immunoprecipitates Daxx protein and, conversely, anti-Daxx antibody co-precipitated DENV C (Fig. 3B). In contrast, loss of nuclear localization of DENV C resulted in loss of interaction between DENV C and Daxx (Fig. 3A). Co-immnoprecipitation of DENV C and Daxx were observed in in HepG2 cells expressing DENV C not in HepG2 cells expressing DENV C (Δ85–100), DENV C (Δ73–100), (Fig. 3B), DENV C (K73A, K74A), or DENV C (R85A, K86A) (Fig. 4). These results show that the interaction of DENV C and Daxx is restricted to a subpopulation of these proteins, those localized to the nucleus, and does not occur between cytoplasmic forms.

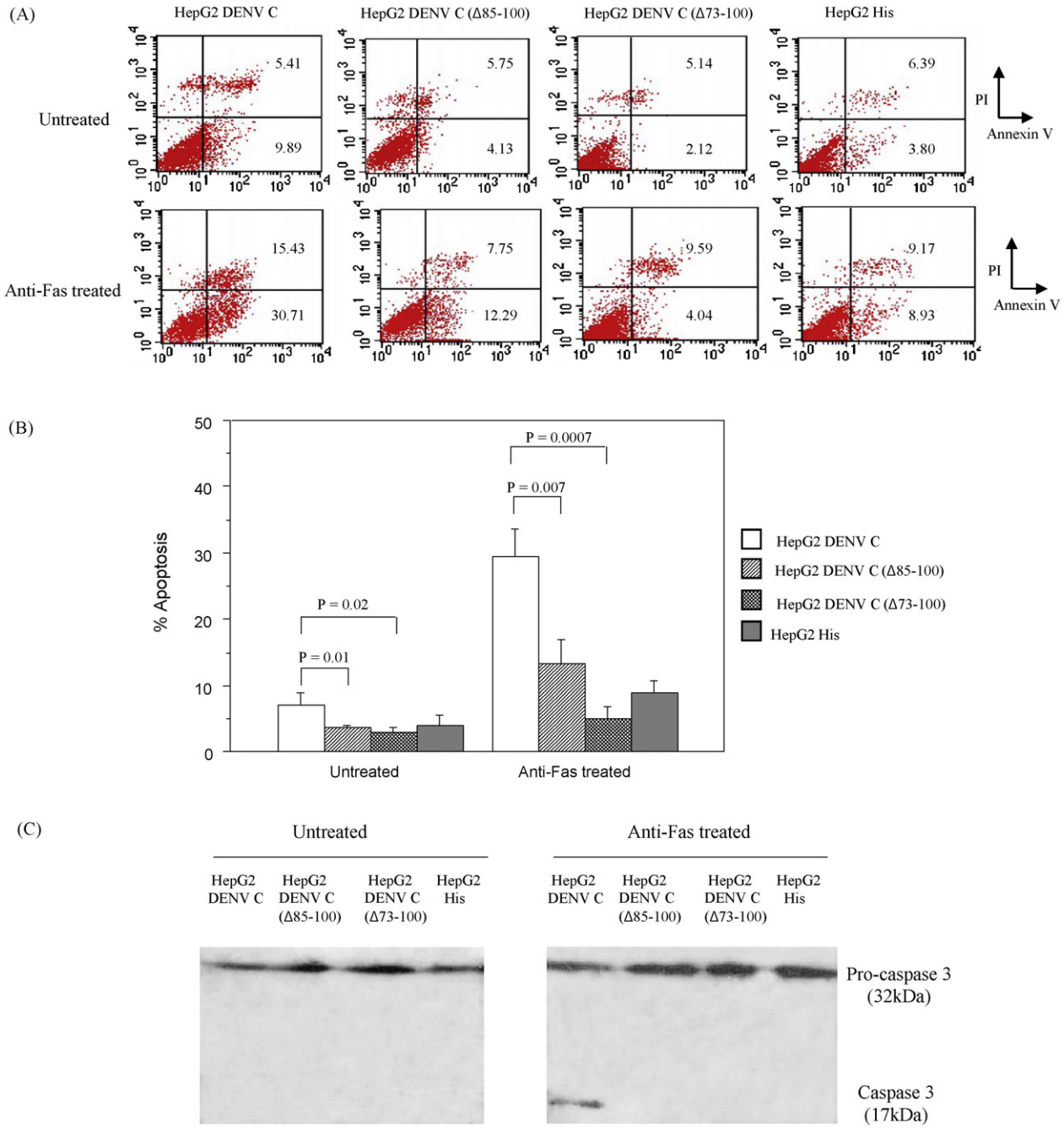

Fig. 5. Induction of apoptosis by DENVC requires its translocation to the nucleus. (A) The untreated and anti-Fas antibody treated HepG2 cells constitutively expressing either wild-type DENV C or mutant DENV C were incubated with FITC-conjugated annexin V. Immediately prior to analysis, propidium iodide was added and samples were analyzed by flow cytometry. The numbers in the lower right boxes represent the percentages of the apoptotic cells. (B) The percentage of cells undergoing apoptosis as measured by the per cent of annexin V positive cells measured in three independent experiments. Stat view was used for statistical tests. *P* stands for the *P* value and the *y*-axis of (B) represents the percentage of cells undergoing apoptosis. (C) Caspase-3 cleavage and activation was assessed in untreated and anti-Fas antibody treated HepG2 cells expressing either wild-type DENV C or mutant DENV C by Western blot analysis using goat anti-caspase 3 antibody.

3.3. Induction of apoptosis by DENV C requires its translocation to the nucleus

HepG2 cells expressing DENV C upon treatment with anti-Fas antibody showed a statistically significant increase in staining with annexin V and propidium iodine in contrast to cells expressing DENV C (Δ85–100), DENV C (Δ73–100) (Fig. 5A and B), DENV C (K73A, K74A), and DENV C (R85A, K86A) (Fig. 6). Apoptosis in response to this treatment was also evident in the activation of caspase 3 in cells expressing DENV C, but not in DENV C (Δ85–100) or DENV C (Δ73–100) expressing cells (Fig. 5C), suggesting the critical role of DENV C in the nucleus in sensitization cells to apoptosis.

4. Discussion

Following dengue virus infection, apoptosis of hepatic cells was observed both *in vitro* and *in vivo* (Catteau et al., 2003; El-Bacha et al., 2007; Limonta et al., 2007; Marianneau et al., 1997, 1998; Matsuda et al., 2005; Thongtan et al., 2004). Both the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway were affected. In the extrinsic pathway, dengue virus infection promotes apoptosis in hepatoma cell lines partly through the induction of TRAIL expression (Matsuda et al., 2005). In addition, DENV C sensitizes HepG2 cells to Fas-mediated apoptosis (Limjindaporn et al., 2007). In the intrinsic pathway, DENV C was shown to induce mitochondrial membrane potential and p53 expression in Huh-7 cells (Nasirudeen and Liu, 2009; Nasirudeen et al., 2008). The associations between dengue virus-induced cell death and mitochondrial function in HepG2 cells was demonstrated by changes in mitochondrial bioenergetics and ultra structural alterations, including mitochondria swelling and other morphological changes typical of the apoptotic process(El-Bacha et al., 2007). A contribution of apoptosis *in vivo* to the pathogenesis of fatal DHF/DSS during a Cuban dengue epidemic was also reported. Apoptotic cells were found in five of the six cases studied and DENV antigens were immunolocalized mainly in hepatocytes (Limonta et al., 2007).

There are two independent signaling pathways downstream of Fas, involving the adapter protein Fas-associated death domain (FADD) and Daxx (Chinnaiyan et al., 1995; Yang et al., 1997). Daxx functions as a pro-apoptotic molecule downstream of FAS through activation of the JNK pathway in a Fas associated death domain (FADD)-independent manner (Chang et al., 1998; Yang et al., 1997) The JNK pathway regulates stress-induced apoptosis in neuronal cells (Weston and Davis, 2002). However, the FAS–Daxx

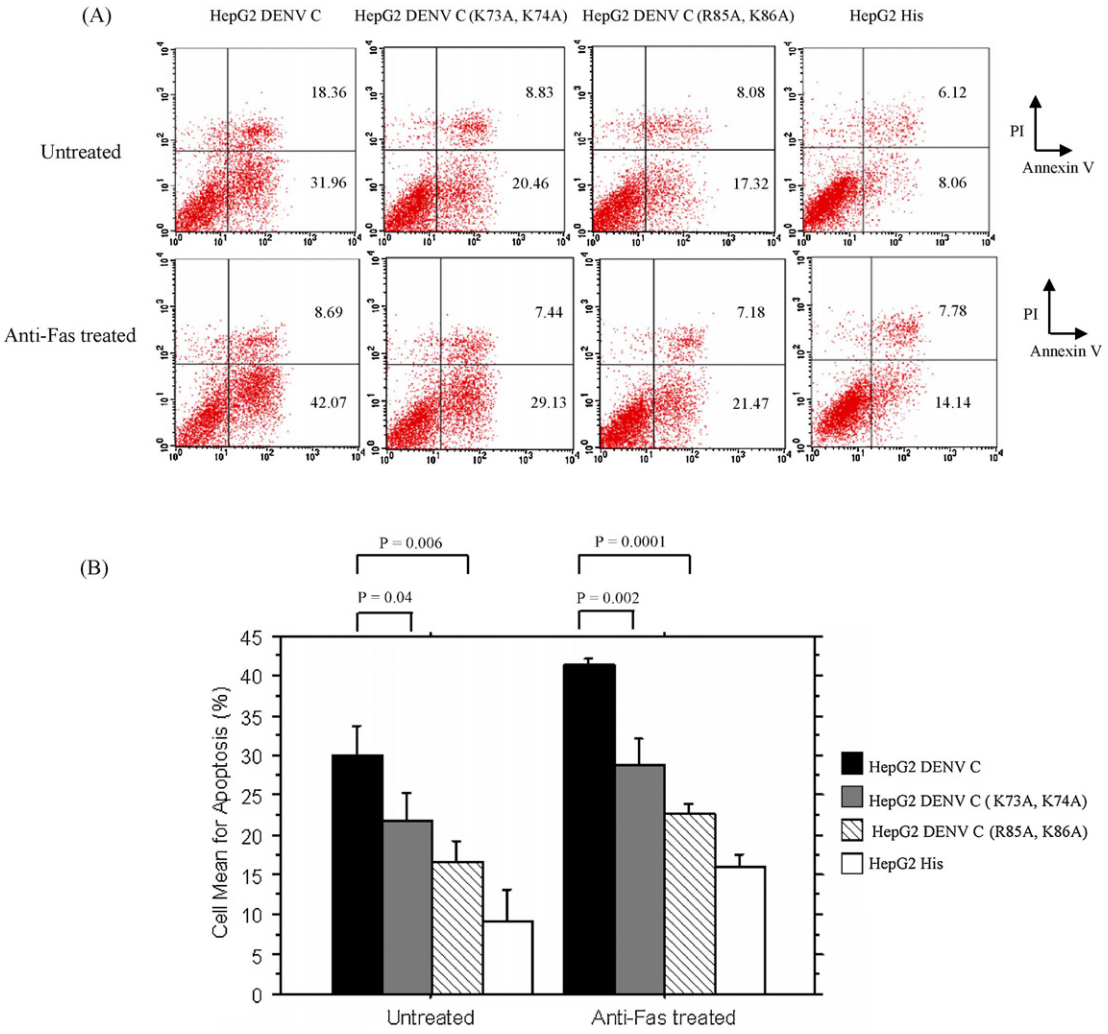

Fig. 6. HepG2 cells expressing DENV C upon treatment with anti-Fas antibody showed a statistically significant increase in staining with annexin V and propidium iodine comparable to HepG2 cells expressing DENV C (K73A, K74A), or DENV C (R85A, K86A). (A) The untreated and anti-Fas antibody treated HepG2 cells constitutively expressing wild-type DENV C or DENV C (K73A, K74A) or DENV C (R85A, K86A) were incubated with FITC-conjugated annexin V. Immediately prior to analysis, propidium iodide was added and samples were analyzed by flow cytometry. The numbers in the lower right boxes represent the percentages of the apoptotic cells. (B) The percentage of cells undergoing apoptosis as measured by the per cent of annexin V positive cells measured in three independent experiments. Stat view was used for statistical tests. *P* stands for the *P* value and the *y*-axis of (B) represents the percentage of cells undergoing apoptosis.

pathway proceeds through activation of the p38 MAP kinase in non-neuronal cells (Raoul et al., 2002). Daxx-mediated potentiation of FAS-induced apoptosis was suggested to occur from its nuclear location (Torii et al., 1999). In the nucleus, Daxx represses several transcription factors including Pax3 (Hollenbach et al., 1999), ETS1 (R. Li et al., 2000), E2F1 (Cermak et al., 2002), NF-kB (Michaelson et al., 1999), p53 (Gostissa et al., 2004; Kim et al., 2003) and p73 (Kim et al., 2003) and the ability of Daxx to repress transcription is inhibited by its localization to the PML-NB (H. Li et al., 2000).

Daxx is ubiquitously expressed throughout the body with particular high expression in the thymus and testes (Yang et al., 1997). In HepG2 cells, the expression of Daxx is relatively low (Tuo et al., 2008). In dengue virus-infected HepG2 cells, the expression of Daxx is up-regulated comparable to non-infected cells (data not shown) and interaction of Daxx and DENV C in the nucleus is critical for Fas-mediated apoptosis. DENV C was previously shown to bind to the carboxyl terminus of Daxx, which is the same region interacting with the tumor suppressor PML (Limjindaporn et al., 2007). The competitive binding between DENV C and PML with Daxx may limit the interaction between Daxx and PML thereby releasing Daxx to the nucleoplasm. Then, activation of apoptosis could occur via down-regulation of anti-apoptotic Bcl2 by Daxx. By the same concept, interaction of Daxx and DENVC in the nucleus may disrupt the Mdm2-Daxx-Hausp complex leading to degradation of Mdm2 by self-ubiquitination, accumulation of p53 and sensitization HepG2 to apoptosis (Tang et al., 2006). West Nile virus capsid protein, which belongs to the *Flaviviridae* family, was also shown to induce p53-mediated apoptosis via the sequestration of Mdm2 in the nucleus (Yang et al., 2008). How DENV C affects PML, Mdm2-Daxx-Hausp complex and Daxx translocation still requires further investigation. However, this work is the first to demonstrate that nuclear localization of DENV C is required for DAXX interaction and apoptosis in HepG2 cells.

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ORIGINAL PAPER

Evidence suggesting a genetic contribution to kidney stone in northeastern Thai population

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Abstract Genetic factor may play a role in the pathogenesis of kidney stone that is found in the northeastern (NE) Thai population. Herein, we report initial evidence suggesting genetic contribution to the disease in this population. We examined 1,034 subjects including 135 patients with kidney stone, 551 family members, and 348 villagers by radiography of kidney–ureter–bladder (KUB) and other methods, and also analyzed stones removed by surgical operations. One hundred and sixteen of 551 family members (21.05%) and 23 of the 348 villagers (6.61%) were affected with kidney stone. The relative risk (λ_R) of the disease among family members was 3.18. Calcium stones (whewellite, dahllite, and weddellite) were observed in about 88% of stones analyzed. Our data indicate familial aggregation of kidney stone in this population supporting that genetic factor should play some role in its pathogenesis. Genetic and genomic studies will be conducted to identify the genes associated with the disease.

Keywords Kidney stone · Nephrolithiasis · Genetic evidence · Genetic factor · Thailand · Thai · Northeastern Thai

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Introduction

Urinary tract stone has previously been recorded to be prevalent in Thailand [1–3]. However, in the past few decades, bladder stone in children has markedly declined as the result of the improvement of nutrition and living standard while kidney stone in adults remains to be a major health problem in the northeastern (NE) Thai population [4–6]. Although the etiology of kidney stone in the NE Thai population is unknown, it is possibly different from what was reported in the western and other ethnic groups because it does not seem to be associated with the conditions of increased urinary solutes such as hypercalciuria, hyperoxaluria, and hyperuricosuria [7]. Interestingly, the analysis of urinary constituents of normal adult villagers and individuals with the previous history of kidney stone showed that their daily urinary excretions of major electrolytes and solutes including sodium, potassium, oxalate, phosphate, and citrate were lower than those of the healthy city dwellers [7, 8]. The predominant abnormalities in these patients were hypocitraturia and potassium deficiency probably attributable to low dietary intake and loss through sweating [9, 10], but these abnormalities were not specific and found to be

associated with several disorders [11, 12]. Previously, the reported prevalence of kidney stone in the NE Thai population was greatly variable [6, 12, 13], which probably depended on the methods of studies and it is not clear whether the genetic factor will play a role in its pathogenesis. The aim of this study is thus to investigate evidence of genetic contribution to kidney stone in the NE Thai population. The result in this study showed that the prevalence of kidney stone among members of the affected families is higher than that of the villagers, implying that genetic factor should play some role in its pathogenesis.

Subjects and methods

The study was conducted in Khon Kaen Province (449 km from Bangkok) in the northeast of Thailand and this project was approved by the Ethics Committees of the Faculty of Medicine Siriraj Hospital and the Ministry of Public Health. A written informed consent was obtained from individual subject before enrolling into the project. The patients with kidney and/or ureteric stone were diagnosed and admitted for surgical intervention at Khon Kaen Regional Hospital during 2004–2006. The ages of all the recruited patients were more than 15 years. The patients' family members were included into the study without selection and enrolled without a prior knowledge whether they had kidney stone or not. Most of the patients and their families were of rural (village) origins. The rural control subjects were the villagers who resided in five villages within Khon Kaen Province but outside the city area; they were randomly chosen from a census registration by two-stage simple random sampling using SPSS 13.0 program for selection of only one individual from each family. The rural population who live in this region of the country is more homogeneous than those who live in other regions of the country [14], although the patients' families lived both inside and outside Khon Kaen Province while the villagers resided only within this province. The exclusion criteria of subjects were the presence of kidney stone secondary to all known causes (including renal tubular acidosis, primary hyperparathyroidism, inflammatory bowel disease, cushing disease, hyperthyroidism, and drug-induced kidney stone) diagnosed by clinical history and symptoms, physical and laboratory examinations, acute acid loading test, blood and urine biochemical and electrolyte analyses.

A total of 1,034 subjects including 135 patients with kidney and/or ureteric stone, 551 family members, and 348 villagers—representing control population, were recruited for the study. Clinical history of kidney stone, associated symptoms (i.e., back and abdominal pain, hematuria, and stone passage), treatments, surgical scars, findings in previous X-ray films, family history, and pedigree were recorded. All subjects were again investigated by roentgenography of kidney–ureter–bladder (KUB) and in some suspicious cases by additional ultrasonography. Urine and blood samples were collected for biochemical and electrolyte analyses. The cases with distal renal tubular acidosis, a known risk factor for kidney stone, were initially identified by spot urine pH > 5.5 and confirmed by short acid loading test [15], which were then excluded from the study. Stones were also collected from a group of 109 patients after removal by surgery for analyses using Nicolet™ 380 Fourier Transform Infrared Spectrometer. All statistical analyses were carried out by SPSS 13.0 program. The prevalence of kidney stone in family members and in villagers, a control population, was determined. The relative risk (λ_R) of kidney stone in family members was determined from the prevalence in family members compared with that of the control group.

Results

From the studies by KUB radiography, ultrasonography, surgical scar observation, and clinical history, we found that 116 of 551 family members (21.05%) and 23 of 348 villagers (6.61%), who were previously unknown for kidney stone status, were affected with kidney stone (Table 1). Thus, the relative risk (λ_R) of kidney stone among the family members, compared with the villagers, a control population, was found to be 3.18. Clinical symptoms were recorded in the patients and family members with kidney

Table 1 Number of studied patients, family members and villagers, methods for diagnosis, prevalence of kidney stone, and relative risk

	Patients	Family members	Villagers
Number (*N*)	135	551	348
Age (years)	48.9 ± 11.4	48.7 ± 12.8	45.9 ± 16.6
Males/females	35/100[a]	225/326	150/198
Diagnosis of kidney stone	135	116	23
By KUB radiography	133	58	13
By ultrasonography	–	1	4
By surgical scar	2	20	2
By clinical history[b]	–	37	4
Prevalence (%)	100	21.05	6.61
Relative risk	–	3.18[c]	–

[a] The female bias was due to that a majority of patients were recruited from female wards

[b] This group of subjects had negative results of KUB radiography or ultrasonography or no record of surgical scar but they had a strong clinical history as justified from the presence of several symptoms associated with kidney stone, especially hematuria and stone passage

[c] Relative risk in the family members was estimated from the prevalence in this group divided by the prevalence in the group of villagers representing a control population

stone ($n = 251$). A majority (~88%) of the patients and affected family members had combined clinical symptoms associated with kidney stone including abdominal and back pain, hematuria, and stone passage. Thirty affected individuals (~12%) had no associated symptoms although all of them had kidney stone as shown by positive KUB radiography and two also had surgical operations.

Figure 1 shows age distributions of the subjects in the groups of patients, affected family members, and affected villagers, respectively. Both patients and affected family members had similar age distributions with the peak ages between 40 and 59 years while the affected villagers had a broadly low distribution with a shift to older ages. The average ages in the first two groups (48.9 ± 11.4 and 50 ± 11.6 years) were significantly less than that in the last group (60.0 ± 13.6 years) with P values of 0.0001 and 0.0006, respectively.

The location and number of stones observed in 192 patients and affected family members were analyzed. Unilateral (either right or left side) and bilateral kidney stones were found in about 72 and 28% of the affected individuals, respectively. The locations of stones were in the kidney

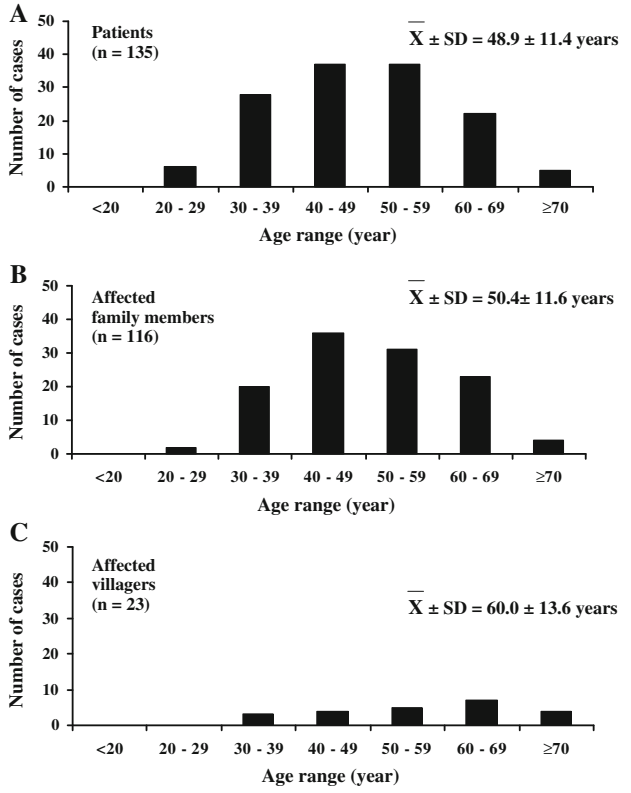

Fig. 1 Age distributions and average ages of three subject groups including patients, affected family members, and affected villagers. The peak ages in the first two groups were between 40 and 59 years while that in the last group were between 50 and 69 years. The average ages (mean ± standard deviation) in the first two groups (48.9 ± 11.4 and 50 ± 11.6 years) were significantly younger than that in the last group (60.0 ± 13.6 years)

(~80%), kidney and ureter (~11%), ureter (~8%), and kidney or ureter and urinary bladder (1%). The percentages of affected individuals with one, two, more than two, and staghorn stones were about 22, 9, 34, and 35%, respectively. The analysis of chemical composition of kidney stones, which were removed by surgical operations from the patients showed that approximately 88% of the stones contained calcium salts (whewellite, dahllite, and weddellite) and the remaining (12%) comprised uric acid, struvite, and ammonium hydrogen urate (Table 2).

Family data analysis showed that 67 of the 135 families (~50%) consisted of two or more affected members while the remaining 68 families (~50%) had only one affected member. Twenty-nine families (~21%) contained three or more affected members in the family. The examples of pedigrees with four or more affected members are shown in Fig. 2.

Discussion

Kidney stone is a major heath problem in the NE Thai population [4–6]. A large number of kidney stone patients were hospitalized [4, 5]. From our own experience and available data, each year several thousands of new kidney stone cases were admitted to our and other regional hospitals for extracorporeal shockwave lithotripsy and surgery. The cause of kidney stone in this population is unknown but may be unique as it is not obviously associated with increased urinary solutes [7, 8], which are known to be stone promoters. To examine the evidence of genetic contribution to kidney stone in this population, we studied the prevalence of kidney stone in members of the affected families to compare with that of villagers representing the rural control population. While the prevalence of the disease in the villagers was 6.6%, comparable to that was previously reported 8.4%

Table 2 Chemical compositions of kidney stones removed from the patients by surgeries

Chemical composition	Number	Percentage
Whewellite or calcium oxalate monohydrate ($CaC_2O_4 \cdot H_2O$)	48	44.04
Dahllite or carbonate–hydroxylappatite [$Ca_5(PO_4,CO_3)_3(OH)$]	43	39.45
Uric acid ($C_5H_4N_4O_3$)	10	9.17
Weddellite or calcium oxalate dihydrate ($CaC_2O_4 \cdot 2H_2O$)	5	4.59
Struvite or magnesium–ammonium phosphate–hexahydrate [$(NH_4)MgPO_4 \cdot 6(H_2O)$]	2	1.83
Ammonium hydrogen urate ($NH_4C_5H_3N_4O_3$)	1	0.92
Total	109	100

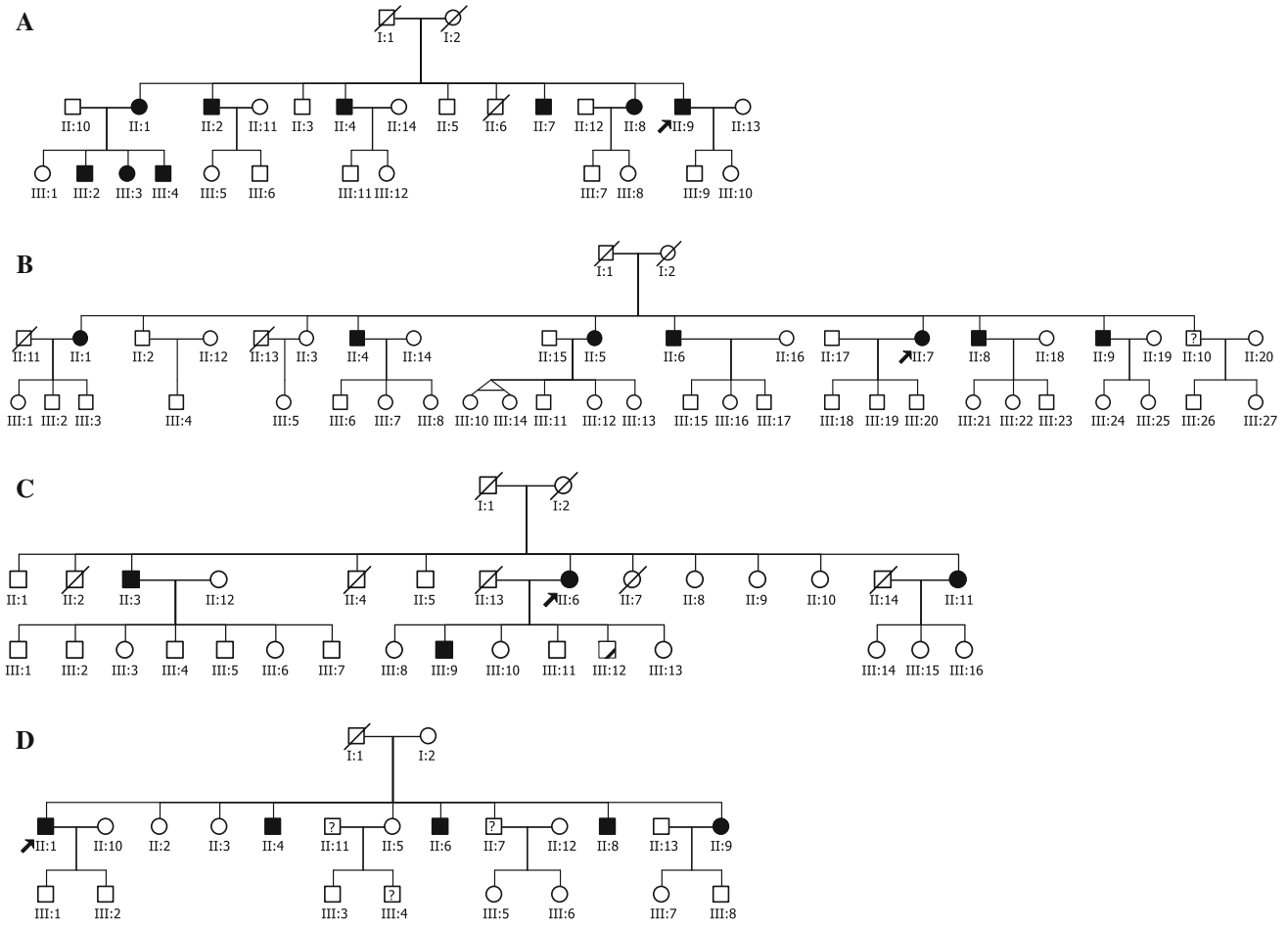

Fig. 2 The examples of pedigrees with four or more members affected with kidney stone. *Symbols* used in the figure are as follows: *open square* unaffected male, *open circle* unaffected female, *filled square* affected male, *filled circle* affected female, *slash* deceased, *arrow* proband

[6], it was as high as 21.0% among members of the affected families (Table 1). Thus, the relative risk (λ_R) of the disease among members of the affected family who were largely first-degree relatives was 3.18-fold higher than that in the villagers, indicating a family clustering of the disease. The relative risk in the second-degree relatives could not be calculated because the number of second-degree relatives was too small for the calculation. Although the family members were recruited without selection and prior knowledge of kidney stone status, it is still possible that there might be some recruitment bias whereby the members with kidney stones could have been preferentially included because they might have clinical symptoms that brought them to be investigated in the project. This would make a relative risk (λ_R) lower than the estimated 3.18. It is not known how large this possible recruitment bias is. However, a majority of the family members were free of the disease and its related clinical symptoms. In addition, the evidence that 30 family members (∼12%) who had no clinical symptoms were affected by kidney stones indicates that at least in this group of affected members their recruitments were not attributable to the associated clinical symptoms. This relative risk is the evidence suggesting a genetic contribution to the disease and the high incidence of hospitalized kidney stone cases in this NE Thailand region may be at least partly due to the high prevalence of familial cases.

The findings that average ages of the patients and affected family members (48.9 ± 11.4 and 50 ± 11.6 years) were significantly less than that of the villagers (60.0 ± 13.6 years) may indicate that although the population was homogeneous, they might carry different types of kidney stones. However, based on the available data, these groups of subjects are likely to carry a similar type of kidney stone because most of them carried opaque stone as detected by KUB radiography indicating calcium-containing stones, although it was not possible for us to obtain the stones from the villagers for the analysis of their compositions. Even though, there are many possible genetic causes for calcium-containing stones (e.g., calcium oxalate stone) resulting from different pathophysiologies.

About a half of the recruited families had one affected member and the other half had more than one affected members. This may indicate the presences of both sporadic and familial cases of kidney stone in this population, which is normally observed for many well-established genetic diseases with monogenic and polygenic causes such as thalassemia, diabetes, etc., especially when the families were small. There were, however, many families with several affected members and the examples are shown in Fig. 2. The presence of several affected members in the same families suggests the role of genetic factor for the disease. Nevertheless, the familial clustering could also be caused by environmental factors such as diet although in our previous study we could not demonstrate the relationship of nutrient intake and kidney stone in this population (unpublished data). Even though there were many families with several affected members, it was difficult to establish the definite mode of inheritance for the disease. One reason is that the onset of the disease was at about midlife and thus before this age the family members who were genetically affected might not have the disease. From our data, we found that there were both autosomal dominant (AD) and autosomal recessive (AR) modes of inheritance with a more prevalence of the families with the AR pattern. All forms of inheritance (AD, AR and X-linked) could be found in the reported monogenic hypercalciuric stone-forming diseases [16]. The development of disease in adulthood indicates that, in addition to the genetic component, environmental factors may also play some role in pathogenesis of the disease.

To further investigate into the role of genetic factor in pathogenesis of kidney stone in the NE Thai population, we will employ genetic or genomic approach, such as candidate-gene and genome-wide association and linkage analyses, to identify the disease or susceptible genes in this group of patients and family members. For example, a case–control association study may be conducted by the analysis of single-nucleotide polymorphisms in candidate genes encoding urinary stone-inhibitor proteins. The result of this genetic or genomic study may lead to the elucidation of molecular pathogenic mechanism of kidney stone in this population.

In conclusion, genetic factor should play some role in the pathogenesis of kidney stone in the NE Thai population as the disease has characteristics of familial aggregation and a high relative risk among members of the affected families. The gene responsible for the disease should be identified by the current genetic or genomic approach.

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Conflict of interest statement The authors declare no conflicts of interest.

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ORIGINAL ARTICLE

Mutations of maturity-onset diabetes of the young (MODY) genes in Thais with early-onset type 2 diabetes mellitus

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Summary

Objective Six known genes responsible for maturity-onset diabetes of the young (MODY) were analysed to evaluate the prevalence of their mutations in Thai patients with MODY and early-onset type 2 diabetes.

Patients and methods Fifty-one unrelated probands with early-onset type 2 diabetes, 21 of them fitted into classic MODY criteria, were analysed for nucleotide variations in promoters, exons, and exon–intron boundaries of six known MODY genes, including *HNF-4α*, *GCK*, *HNF-1α*, *IPF-1*, *HNF-1β*, and *NeuroD1/β2*, by the polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) method followed by direct DNA sequencing. Missense mutations or mutations located in regulatory region, which were absent in 130 chromosomes of non-diabetic controls, were classified as potentially pathogenic mutations.

Results We found that mutations of the six known MODY genes account for a small proportion of classic MODY (19%) and early-onset type 2 diabetes (10%) in Thais. Five of these mutations are novel including *GCK* R327H, *HNF-1α* P475L, *HNF-1α* G554fsX556, *NeuroD1* –1972 G > A and *NeuroD1* A322N. Mutations of *IPF-1* and *HNF-1β* were not identified in the studied probands.

Conclusions Mutations of the six known MODY genes may not be a major cause of MODY and early-onset type 2 diabetes in Thais. Therefore, unidentified genes await discovery in a majority of Thai patients with MODY and early-onset type 2 diabetes.

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Introduction

Maturity-onset diabetes of the young (MODY) is a genetically heterogeneous form of diabetes characterized by an early onset (usually before 25 years), frequent insulin-independence at the beginning of the disease, absence of ketosis, and an autosomal dominant pattern of inheritance. MODY can result from mutations in any of six different genes. One of these genes encodes the enzyme glucokinase (*GCK*/MODY2) whereas the other five genes encode transcription factors, including *hepatocyte nuclear factor* (*HNF*)*-4α* (MODY1), *HNF-1α* (MODY3), *insulin promoter factor-1* (*IPF-1*) (MODY4), *HNF-1β* (MODY5), and *neurogenic differentiation 1/β-cell E-box transactivator 2* (*NeuroD1/β2*) (MODY6).[1]

Glucokinase is an enzyme that functions as a glucose sensor and plays a key role in regulating insulin secretion from pancreatic β-cells. Mutations of the *glucokinase* (*GCK*) gene lead to impairment of β-cells' sensitivity to glucose.[2] However, the subjects with *GCK* mutations generally have a milder form of diabetes[3,4] due to the presence of compensatory mechanism that increases insulin secretion.[5] In contrast, subjects carrying mutations of transcription factors usually exhibit more severe hyperglycaemia.[6] Three hepatocyte nuclear factors (HNF-4α, HNF-1α, and HNF-1β) function together to regulate insulin gene expression as well as expression of gene encoding proteins involved in glucose transport and metabolism.[7] IPF-1 is a transcription factor that plays a critical role in the development of the pancreas and in the regulation of expression of insulin, glucokinase and glucose transporter genes.[8] However, the clinical characteristics of *IPF-1* (MODY4) mutations are generally much less severe than those seen in mutations of *HNF* genes due to a compensatory increase in insulin sensitivity.[9]

The prevalence of each MODY subtype varies among various ethnic groups. Mutations of *GCK*/MODY2 are the most common cause of MODY in France, accounting for more than 60% of studied families[10,11] whereas the prevalences of this MODY subtype in United Kingdom and Germany were 11%[12] and 8%,[13] respectively. In general, *HNF-1α*/MODY3 mutations are the most common cause of MODY in Caucasians and the prevalence varies from 21% to 64%.[14–19] The other four types of MODY are rare and have been described in few families. Overall, sequence variations of these six genes account for 75–80% of the cause of MODY in Caucasians