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No Evidence for Role of Common Anion Exchanger 1 Mutations on the Severity Difference in HB E-β-Thalassemia Disease in Northeast Thailand

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ABSTRACT In a distal renal tubular acidosis, a patient with coexistence of anion exchanger 1 (AE1) mutation and α-thalassemia had more severe clinical phenotype than usually observed. AE1 mutation might therefore modify the hematological and clinical presentations of the hemoglobin (Hb) E-β-thalassemia disease. To examine the role of AE1 mutation on severity difference in Hb E-β-thalassemia disease in northeast Thailand, three common AE1 mutations were investigated in Hb E-β-thalassemia patients with thalassemia major (TM) and thalassemia intermedia (TI) phenotypes. One hundred forty-eight patients including 103 TM and 45 TI were studied. Three common AE1 mutations including Southeast Asian Ovalocytosis (SAO), G701D and A858D were screened for using allele specific polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism (RFLP) assays. No any AE1 mutation was detected in all these Thai patients with Hb E-β-thalassemia. This result indicates that AE1 mutation is rare and should not be an important phenotypic modifier in the Hb E-β-thalassemia disease. Therefore, screening of common AE1 mutation in thalassemia patient is not necessary.

INTRODUCTION

Hemoglobin (Hb) E-β-thalassemia is the most important thalassemia syndrome in Southeast Asian populations. The disease exhibits varied clinical phenotypes ranging from severe transfusion dependent thalassemia major (TM) to milder thalassemia intermedia (TI) (Weatherall 2010). Understanding of the basis for this clinical diversity of the disease is very important for a prevention and control program and genetic counseling. Studies have shown that the phenotypic heterogeneities are modulated by different modifying factors which could be classified into primary, secondary and tertiary modifiers (Nienhuis and Nathan 2012; Thein 2013) Despite the ability to define accurately the primary modifier that is, β-thalassemia mutations and secondary modifiers that is, the ability to produce α- and γ- globin chains, prediction of patient phenotype remains a problem in genetic counseling and prenatal diagnosis (Ho et al. 1998; Nuinoon et al. 2009; Lettre 2012). In a previous study of Hb E-β-thalassemia disease in northeast Thailand, we have evaluated the effects of several primary and secondary factors including β-thalassemia mutations, the pre-sence of α-thalassemia, -158 $^{G}\gamma$-*Xmn*I polymorphism, β-globin gene haplotype and the polymorphic (TG)n(CG)m dinucleotide repeats within $^{G}\gamma$ and $^{A}\gamma$-globin genes on the hematological and clinical phenotypes of the patients. No different proportions of these genetic factors among TM and TI were observed except for the -28 A-G β^{+}-thalassemia mutation which was found only in the TI group. This data indicated that it is very hard to predict the clinical pheno-type in most of the patients from these primary and secondary genetic modifiers. The effect of tertiary modifying factor has not yet been examined. (Nuntakarn et al. 2009).

Anion exchanger 1 (AE1), also known as Band 3 is predominantly expressed in erythrocyte and the kidney. Erythroid AE1 is a major integral protein of human erythrocyte that plays an important role in mediated the anion

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transport function and maintaining the shape, stability, and flexibility of erythrocyte (Peters et al. 1996). Interaction of AE1 mutations could lead to a recessive distal renal tubular acidosis (dRTA) and hemolytic anemia (Shmukler et al. 2010). It has been thought therefore that AE1 mutation could be one of the tertiary phenotype modifiers in β-thalassemia disease. A more severe hemolysis with hepatosplenomegaly has been noted for a genetic compound heterozygosity of AE1 mutation (SAO/G701D) and α^{+}-thalassemia than that usually observed in a carrier of each defect (Khositseth et al. 2008). Unfortunately, this has not been investigated in Hb E-β-thalassemia disease, the most common thalassemia syndrome in the regions with highly phenotypic heterogeneity.

Objective

AE1 mutation might modify the hematological and clinical presentations of the Hb E-β-thalassemia disease. We have examined the possible role of AE1 mutation on the severity difference in Thai Hb E-β-thalassemia disease.

MATERIALS AND METHODS

Subjects

Ethical approval of the study protocol was obtained from the Institutional Review Board (IRB) of Khon Kaen University, Thailand. Archival DNA specimens of northeast Thai patients with Hb E-β-thalassemia were obtained from our earlier study (Nuntakarn et al. 2009). A total of 148 patients including 103 TM and 45 TI based on criteria including age at presentation of symptoms, average steady state Hb level, transfusion history, degree of hepatosplenomegaly, the presence of bone change and growth and development deficiency (Ho et al. 1998). Baseline hematological characteristics of these Hb E-β-thalassemia patients were as follows: (Hb 6.8±1.0 g/dl, MCV 71.9±8.8 fl, MCH 22.4±2.6 pg, Hb E 39.5±16.2 % and Hb F 19.5±12.1 %) for TM and (Hb 8.4±0.9 g/dl, MCV 63.1±6.4 fl, MCH 20.2±2.1 pg, Hb E 46.1±14.0 % and Hb F 19.8±11.5 %) for TI, respectively. In addition, another 246 unrelated Thai individuals of northeast Thailand were also examined.

Hematological and DNA Analysis

Hematological parameters, thalassemia screening, Hb analysis and DNA diagnostics for common forms of thalassemia in Thailand were performed routinely in our laboratory using standard hematological methods and PCR assays (Fucharoen et al. 2004; Sanchaisuriya et al. 2005). Data on other genetic markers including the $\alpha\alpha\alpha^{anti3.7}$ triplication, -158 $^{G}\gamma$-*Xmn*I polymorphism, β-globin gene haplotype and the (TG)n(CG)m dinucleotide repeat polymophisms in IVS2 of $^{G}\gamma$ and $^{A}\gamma$ globin genes were described in the previous study (Nuntakarn et al. 2009).

Analysis of Common AE1 Gene Mutations

In this study, three most common AE1 mutations found in Thai population including the 27 bp deletion in band 3 of Southeast Asian Ovalocytosis (SAO), the G701D and the A858D mutations (Yenchitsomanus et al. 2003) were investigated in 148 patients with Hb E-β-thalassemia. The **SAO** specific 27 bp deletion in exon 11 of AE1 gene was examined by PCR amplification exactly as described (Fucharoen et al. 2007). The **G701D** mutation in exon 17 was identified by PCR-restriction digestion using *Hpa*II. Two PCR primers, 5'TGGAGGA GGCAGGGGAGAACCCT3' and 5' GGGG CAGGAGGATGGTGAAGACG 3' were used to generate the amplified fragment of 347 bp in length. The G701D mutation eliminates the *Hpa*II site within this amplified fragment. After digestion with *Hpa*II, normal subject would have two digested fragments with 254 bp and 93 bp whereas the 347 bp fragment indicates the presence of G701D mutation. The same approach was done to identify the **A858D** mutation in exon 19 but with two other specific PCR primers; 5'GGTACAGGACCCTTTT CTGG 3' and 5' GCCTGCCCTAGTTCTGA GAC 3' followed by *Bgl*I digestion. In the absence of A858D mutation, the 334 bp amplified fragment would be digested into three smaller fragments of 175 bp, 121 bp and 38 bp. The A858D mutation eliminates one of the *Bgl*I restriction site within the amplified fragment and would alternatively generate two fragments of 213 bp and 121 bp. Confirmation for the accuracy of these two PCR-restriction assays developed was done by direct DNA sequencing

using ABI Prism 377 automated DNA sequencer (Perkin-Elmer Biosystems Co. Ltd.).

RESULTS

Nuntakarn et al. (2009) have previously demonstrated a wide variation on hematological data for the 148 Hb E-β- thalassemia patients, including 103 TM and 45 TI. We further examined a possibility of AE1 gene mutations in determining the disease severity in these Thai patients (Table 1). Figure 1 demonstrated that the presence of three most common AE1 gene mutations; SAO, G701D and A858D could be screened for using PCR assays developed. DNA sequencing of the amplified fragments of representative samples revealed the accuracy of the assays. Unexpectedly, among 148 Hb E-β-thalassemia patients with TM and TI, no any *AE1* mutation was detected. Co-inheritance of AE1 mutation as a phenotypic modulating factor was not observed in this study of Thai patients with Hb E-β- thalassemia disease. Further investigation on another 246 unrelated Thai subjects revealed no any common AE1 mutation among them either.

Table 1: Existence of three common AE1 mutations among 148 Thai Hb E-β-thalassemia patients including 103 TM and 45 TI phenotypes.

AE1 mutations	*Hb E-β-thalassemia phenotype*	
	TM	*TI*
Number	103	45
SAO	N / N	N / N
G701D	N / N	N / N
A858D	N / N	N / N

N indicates normal allele

DISCUSSION

The hematological parameters presented in Table 1 indicated the phenotypic heterogeneity of this common Hb E-β-thalassemia disease in Thailand. Our previous study has indicated that only the -28 A-G β^+-thalassemia mutation which was found in only in TI group of patient is a useful marker for a mild β-thalassemia phenotype. No difference on other genetic markers including coexistence of α-thalassemia, -158 $^{G}\gamma$- *Xmn*I polymorphism, β-globin gene haplotypes and (TG)n(CG)m dinucleotide repeat polymophisms in IVS2 of the $^{G}\gamma$ and $^{A}\gamma$ -globin genes was observed between TM and TI

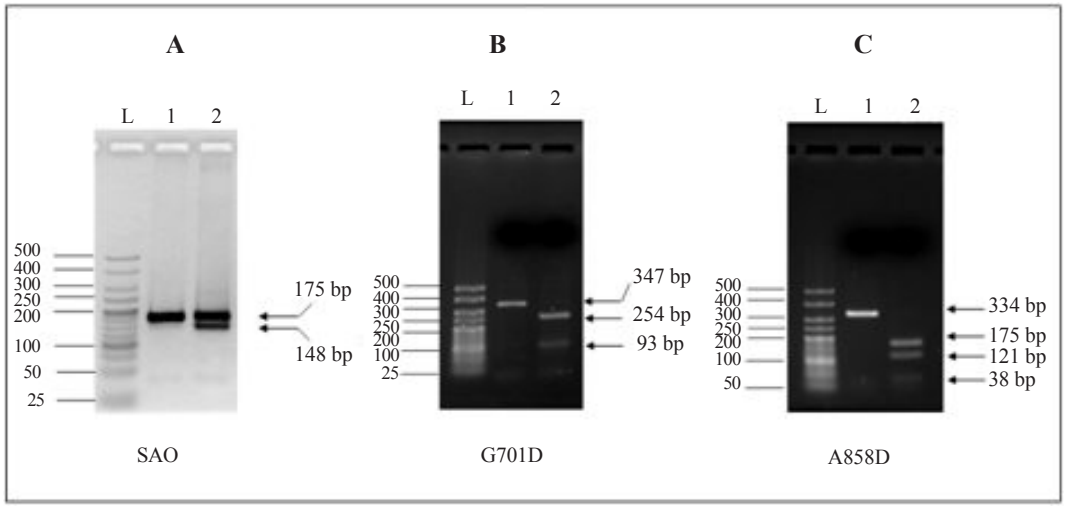

Fig. 1. (A): Identification of the SAO mutation. The amplified fragment of 175 bp and 148 bp represent normal allele and mutant allele, respectively. Lane 1 is normal. Lane 2 is heterozygous SAO. L is HyperLadder V markers.
(B): Identification of the G701D mutation by PCR-RFLP. The normal 347 amplified DNA fragment was digested with *Hpa*II enzyme into two smaller fragments with 93 bp and 254 bp. Lane 1 is undigested amplified DNA whereas lane 2 is *Hpa*II digested fragments of normal subject. L is HyperLadder V markers.
(C): Identification of the A858D by PCR-RFLP with *Bgl*I enzyme. After digestion the normal 334 amplified DNA is digested into three fragments with 175 bp, 121 bp and 38 bp while the fragments of 213 bp and 121 bp present mutant allele. Lane 1 is amplified DNA of normal subject whereas lane 2 showed the BglI digested fragment of normal individual. L is HyperLadder V markers.

groups of these patients; the data indicating of other undiscovered factors especially those of tertiary genetic modifiers (Nuntakarn et al. 2009).

The researcher have investigated for the first time the possibility of AE1 mutation in modification of severity in Hb E-β-thalassemia disease in northeast Thailand, the most common form of thalassemia in the region (Yamsri et al. 2010). However, no any common AE1 mutations investigated (namely SAO, G701D and A585D) was detected among 103 TM and 45 TI patients with Hb E-β-thalassemia. Although the G701D and A858D mutations have only been documented in northeast Thai patient with distal renal tubular acidosis (Yenchitsomanus et al. 2003), SAO has been found to be relatively common among southern Thai population (Laosombat et al. 2010). SAO is a clinically asymptomatic hereditary disorder of red blood cell characterized by the presence of macro-ovalocytes and stomatocytes on blood film examination (O'Donnell et al. 1998). Although, we have observed no additional clinical phenotype in a sporadic case of double heterozygote for SAO and β-thalassemia (Fucharoen et al. 2007), it is possible that co-existence of SAO with Hb E-β-thalassemia disease may lead to significant changes in clinical phenotype of the patient. In another study we have detected the prevalence of 2-3 % SAO mutation among southern Thai population but no SAO was observed among 246 northeastern Thai individuals (Ngouprommin et al. 2013). As no common AE mutations were observed in this study, the clinical severity of Hb E-β-thalassemia patient with AE1 mutation could not be evaluated and it is hard to conclude that AE1 mutation is not one of the genetic modifiers for the disease. However, all these results indicate that the prevalence of AE1 mutation is low and it is unlikely that AE1 mutation is an important phenotype genetic modifier of Hb E-β-thalassemia disease among northeast Thai population. Recent information obtained by the genome wide association study (GWAS) has indirectly supported this. It has been found that three quantitative trait loci (QTL) could influence Hb F level and disease severity in β-thalassemia disease i.e. the $^{G}\gamma$-*Xmn*I polymorphism in β-globin gene cluster and multiple SNPs in *HBS1L-MYB* intergenic region and *BCL11A* gene (Menzel et al. 2007; Sedgewick et al. 2008; Nguyen TKT et al. 2010; Neishabury et al. 2013; Lettre 2012). Yet it is conceivable that substantial genetic markers contributing to the disease severity in Hb E-β-thalassemia disease are still undiscovered. Further study on other genetic markers is required to provide a more insight into the phenotypic heterogeneity of this common genetic disorder in the region. Nonetheless, the result from this study indicates that screening of common AE1 mutations in Thai patients with Hb E-β-thalassemia disease with different severity is not necessary.

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