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ORIGINAL ARTICLE

Genetic origin of α^0-thalassemia (SEA deletion) in Southeast Asian populations and application to accurate prenatal diagnosis of Hb Bart's hydrops fetalis syndrome

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α^0-thalassemia of SEA deletion (--^{SEA}) is common among Southeast Asian and Chinese. Using haplotype and phylogenetic analyses, we examined the origin of this defect in Southeast Asian populations. Study was done on both normal and α^0-thalassemia alleles in 3 ethnic groups including 96 Thai, 52 Laotian and 21 Cambodian. Five SNPs encompassing the (--^{SEA}) including (rs3760053 T>G), (rs1211375 A>C), (rs3918352 A>G), (rs1203974 A>G) and (rs11248914 C>T) were examined using high-resolution melting assays. It was found that 94.0% of Thai, 100% of Laotian and 100% of Cambodian α^0-thalassemia alleles were linked to the same haplotype: the haplotype H4 (AAGC), representing an Asian specific origin. An G allele of the (rs3760053) was found to be in strong linkage disequilibrium with the α^0-thalassemia allele in these populations. A multiplex PCR assay was developed to detect simultaneously the (--^{SEA}) allele and genotyping of a linked (rs3760053) to improve accuracy of prenatal diagnosis of α^0-thalassemia. Application of this multiplex PCR assay for routine prenatal diagnosis of α^0-thalassemia in 12 families revealed a 100% concordant result with conventional gap-PCR assay. Therefore, a single genetic origin is responsible for the spread and high prevalence of the (--^{SEA}) in the region. The multiplex PCR assay developed should provide a double-check PCR system for more accurate diagnosis and allow the monitoring of possible maternal contamination at prenatal diagnosis of this important genetic disorder.
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INTRODUCTION

The Southeast Asian deletion α^0-thalassemia (--^{SEA}) is the most common and severe form of α-thalassemia found in Southeast Asia and south China.[1,2] Association of this severe form with a milder form of α^+-thalassemia leads to the Hb H disease (--^{SEA}/-α), commonly encountered in the region. Homozygous α^0-thalassemia (--^{SEA} /--^{SEA}) can cause severe thalassemia syndrome known as Hb Bart's hydrops fetalis. Pregnancy with this Hb Bart's hydrops fetalis has increased risk of severe maternal complications and infant with this syndrome usually die *in utero*, or soon after birth.[3] Prenatal diagnosis of homozygous state of α^0-thalassemia is therefore preferable in routine practice. Generally, this can be done by using gap-PCR analysis of fetal tissues collected during the first or second trimesters of pregnancy. Although this is simple, allele dropout or maternal contamination leading to a misdiagnosis can happen and diagnosis using two different methods is usually employed.[4–6] Alternatively, analysis of fetal blood obtained by cordocentesis for Hb Bart's quantification could also provide accurate diagnosis of the disease.[7] However, cordocentasis is not practical and is relatively outdate, DNA analysis of fetal tissues is preferred method.

Qiu *et al.* reported an evidence of recent natural selection on the α^0-thalassemia (SEA deletion) in south China triggered by malaria.[8] A single origin of α^0-thalassemia (SEA deletion) as characterized by haplotype analysis was found among southern Chinese, the haplotype which was likely constructed from an Asian specific haplotype. Unfortunately, no haplotype study has been documented for other Asian populations with high prevalence of this genetic disorder. Here we reported the results of studying genetic backgrounds of α^0-thalassemia (SEA deletion) in Southeast Asian populations using haplotype and phylogenetic analysis. An accurate method for prenatal diagnosis of this α^0-thalassemia (SEA deletion) based on simultaneous detection of the SEA deletion and analysis of a linked single-nucleotide polymorphism (SNP) to the deletion was developed.

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MATERIALS AND METHODS

Samples

Ethical approval of the study protocol was obtained from the Ethical Committee of Khon Kaen University, Thailand (HE592190). Archival DNA samples with α^0-thalassemia at the Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, were obtained from the previous study.[9] Identification of α^0-thalassemia is routinely performed in our laboratory using gap-PCR described elsewhere.[9,10] A total of 169 DNA specimens obtained from 96 Thai, 52 Laotian and 21 Cambodian individuals were recruited. Among 96 Thai individuals, 59 were α^0-thalassemia carriers ($--^{SEA}/\alpha\alpha$), 7 were homozygotic fetuses with Hb Bart's hydrops fetalis ($--^{SEA}/--^{SEA}$) and the remaining 30 had wild-type ($\alpha\alpha/\alpha\alpha$). Of the 52 Laotian individuals, 28 were heterozygous ($--^{SEA}/\alpha\alpha$) and 24 had wild-type ($\alpha\alpha/\alpha\alpha$). Cambodian subjects included 7 heterozygous ($--^{SEA}/\alpha\alpha$) and 14 wild-type ($\alpha\alpha/\alpha\alpha$).

SNPs genotyping

Five SNPs encompassing the SEA deletion α^0-thalassemia breakpoints were examined. These SNPs, spanning about 82 kb in length, included a SNP located upstream of the SEA deletion breakpoint (rs3760053 T>G) and four other SNPs located downstream; [(rs1211375 A>C), (rs3918352 A>G), (rs1203974 A>G) and (rs11248914 C>T)] as shown in Figure 1.[8] All of them were genotyped using the high-resolution melting (HRM) assays described[8] and further confirmed by DNA sequencing. Oligonucleotide primers used in this study were listed in Supplementary Table S1. PCR was carried out using the KAPA HRM FAST PCR kit (KAPA Biosystems, Wimington, MI, USA). HRM analysis was performed on a Light Scanner System (Idaho Technology Inc., Salt Lake City, UT, USA) and the data was analyzed with the Light Scanner System 2.0 Software program (Idaho Technology Inc.).

Data analysis

The basic statistics including allele frequency, genotype frequency, minor allele frequency and Hardy–Weinberg Equilibrium (P-value >0.05) were calculated using the PEAS V1.0: a package for elementary analysis of SNP data.[11] Haplotype patterns, constructed using data from the four SNPs located downstream of the SEA deletion breakpoint, and pairwise linkage disequilibrium (LD) test were determined using the HAPLOVIEW 4.2 software, which examined haplotypes $>1\%$ observed in the study.[12] The D' value of the linkage disequilibrium test was calculated and shown in LD pattern. Using haplotype data, the phylogenetic tree (Figure 2) was generated using the online DendroUPGMA software (http://genomes.urv.cat/UPGMA) applying Jaccard (Tanimoto) coefficient with default settings.[13]

Development of multiplex PCR assay for simultaneous detection of α^0-thalassemia and genotyping of SNP rs3760053

A novel method based on HRM analysis was developed. Two primer pairs with sequences shown in Supplementary Table S1 were designed and used to produce specific amplicons for detecting the SEA deletion α^0-thalassemia and genotyping of the SNP rs3760053. Detection of the SEA deletion α^0-thalassemia allele was by gap-PCR but genotyping of the (rs3760053) was done by HRM analysis on the Eco Real-Time PCR system (Illumina Co., Ltd., San Diego, CA, USA; Figure 3). The multiplex HRM reaction mixture (20 µl) contained 50–100 ng DNA in a buffer consisting of 60 $mmol\,l^{-1}$ Tris HCl, pH 9.0, 40 $mmol\,l^{-1}$ 2 M KCl, 16 $mmol\,l^{-1}$ $(NH_4)_2SO_4$, 3 $mmol\,l^{-1}$ $MgCl_2$, 50 $\mu mol\,l^{-1}$ each dNTP, 480 $nmol\,l^{-1}$ of each specific primer for SEA deletion, 900 $nmol\,l^{-1}$ of each primer for SNP (rs3760053), 1 $\mu mol\,l^{-1}$ SYBR green and 0.02 units *Taq* DNA polymerase (Biolabs Co., Ltd., Ipswich, MI, USA). The PCR amplification started with heating at 95 °C (15 min) followed by 32 cycles of (95 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min 20 s). After PCR, HRM analysis was carried out from 55 to 95 °C at 0.1 °C per second by the Eco Real-Time PCR system and data was analyzed using HRM analysis software. A total of 221 left-over DNA samples randomly collected at our routine service for thalassemia screening were examined in blinded trials with this new technique and the results were compared with conventional gap-PCR assay performed routinely.

Development of multiplex PCR assay for simultaneous detection of α^0-thalassemia and a T allele of SNP rs3760053

In this development, three primer pairs (listed in Supplementary Table S1) were used to generate specific fragments for normal allele (836 bp), the SEA deletion α^0-thalassemia (660 bp) and a T allele of SNP rs3760053 with size ranging from

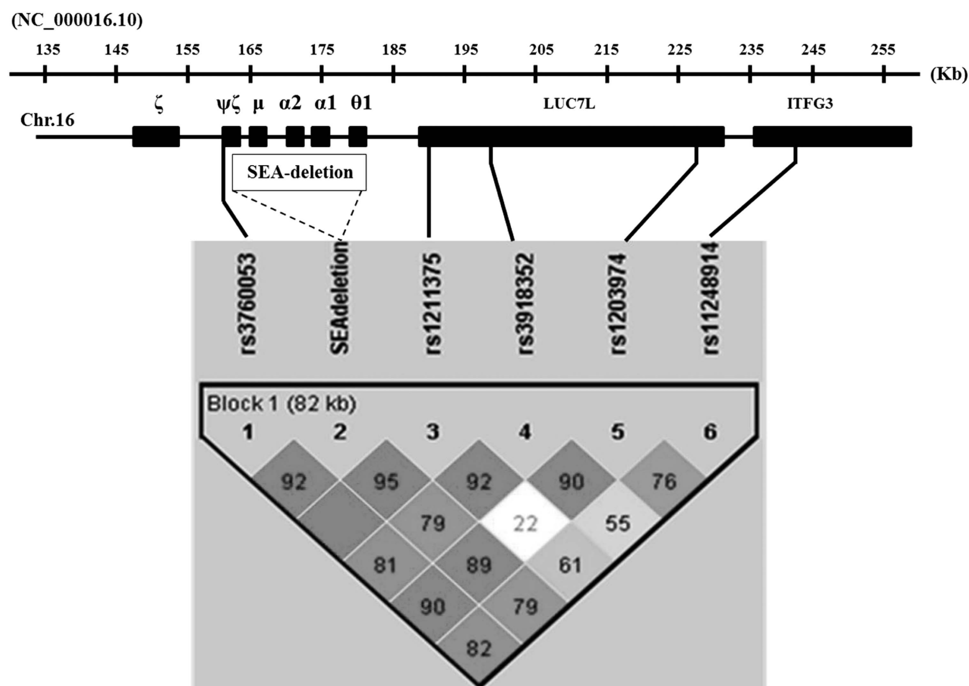

Figure 1 Structure of linkage disequilibrium (LD) constructed from 5 SNPs and $-^{SEA}$ deletion α^0-thalassemia allele in sample pooled of 338 chromosomes of Southeast Asian origin. Number in each square is the percentage of D'-value between the pair of loci. The SEA deletion α^0-thalassemia allele showed strong LD with all single-nucleotide polymorphisms (SNPs) investigated. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

800 bp to 2.7 kb depending on the inter-ζ hypervariable region (HVR) that is, S = 900–1000 bp, M = 1.7–1.8 kb, L = 2.4–2.7 kb, respectively. Again, identification of an α^0-thalassemia allele was straightforward by using gap-PCR but detection of a T allele of the SNP rs3760053 was based on allele specific PCR (Figure 4). The multiplex PCR reaction mixture (50 μl) contained 50–100 ng DNA in a buffer containing 10 mmol l^{-1} Tris HCl, pH 8.3, 50 mmol l^{-1} KCl, 1.5 mmol l^{-1} $MgCl_2$, 50 μmol l^{-1} each dNTP, 1.02 pmol of primer A7, 1.32 pmol of primer A9, 480 nmol of primer αG29, 240 nmol each of primers αG72, αG42 and αG43, and 0.03 units *Taq* DNA polymerase (Biolabs Co. Ltd.). The multiplex PCR was carried out on a Biometra T Personal Thermocycler (Biometra GmbH Co., Ltd., Göttingen, Germany) by initial heating at 95 ºC (5 min), followed by a 35 cycles of (95 ºC, 1 min - 58 ºC, 1 min - 72 ºC, 1 min) and a final step of 72 ºC for 5 min. PCR product was then analyzed by agarose gel electrophoresis after ethidium bromide staining. A total of 155 left-over DNA samples randomly collected at our routine service were examined in blinded trials with this new technique and results were compared with conventional gap-PCR assay performed routinely.

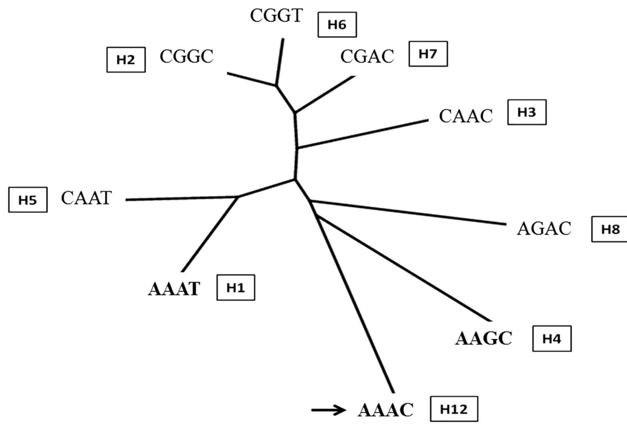

Figure 2 Phylogenetics (cladogram) analysis of haplotypes found in Asian populations, constructed using DendroUPGMA software with Cophenetic Correlation Coefficient = 0.7759. The H1, H4 and H12 are haplotypes associated with the SEA deletion α^0-thalassemia found in Southeast Asian populations and H4 (AAGC) is the most common one. Arrow indicates an ancestral haplotype H12.

Prenatal diagnosis of α^0-thalassemia using a double-check system

Prenatal diagnosis of α^0-thalassemia was done in 12 families at risk of having fetuses with Hb Bart's hydrops fetalis. Fetal DNA was prepared from fetal tissues obtained routinely by chorionic villus sampling or amniocentesis.[14] Prenatal diagnosis was carried out with the two newly developed systems (multiplex HRM analysis and multiplex PCR amplification) and results compared with conventional gap-PCR assay run routinely at our center.

RESULTS

Genetic background of the SEA deletion α^0-thalassemia among Southeast Asian populations

From a pool of 169 Southeast Asian subjects examined (including 96 Thai, 52 Laos and 21 Cambodian, that is, 338 chromosomes in total), DNA analysis for α^0-thalassemia (SEA deletion) identified 7 cases with homozygous (--^{SEA}/--^{SEA}), 94 heterozygous (--^{SEA}/αα) and 68 wild-type (αα/αα). This led to a total number of 230 wild type (αα) and 108 mutant alleles (--^{SEA}). Analysis of the 5 SNPs surrounding the SEA deletion as shown in Figure 1 were performed on all these 169 subjects. The results of this analysis including allele frequencies, genotype frequencies and minor allele frequencies as well as the χ^2-test for Hardy–Weinberg Equilibrium were summarized in

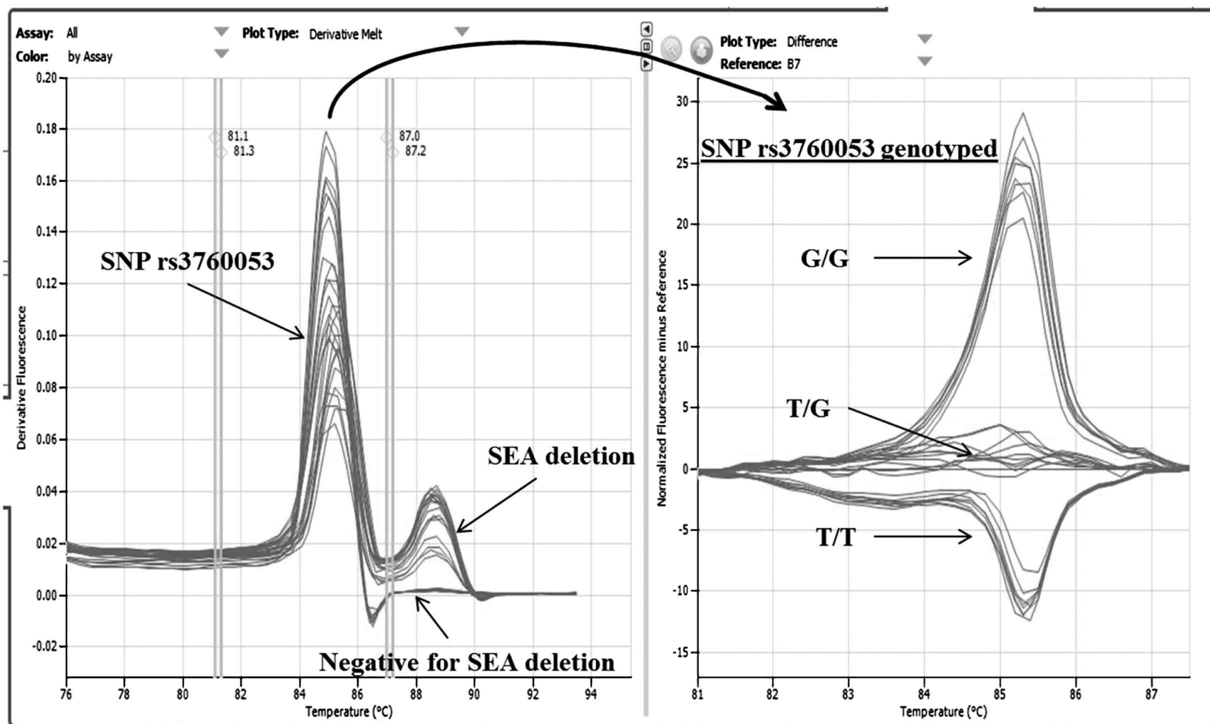

Figure 3 A multiplex high-resolution melting (HRM) analysis for simultaneous detection of SEA deletion α^0-thalassemia and genotyping of single-nucleotide polymorphism (SNP) rs3760053. On the left side, the specific amplicon of SNP rs3760053 was detected at Tm 85.09 ± 0.12 ºC whereas that of the SEA deletion α^0-thalassemia was identified at Tm 88.64 ± 0.09 ºC. Further differentiation of the specific SNP rs3760053 amplicon for genotyping (T/T, T/G and G/G) was shown on the right hand side. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

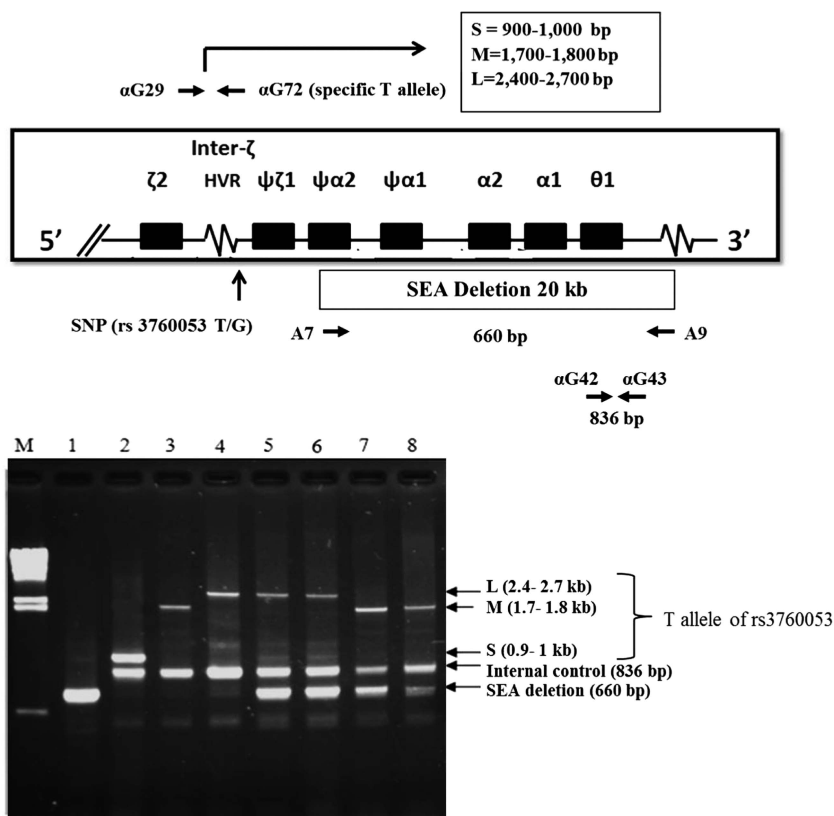

Figure 4 A multiplex PCR assay for simultaneous detection of the SEA deletion α^0-thalassemia and a T allele of the single-nucleotide polymorphism (SNP) rs3760053 using combined gap-PCR (for SEA deletion) and allele specific PCR (for T allele). The locations and orientations of primers used were depicted. Gel electrophoresis represented results of the multiplex PCR amplification. 'M' is the λ/*Hind*III size markers, whereas 1 = homozygote α^0-thalassemia (Hb Bart's hydrops fetalis); 2, 3, 4 = non α^0-thalassemia carriers carrying T alleles with S, M, L polymorphisms, respectively; 5 and 6, and 7 and 8 = α^0-thalassemia carriers with T allele of L and M polymorphisms, respectively.

Supplementary Table S2. As shown in the table, χ^2-test indicated in all SNPs, the assumption of Hardy–Weinberg Equilibrium of the studied population ($P > 0.05$) and minor allele frequency of all SNPs were found to be > 0.1. Because of having the highest number of subjects, data from a group of Thai individuals including 59 SEA deletion α^0-thalassemia carriers and 30 normal subjects were analyzed further. The derived allele frequency (DAF) of each SNP (G of rs3760053, C of rs1211375, G of rs3918352, G of rs1203974 and T of rs11248914) was calculated and compared between α^0-thalassemia carriers ($n = 59$) and wild type ($n = 30$). For instance, a G allele of rs3760053 was found to be more common in α^0-thalassemia carrier (with DAF of 0.4831) than that of a normal subject (with DAF of 0.0333). This is also the case for a G allele of the rs1203974 (with DAF of 0.6694). For the rs11248914, however, a T allele is less common in α^0-thalassemia carrier (with DAF of 0.2712) than that of a normal subject (with DAF of 0.5000). Fisher's exact test revealed significant differences for the rs3760053 ($P < 0.001$), rs1203974 ($P = 0.002$) and rs11248914 ($P = 0.002$) but not the two remaining SNPs.

As shown in Figure 1, pairwise analysis of linkage disequilibrium (LD) between these SNPs and the SEA deletion α^0-thalassemia in a pooled of 338 subjects demonstrated strong LD of the SEA deletion allele with all 5 SNPs with the D'-values ranging from 0.79 to 0.95 (79–95%). In addition, this analysis also indicated the strongest LD between rs3760053 & rs1211375 and the lowest LD with D'-value of 0.22 (22%) between rs1211375 and rs1203974.

The four SNPs located downstream of the SEA deletion breakpoint including rs1211375, rs3918352, rs1203974 and rs11248914 were used to construct haplotype using the HAPLOVIEW 4.2 software. Frequencies of these haplotypes of Southeast Asian populations were compared to those of other populations described in the HapMap project including CHB (Han Chinese in Beijing, China), JPT (Japanese in Tokyo, Japan), GIH (Gujarat Indians in Houston, Texas), CEU (Utah residents with Northern and Western European ancestry), TSI (Toscans in Italy), YRI (Yoruba Ibadan, Nigeria) and MEX (Mexican ancestry in Los Angeles) as shown in Table 1. Haplotype H12 (AAAC), an ancestral haplotype, was rarely found in Thai, Cambodian and MEX populations. The three most common haplotypes for a wild-type allele in Southeast Asian populations were H1 (AAAT), H2 (CGGC) and H3 (CAAC) whereas H4 (AAGC) was found to be Asian specific haplotype. Interestingly, we found that most of the SEA deletion α^0-thalassemia alleles identified in Southeast Asian populations were associated with haplotype H4 (AAGC; 94.05% in Thai and 100% in Laos & Cambodian), similar to that described previously in Chinese population (100%).[8] This finding likely indicates the same origin of the SEA deletion α^0-thalassemia in Asian populations and could partly explain why this common α^0-thalassemia in Asian populations had never been found in other populations lacking the H4 (AAGC) haplotype. Further phylogenetic analysis using the DendroUPGMA software indicated that this haplotype H4 (AAGC) arose from the ancestral haplotype H12 (AAAC) by a mutational event (A>G) at

Table 1 Haplotypes generated using 4 SNPs located downstream of the SEA deletion α^0-thalassemia in Southeast Asian populations investigated as compared to other populations described in the HapMap project

	rs1211375	*rs3918352*	*rs1203974*	*rs11248914*	*(αα) Allele frequency, %*										*(–SEA) Allele frequency, %*			
					Asia						*Europe*		*Africa*	*America*	*Asia*			
					THA[a]	*LAO*[a]	*CAM*[a]	*CHB*[b]	*JPT*[b]	*GIH*[b]	*CEU*[b]	*TSI*[b]	*YRI*[b]	*MEX*[b]	*THA*[a]	*LAO*[a]	*CAM*[a]	*CHN*[c]
Haplotype	*A>C*	*A>G*	*A>G*	*C>T*	(n = *119)*	(n = *76)*	(n = *35)*	—	—	—	—	—	—	—	(n = *73)*	(n = *28)*	(n = *7)*	(n = *30)*
H1	A	A	A	T	44.9	32.9	39.6	34.5	36.6	31.2	26.1	29		19.2	3.12			
H2	C	G	G	C	24.4	25.3	14.2	35.7	30.8	11.4	9.8	11.4	3.9	26				
H3	C	A	A	C	17.2	12.4	17.2	14.3	22.1	24.4	10.5	11.9	27.8	17.3				
H4	A	A	G	C	6.7	11.9	14.3	10.7	5.8	1.1					94.05	100	100	100
H5	C	A	A	T	2.8	5.6	5.7	2.4		2.3	1.5	1.1	23					
H6	C	G	G	T	2.3	6.2	5.7	1.2	1.7	18.7	35.6	31.2	10	26.9				
H7	C	G	A	C		5.2			1.2	6.8	7.1	6.8		4.8				
H8	A	G	A	C						2.3	6.3	5.7						
H9	A	A	G	T									27					
H10	C	G	A	T									3					
H11	A	G	G	T									2.6					
H12[d]	A	A	A	C	1.8		3							1.9	2.83			

[a]Populations under study; THA (Thai), LAO (Laotian), CAM (Cambodian).
[b]Data taken from the HapMap project; CHB (Han Chinese in Beijing, China), JPT (Japanese in Tokyo, Japan), GIH (Gujarat Indians in Houston, TX, USA), CEU (Utah residents with Northern and Western European ancestry), TSI (Toscans in Italy), YRI (Yoruba Ibadan, Nigeria) and MEX (Mexican ancestry in Los Angeles).
[c]From Qiu QW, *et al.*, *BMC Evolutionary Biology* 2013; 13: 63. CHN (Chinese).
[d]Ancestral haplotype.

Table 2 Derived allele frequencies of five SNPs surrounding the SEA deletion α^0-thalassemia in normal and α^0-thalassemia carrier in Thai population

			Derived allele frequencies		
SNP ID	Polymorphism[a]	Position[b]	SEA carrier (n = 59)	Normal (n = 30)	P-value[c]
rs3760053	T>G	161244	0.4831	0.0333	< 0.001
rs1211375	A>C	190281	0.2881	0.4667	0.100
rs3918352	A>G	197889	0.2034	0.2667	0.511
rs1203974	A>G	227459	0.6694	0.3333	0.002
rs11248914	C>T	243563	0.2712	0.5000	0.002

Abbreviation: SNP, single-nucleotide polymorphism.
[a]Allele on the positive strand and the first character represents an ancestral allele.
[b]NCBI position in chromosome 16.
[c]By Fisher's exact test.

Table 3 Validation of a multiplex HRM analysis for simultaneous identification of the SEA deletion α^0-thalassemia and genotyping of the SNP rs3760053 in 211 individuals as compared to the conventional gap-PCR assay

Multiplex HRM analysis				
SEA deletion α^0-thalassemia	SNP rs3760053 genotyping	No	Gap-PCR assay for SEA deletion α^0-thalassemia	No
Negative	T/T	126	αα/αα	134
Negative	T/G	8		
Positive	T/G	66	αα/--SEA	71
Positive	G/G	5		
Positive	G/G	6	--SEA/--SEA	6
Total		211	Total	211

Abbreviations: HRM, high-resolution melting; SNP, single-nucleotide polymorphism.

the SNP rs1203974 before an introduction of the SEA deletion α^0-thalassemia mutation onto this haplotype (Figure 2).

Validation of multiplex PCR assays for simultaneous detection of α^0-thalassemia and SNP rs3760053

As shown in Table 2, a highly significant difference ($P<0.001$) in DAF between α^0-thalassemia carrier and normal subject for the rs3760053 (T>G) was observed and G allele was strongly linked to the SEA deletion α^0-thalassemia in this population. In order to improve accuracy for prenatal diagnosis of α^0-thalassemia (SEA deletion) in routine practice, we have developed two different multiplex assays to detect the SEA deletion α^0-thalassemia and the SNP rs3760053, simultaneously.

The first assay based on HRM analysis was shown in Figure 3. As shown in the figure, a specific peak of the SEA deletion α^0-thalassemia was detected at Tm; 88.64 ± 0.09 °C. A peak corresponding to the SNP rs3760053 was found separately at Tm; 85.09 ± 0.12 °C from which with further differentiation could demonstrate the GG, TG and TT genotypes of this SNP. The method was applied to 211 blind specimens and the results shown in Table 3. As shown in the table, a 100% concordant result for detection of the SEA deletion α^0-thalassemia as compared to the conventional gap-PCR was obtained for all 134 normal, 71 heterozygous and 6 homozygous subjects. In addition, genotypes of the SNP rs3760053 of all subjects were readily identified. Among 134 normal subjects, 126 had T/T and 8 had T/G genotypes. In heterozygotes, 66 of 71 had T/G and 5 had G/G genotypes. Interestingly, all the 6 homozygotes carried G/G genotype; the data confirmed that most of the SEA deletion α^0-thalassemia in Southeast Asian population was linked to a G allele of the SNP rs3760053.

We also used another method to detect the SEA deletion α^0-thalassemia by gap-PCR and identification of a T allele of the SNP rs3760053 by allele specific PCR. As shown in Figure 4, the amplified DNA fragments specific for SEA deletion (660 bp), normal allele (836 bp) and a T allele of the SNP rs3760053 with size polymorphism of the inter-ζ HVR (S, M or L) could be demonstrated on the gel electrophoresis. Since the SEA deletion α^0-thalassemia was linked to a G allele of the SNP rs3760053, homozygous α^0-thalassemia would generate only one fragment at 660 bp, but not 836 bp and T allele specific fragments. This should provide an effective double-check system for the diagnosis of a homozygotic condition of this most severe thalassemia disease. Validation of this method was done in blinded trials on 155 subjects. A 100% concordant result with routine gap-PCR assay was obtained by this validation. As shown in Table 4, wild-type subjects ($n=41$) had no SEA-deletion-specific fragment (660 bp) but had internal control fragment (836 bp) and T allele with inter-ζ HVR (S = 10, M = 26, and L = 5). Heterozygotes ($n=104$) had specific SEA-deletion fragment (660 bp), and either T allele of the SNP rs3760053 with inter-ζ HVR (S = 14, M = 58, L = 31) or G allele of the SNP rs3760053 ($n=1$) as well as internal control fragment (836 bp). In contrast, all 10 homozygotes (--SEA/--SEA) presented, as expected, only one specific fragment at 660 bp without fragments specific for a T allele of SNP rs3760053 and normal control.

The two new methods developed have been applied to prenatal diagnosis of Hb Bart's hydrops fetalis in routine practice on 12 at-risk families. Again the concordant results of diagnosis using conventional gap-PCR method was obtained as shown in Supplementary Table S3. The two developed methods for simultaneous detection of α^0-thalassemia, genotyping of SNP rs3760053 and identification of a T allele could improve the accuracy of diagnosis provided by gap-PCR alone. The method can also help in detecting maternal contamination of fetal specimen by looking at the pattern of size polymorphism associated with the T allele of the fetus. Analysis of the 12 families revealed 3 fetuses with normal (αα/αα), 5 fetuses with heterozygous α^0-thalassemia (--SEA/αα) and 4 fetuses with homozygous α^0-thalassemia (--SEA/--SEA) leading to the Hb Bart's hydrops fetalis syndrome.

DISCUSSION

The SEA deletion α^0-thalassemia represents one of the most common α-thalassemia found among Southeast Asian and Chinese populations. It results from a DNA deletion on chromosome 16 spanning approximately 20.5 kb which removes the Ψα2-, Ψα1-, α2-, α1- and θ1 globin genes.[15] Parents who are both carriers of this α^0-thalassemia would have a 25% risk of having fetus with homozygous α^0-thalassemia causing Hb Bart's hydrops fetalis syndrome. Although some survivors with Hb Bart's hydrops fetalis syndrome have been documented, the disease can lead to serious maternal complications during pregnancy such as hypertension, pre-eclampsia, polyhydramnios and severe postpartum hemorrhage.[16,17] Therefore, it is necessary to provide accurate diagnostic test for carrier screening and prenatal diagnosis. This SEA deletion α^0-thalassemia has limited geographical distribution and has only been found among Chinese and Southeast Asian populations. High prevalence of this mutation has been reported among northeast Thai, Laos and Cambodian populations.[18,19] Although the evidence of malaria selection in α^0-thalassemia at the

Table 4 Validation of a multiplex PCR assay for simultaneous detection of the SEA deletion α^0-thalassemia and a T allele of SNP rs3760053 with size polymorphism of inter-ζ HVR in 155 individuals as compared to the conventional gap-PCR assay

Inter-ζ HVR with T allele of rs3760053	*SEA α^0-thal (660 bp)*	*Internal control (836 bp)*	*No*	*Diagnosis by multiplex PCR*	*Gap-PCR*	*No*
S	–	+	10	Normal	αα/αα	41
M	–	+	26			
L	–	+	5			
S	+	+	14	Heterozygote (αα / –^{SEA})	αα/–^{SEA}	104
M	+	+	58			
L	+	+	31			
—	+	+	1			
—	+	–	10	Homozygote (–^{SEA} / –^{SEA})	–^{SEA}/–^{SEA}	10
Total			155			155

Abbreviations: HVR, hypervariable region; SNP, single-nucleotide polymorphism.

cellular level is still unclear, this mutation is primarily found in malarious regions. In the study of Chinese population, Qiu *et al.*[8] have hypothesized that this SEA deletion α^0-thalassemia has been subjected to recent balancing selection, triggered by malaria and suggested a single origin of this mutation in Chinese population.

We reported for the first time the genetic background of this SEA deletion α^0-thalassemia among three Southeast Asian populations. Five SNPs surrounding the SEA deletion α^0-thalassemia spanning about 82 kb in length were examined in Thai, Laos and Cambodian subjects with and without the mutation. As shown in Figure 1, all these five SNPs were found to have linkage disequilibrium with the SEA deletion α^0-thalassemia allele in these three Southeast Asian populations. When the data of these SNPs were compared between the α^0-thalassemia carriers and normal controls, we found that the SNP rs3760053 T > G located upstream of SEA deletion breakpoint was uncommon in the normal population (with a DAF of 0.033) but was predominant in carriers (with DAF of 0.4831) (Table 2). The G allele of this SNP was mostly linked to a chromosome with the SEA deletion α^0-thalassemia in the three populations. We observed no hematological difference between α^0-thalassemia carriers with this most common G-linked and a rarer T-linked alleles. The difference in DAF between normal controls and carriers allowed us to develop the more reliable assays for screening of the SEA deletion α^0-thalassemia as shown in Figures 3 and 4. In Figure 3, the SEA deletion α^0-thalassemia was detected along with SNP rs3760053 genotyping on the HRM format. Analysis of 211 individuals confirmed that most of the normal subjects (αα/αα) had T/T genotype while heterozygotes (αα/--^{SEA}) had T/G or G/G genotypes. In addition, only G/G genotype was observed for the 6 cases with homozygous α^0-thalassemia (--^{SEA} /--^{SEA}; Table 3). Therefore, a T allele was not detected on a chromosome with SEA deletion α^0-thalassemia in these Southeast Asian populations. This was also the case for the southern Chinese population in whose the DAF for SNP rs3760053 T > G were found to be 0.520 and 0.098 for α^0-thalassemia carriers and normal population, respectively.[8] Undetectable T allele at the SNP rs3760053 T > G in case with Hb Bart's hydrops fetalis caused by homozygosity of the SEA deletion α^0-thalassemia should provide additional confirmation for diagnosis of this important thalassemia syndrome as shown in Figure 4 and Table 4. This is very important in a routine prenatal diagnosis practice because misdiagnosis of Hb Bart's hydrops fetalis can occur with maternal contamination and allelic dropout (a mechanism in which one of the alleles fails to be amplified) on a small amount of fetal DNA. In our laboratory, maternal contamination is usually monitored by PCR analysis of VNTR.[14,20] When a wild-type allele fails to be amplified in heterozygote, PCR will show homozygosity for the mutant allele.[21] It is therefore necessary to perform at least two different PCR methods for accurate prenatal diagnosis of the case.[4,22] Although the SNP genotyping is not less prone to allelic dropout than gap-PCR method, diagnosis using a double-check system is preferred. The methods described in Figure 4 for simultaneous detection of the SEA deletion α^0-thalassemia and a T allele at SNP rs3760053 should provide an effective system for this.

With a relatively high prevalence and spread of the SEA deletion α^0-thalassemia among Southeast Asian and Chinese populations, information regarding the origin of this common thalassemic allele has been limited. Study on the inter-ζ HVR and IVS1 of Ψζ gene in a small number of patients has pointed to a single origin in Thailand. No haplotype data was reported.[23] A more detailed study using pattern of linkage disequilibrium and a long-range haplotype based on 28 SNPs encompassing the SEA deletion, covering around 410 kb region on chromosome 16 has recently been conducted in southern Chinese population. The study has also suggested a single origin of the SEA deletion α^0-thalassemia allele in Chinese.[8]

Data on the 5 SNPs flanking the deletion breakpoints and haplotypes reported herein are the first time this α^0-thalassemia allele has been addressed for Southeast Asian populations. The results shown in the Table 1 for Thai, Laos and Cambodian demonstrated that while normal allele (αα) could be presented on diverse haplotypes; H1-H7 and H12, most of the SEA deletion α^0-thalassemia (--^{SEA}) were associated with haplotype H4 (AAGC), the data presented for the first time among Southeast Asian populations. Interestingly, as shown in the table, this haplotype H4 was found only in Asian populations including Thai, Laos, Cambodian, Chinese, Japanese and Asian Indians but not in European, African and American. In addition, phylogenetic analysis indicated that this haplotype H4 (AAGC) arose from the ancestral haplotype H12 (AAAC) before introduction of the SEA deletion α^0-thalassemia mutation (Figure 2). All these data should partly explain why the SEA deletion α^0-thalassemia has never been found among the non-Asian populations. This haplotype analysis pointed to a single origin of the SEA deletion α^0-thalassemia in human population and further indicates that a single origin was responsible for the high prevalence and spread of SEA deletion α^0-thalassemia in the regions. As for Chinese population, this should support the potential effect of natural selection on the SEA deletion α^0-thalassemia in Southeast Asia especially the susceptibility to malaria infection.[8,24] A single origin of the Filipino β^0-thalassemia in Asian population has been documented.[25] However, these are in contrast with other hemoglobinopathies found in Southeast Asia like Hb E, Hb Constant Spring and Hb Q-Thailand in which multiple origins existed.[26–29]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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