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Effect of the *XmnI*-^{G}γ polymorphism on HbE and red blood cell parameters of Hb E carriers with and without SEA-α thalassemia 1

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ABSTRACT

Background: *XmnI*-^{G}γ polymorphism has been found to be the major *cis*-acting factor responsible to increase γ-globin gene activation and closely linked to HbE. It was expected that the polymorphism might modify HbE and RBC indices in HbE carriers.

Objectives: To determine the effect of *XmnI*-^{G}γ polymorphism on RBC indices and HbE levels in single HbE carriers and double HbE/SEA-α thalassemia 1 carriers.

Materials and methods: Samples comprised 160 EDTA blood collected from routine Hemoglobin Typing Laboratories of Sawan Pracharak Hospital, Nakorn Sawan Province and Lampang Regional Hospital, Lampang Province. *XmnI*-^{G}γ polymorphism was determined by PCR-RFLP. SEA-α thalassemia 1 was genotyped by Gap-PCR. HbE level was determined by cation-exchange HPLC, and RBC indices by automated hematology analyzer. Mann-Whitney U test was computed to analyze the data. *P* value of less than 0.05 is considered to be statistically significant.

Results: The prevalence of *XmnI*-^{G}γ (+/+) was 13.9% and 6.2%, of *XmnI*-^{G}γ (+/-) was 66.0% and 81.3%, and of *XmnI*-^{G}γ (-/-) was 20.1% and 12.5% in single HbE carriers and double HbE/SEA-α thalassemia 1 carriers, respectively. Presence of the *XmnI*-^{G}γ site did not affect HbE levels and RBC indices in both single HbE carriers and double HbE/SEA-α thalassemia 1 carriers.

Conclusion: *XmnI*-^{G}γ polymorphism did not affect RBC indices and HbE levels in HbE carriers. It was not a confounding factor to be concerned when considering RBC parameters in screening for HbE/SEA-α thalassemia 1 double carriers and can be ignored.

Introduction

Hemoglobin E (Hb E) ($\alpha_2\beta^E_2$) is an abnormal hemoglobin commonly found in Thailand with the national prevalence of approximately 13% and 50-60% at Thai-Laos-Kampuchea border.[1] This abnormal hemoglobin is resulted from missense mutation (G to A substitution) at codon 26 of β-globin gene, causing change from glutamic acid to lysine. This missense mutation was found to activate cryptic splice site at codon 25 which reduces normal splicing of messenger RNA (mRNA).[2] Therefore, β^E globin chain is synthesized at a reduced rate, and acts as β^+-thalassemia. Compound heterozygosity of β^E gene and $\beta^{Thalassemia}$ gene results in the life-threatening chronic anemia namely HbE/β-thalassemia.[3] To prevent birth of new patient of HbE/β-thalassemia, detecting HbE in parents is essential.

HbE carrier is an individual having HbE gene or β^E-gene in heterozygous form. Clinically, HbE carrier is asymptomatic.[4] Thus, detecting HbE carrier entirely requires laboratory information. Laboratory tests are conventionally employed

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for detecting HbE carrier include one-tube osmotic fragility test, red blood cell indices especially MCV, dichlorophenol indophenol precipitation (DCIP) test, cation-exchange high performance liquid chromatography (HPLC), and capillary zone electrophoresis (CZE).[5-7]

Our recent survey showed that beside single or pure HbE carriers, the double HbE/SEA- α thalassemia 1 can also be found in Thailand with the approximate prevalence of 11.0% in pregnant women[8] and 11.2% in general population.[9] SEA-α thalassemia 1 is a severe form of α-thalassemia. Co-existence of SEA-α thalassemia 1 in the HbE carrier reduces MCV, MCH, and HbE to the levels lower than those observed in the single HbE carrier.[10] Our survey, however, had found that anemic status of HbE carriers substantially affected the MCV, MCH, and HbE levels, and different cutoff points were established.[10]

XmnI-$^{G}\gamma$ polymorphism (rs 7482144) is the C-T substitution at nucleotide position -158 in promoter region of $^{G}\gamma$ gene. This polymorphism was shown by twin study to be the major cis factor involved in increased F cell production, accounting for 13% of F cell variance.[11] The presence of thymidine nucleotide (T) instead of wild type cytosine nucleotide (C) at this position creates cutting site for the endonuclease *XmnI*, thus so-called *XmnI*-$^{G}\gamma$ site. The *XmnI*-$^{G}\gamma$ site has been shown to be involved in augmentation of the γ-globin gene expression in normal individuals and in those having erythropoietic stress.[12,13] The presence of *XmnI*-$^{G}\gamma$ site was fairly common in Thai population, attaining the frequency of 24.7% and 2.5% for heterozygote and homozygote, respectively (unpublished data). Survey in Thai HbE/β^{0}-thalassemia patients showed that *XmnI*-$^{G}\gamma$ site was closely linked to β^{E} gene, and mild clinical symptoms.[14] Our recent survey in HbE carriers confirmed this study by showing high prevalence of *XmnI*-$^{G}\gamma$ site and its impact on HbE, HbF, and RBC indices levels.[15] However, the relationship of *XmnI*-$^{G}\gamma$ polymorphism on HbE, HbF, and RBC indices in HbE carrier with SEA-α thalassemia 1, so far, has never been evaluated.

Blood samples

One hundred and sixty EDTA blood samples of HbE carriers were collected from routine Hemoglobin Typing Laboratories at Sawan Pracharuk Hospital, Nakorn Sawan Province, and Lampang Hospital, Lampang Province, Thailand. Blood samples were processed anonymously. The protocol of this study was reviewed and approved by the Research Ethics Committee, Faculty of Associated Medical Sciences, Chiang Mai University (Approved number 103/2556)

Determination of RBC parameters and types plus quantities of hemoglobins

Red blood cell parameters including red blood cell count (M/μL), Hb (g/dL), Hct (%), MCV (fL), MCH (pg), MCHC (gm/dL) and RDW (%) were analyzed by automated blood cell counter (Beckman Coulter, Inc. California, USE). Types and quantities of hemoglobins were determined by cation-exchange HPLC (VARIANT™ Hemoglobin Testing System: BioRad Laboratories, Hercules, CA) (Figure 1).

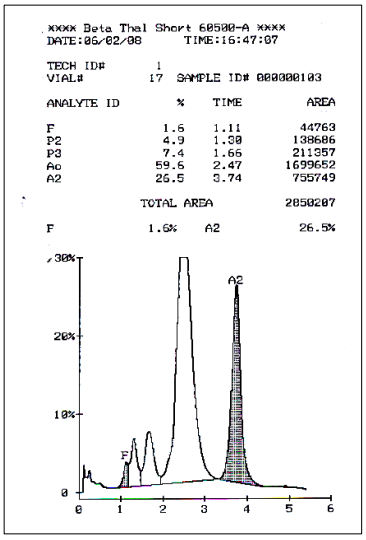

Figure 1. *Hemoglobin pattern and quantities in HbE carrier obtained from the cation-exchange HPLC. HbE is co-eluted with HbA_2 and nominated as "A2". Percentage of 26.5 is a combination of HbA_2 and HbE.*

Preparation of genomic DNA

Genomic DNA was directly prepared from buffy coat using Chelex-100 resin with some modification.[16] Technically, 100 μL of buffy coat was washed consecutively in 1 mL of 0.5% (v/v) Trion X-100 and 1 mL of deionized water (DI). Thereafter, 110 μL of DI and 1-2 drops of 5% (v/v) Chelex-100 suspension (Chelex® 100 Molecular Biology Grade Resin, BioRad Clinical Diagnostics, Hercules, CA) was mixed with pellet and incubated overnight 56°C. The reaction was then heated in boiling water bath for 20 minutes and centrifuged at 12,000g for 1 minute. Finally, supernatant containing genomic DNA was collected and stored at -20°C until use.

Determination of SEA-α thalassemia 1 gene

SEA-α thalassemia 1 gene was determined by Gap-PCR established in our laboratory.[9] This Gap-PCR was able to detect both wild type and SEA deletion in a single reaction with the amplified products sized of 652 bp were wild type and of 762 bp were SEA deletion. Therefore, SEA-α thalassemia 1 carriers had the amplified products of both sizes.

Determination of *XmnI*-$^{G}\gamma$ polymorphism

XmnI-$^{G}\gamma$ polymorphic site (C/T) was determined by PCR-RFLP analysis following the procedure described previously in our laboratory.[15] *XmnI*-$^{G}\gamma$ polymorphism was annotated as *XmnI*-$^{G}\gamma$ (+) for the presence of *XmnI*-$^{G}\gamma$ site ("T" at this point) and as *XmnI*-$^{G}\gamma$ (-) for the absence of *XmnI*-$^{G}\gamma$ site ("C" at this point). By this pattern, *XmnI*-$^{G}\gamma$ (+/+) and (-/-) is indicated homozygote for presence and absence of *XmnI*-$^{G}\gamma$ site, respectively. The heterozygous state of this site would thus have *XmnI*-$^{G}\gamma$ (+/-) genotype.

Statistical analysis

Descriptive statistics, median (min-max), and inferential statistics (Mann-Whitney U test) were analyzed using statistical software. The p value $p<0.05$ was considered statistically significant.

Results

Demographic data

All 160 blood samples were proven to be HbE carriers by cation-exchange HPLC as they all had hemoglobin typing of AE with HbE ranging from 14.1% to 32.5%. All were not anemic as shown by Hb levels >10.0 gm/dL. RBC parameters as well as HbE levels are shown in Table 1. Sixteen samples were found to be double HbE/SEA-α thalassemia 1 carriers, accounting for the prevalence of 10%, while 144 samples were single HbE carriers. Comparing RBC parameters between single HbE carriers and double HbE/SEA-α thalassemia 1 carriers showed that levels of MCV, MCH, and HbE were less in double HbE/SEA-α thalassemia 1 than those in the single HbE carriers (Table 1).

Table 1 RBC parameters and HbE (median, min-max) in all HbE carriers, double HbE/SEA-α thalassemia 1 carriers, and single HbE carriers. The p value of less than 0.05 was considered statistically significant.

RBC parameters	Groups			*p* values
	HbE carriers (n=160)	HbE/SEA-α thalassemia 1 carriers (n=16)	Single HbE carriers (n=144)	
	11.7	11.3	11.7	
Hb (g/dL)	(3.3-25.9)	(6.8-15.3)	(3.3-25.9)	0.325
	35.1	34.0	35.1	
Hct (%)	(9.9-77.7)	(20.4-45.9)	(9.9-77.7)	0.358
	77.3	68.8	77.7	
MCV (fL)	(48.5-114.6)	(48.5-83.5)	(52.0-114.6)	0.001
	25.6	22.6	25.7	
MCH (pg)	(15.0-38.8)	(15.5-27.2)	(15.0-38.8)	0.001
	14.5	15.2	14.5	
RDW (%)	(12.1-43.0)	(14.0-43.0)	(12.1-29.8)	0.424
	27.7	20.1	27.9	
HbE (%)	(14.1-32.5)	(14.1-29.9)	(16.3-32.5)	0.001

Prevalence of *XmnI*-$^{G}\gamma$ site in single Hb E carriers and double Hb E/SEA-α thal 1 carriers

Presence of *XmnI*-$^{G}\gamma$ site (T at nucleotide -158 of $^{G}\gamma$ promoter) found in 150 of 320 chromosomes indicated 0.47 gene frequency. Heterozygote for the presence of this site (*XmnI*-$^{G}\gamma$; +/-) was the most common genotype while homozygote of presence and absence of this site was less common in both single HbE carriers and double HbE/SEA-α thalassemia 1 carriers (Table 2).

Table 2 Prevalence of *XmnI*-$^{G}\gamma$ polymorphism in HbE carriers with and without SEA α-thalassemia 1.

Groups	*XmnI*-$^{G}\gamma$ genotype	Number of cases	Prevalence [%]
HbE/SEA-α thalassemia 1 carriers (n=16)	-/-	2	12.5
	+/-	13	81.3
	+/+	1	6.2
Single HbE carriers (n=144)	-/-	29	20.1
	+/-	95	66.0
	+/+	20	13.9

Comparing of HbE levels and red blood cell parameters in single HbE carriers in the presence and absence of *XmnI*-$^{G}\gamma$ site

Single HbE carriers were divided according to the presence and absence of *XmnI*-$^{G}\gamma$ site. Twenty nine samples were found to be homozygote for absence of the site (*XmnI*-$^{G}\gamma$; -/-). Ninety-five and twenty samples were found to be heterozygote (+/-) and homozygote (+/+) of *XmnI*-$^{G}\gamma$ site, respectively. All red blood cell parameters analyzed and HbE levels were not different among samples with and without *XmnI*-$^{G}\gamma$ site (Table 3). However, trend of low Hb, Hct, HbE, and high MCV, MCH, RDW was observed in group having *XmnI*-$^{G}\gamma$ site (Figure 2).

Table 3 Comparison of RBC parameters and HbE levels (median, min-max) in single HbE carrier without (-/-) and with (+/- and +/+) the ***XmnI***-$^{G}\gamma$ site. The p value of less than 0.05 was considered statistically significant.

RBC parameters	Groups		*p* values
	Single HbE carriers without *XmnI*-$^{G}\gamma$ site (-/-) (n=29)	Single HbE carriers with *XmnI*-$^{G}\gamma$ site (+/-, +/+) (n=115)	
	12.2	11.6	0.237
Hb (g/dL)	(3.3-15.6)	(3.7-25.9)	
	35.9	34.9	0.221
Hct (%)	(9.9-46.2)	(11.1-77.7)	
	76.7	77.9	0.489
MCV (fL)	(57.2-89.3)	(52.0-114.6)	
	25.6	25.8	0.353
MCH (pg)	(16.0-28.8)	(15.0-38.8)	
	14.1	14.5	0.190
RDW (%)	(12.4-21.6)	(12.1-29.8)	
	27.8	27.9	0.696
HbE (%)	(23.3-30.5)	16.3-32.5)	

Comparison of RBC parameters and HbE levels in double HbE/SEA-α thalassemia 1 carriers in the presence and absence of *XmnI*-$^{G}\gamma$ site

Double HbE/SEA-α thalassemia 1 carriers were divided into 2 groups according to the presence and absence of *XmnI*-$^{G}\gamma$ site. Two samples having *XmnI*-$^{G}\gamma$ site in both heterozygous and homozygous form; (+/-, +/+). No difference of both RBC parameters and HbE levels was also observed between these two groups (Table 4). However, trend of low Hb, Hct, HbE, and high MCV, MCH, RDW was observed in group having XmnI-$^{G}\gamma$ site (Figure 2)

Table 4 Comparison of RBC parameters and HbE levels (median, min-max) in double HbE/SEA-α thalassemia 1 carriers without (-/-) and with (+/-, +/+) the *XmnI*-$^{G}\gamma$ site. The p value of less than 0.05 was considered statistically significant.

RBC parameters	Groups		*p* values
	HbE/SEA-α thalassemia 1 without *XmnI*-$^{G}\gamma$ site (-/-) (n=2)	HbE/SEA-α thalassemia 1 with *XmnI*-$^{G}\gamma$ site (+/-, +/+) (n=14)	
Hb (g/dL)	12.2	10.4	0.525
	(12.1-12.3)	(6.8-15.3)	
Hct (%)	36.8	31.1	0.634
	(36.4-37.2)	(20.4-45.9)	
MCV (fL)	68.0	69.0	0.525
	(66.8-69.2)	(48.5-83.5)	
MCH (pg)	22.5	22.6	0.874
	(22.0-23.0)	(15.5-27.2)	
RDW (%)	14.5	15.5	0.203
	(14.0-15.0)	(14.1-43.0)	
HbE (%)	21.8	20.0	0.340
	(21.1-22.5)	(14.1-29.9)	

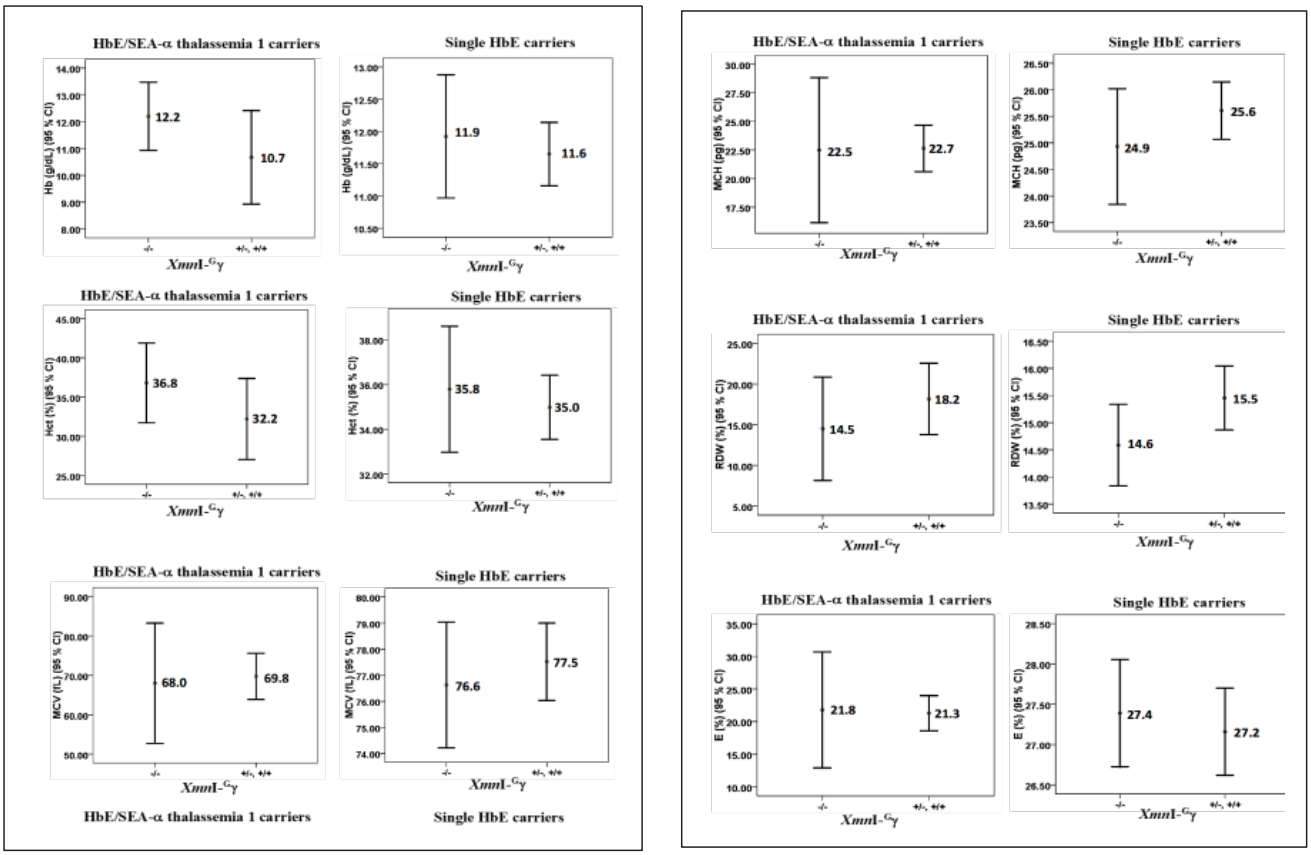

Figure 2. *RBC parameters and HbE levels in single HbE carriers and HbE/SEA-α thalassemia 1 carriers with and without XmnI-$^{G}\gamma$ site. Note that identical relationships of RBC parameters, HbE levels and XmnI-$^{G}\gamma$ polymorphism in single HbE carriers and HbE/SEA-α thalassemia 1 carriers are observed. Numbers at the middle of each bar indicate mean values.*

Discussion

Both HbE and SEA-α thalassemia 1 are common in Thailand and coexisting of these disorders is inevitable. Coexisting of SEA-α thalassemia 1 in HbE carrier causes reduction of MCV, MCH, and HbE levels to the level atypical for traditional or single HbE carrier, *i.e.* HbE carrier without coexisting SEA-α thalassemia 1. This phenomenon has been well described by several authors[8, 17-19] and also by our previous survey.[10]

XmnI-$^{G}\gamma$ site is involved in increased HbF/F cell productions and improvement of clinical phenotype. Our present results showed that RBC indices and HbE levels were not different between samples with or without *XmnI*-$^{G}\gamma$ site in both single HbE carriers and double HbE/SEA-α thalassemia 1 carriers. This meant that *XmnI*-$^{G}\gamma$ site did not have the modifying effect on RBC indices and HbE levels in both groups of HbE carriers. This result was similar to that shown in our previous survey that presence of the *XmnI*-$^{G}\gamma$ site was mildly related to increased MCV, MCH, HbF and lowered HbE levels in HbE carriers, but the levels of these parameters were substantially overlapped.[15] This further confirmed that *XmnI*-$^{G}\gamma$ had the minimal impact on this phenotype in HbE carriers in this cohort. This should be explained by the fact that HbE carrier is a mild form of β-hemoglobinopathy having small degree of erythropoietic stress, which was not appropriate for maximal *XmnI*-$^{G}\gamma$ action.[4, 20]

Although the sample size of double HbE/SEA-α thalassemia 1 carriers and those having no *XmnI*-$^{G}\gamma$ site were quite small, the results of this study should preliminarily establish the conclusion that *XmnI*-$^{G}\gamma$ polymorphism did not have significant effect on all RBC parameters and HbE levels in HbE carriers. Therefore, *XmnI*-$^{G}\gamma$ polymorphism may be ignored in screening for the double carriers of HbE and SEA-α thalassemia 1. Continued study with increased sample size would greatly be invaluable.

Conflicts of interests

Authors declared no conflict of interest.

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