The Associations of SEA-α Thalassemia 1, *Xmn*I-^{G}γ Polymorphism and β-Globin Gene Mutations with the Clinical Severity of β-thalassemia Syndrome in Northern Thailand

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Background: *At least three genetic factors including β-thalassemia mutations, α-thalassemia, and XmnI-^{G}γ polymorphism were shown to modify clinical symptoms in β-thalassemia disease.*
Objective: *To determine associations of β-thalassemia mutations, SEA-α thalassemia 1, and XmnI-^{G}γ polymorphism, and clinical severity of β-thalassemia in northern Thailand.*
Material and Method: *Thirty-two β-thalassemia major and 28 β-thalassemia intermedia attending the Thalassemia Clinic at Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand were recruited. The β-globin gene mutations and SEA-α thalassemia 1 were determined by MS-PCR and Gap-PCR, respectively. The XmnI-^{G}γ polymorphism was identified by RFLP analysis. Odds ratio was calculated to evaluate the associations of these three genetic factors and clinical symptoms.*
Results: *Eight β-globin gene mutations (both β^{O} and β^{+}) were found. Twenty-nine point one percent of the patients had at least one XmnI-^{G}γ site (XmnI-^{G}γ: +) and 4.1% of the patients were heterozygote for the SEA-α thalassemia 1. The β-globin gene mutations showed maximal impact and the XmnI-^{G}γ polymorphism had minimal influence on clinical severity in this cohort. The SEA-α thalassemia 1 had the least effect on the clinical severity due to its low prevalence in these patients.*
Conclusion: *Although these three genetic factors play roles in modifying clinical symptoms of β-thalassemia, the β-thalassemia mutations should be considered first, followed respectively by the XmnI-^{G}γ polymorphism and the SEA-α thalassemia 1, in management and prenatal diagnosis of β-thalassemia in northern Thailand.*

Keywords: *β-thalassemia, HbE/β-thalassemia, β-thalassemia mutations, SEA-α thalassemia 1, XmnI-^{G}γ polymorphism, HbE*

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β-thalassemia is the syndrome characterized by reduction or absence of the β-globin chain. The affected individuals suffer from chronic anemia due mainly to in effective erythropoiesis in combination with several complications resulting from an iron overload, hyperbilirubinemia, osteoporosis, and infections[1]. Three types of β-thalassemia are clinically classified, β-thalassemia major, β-thalassemia intermedia, and β-thalassemia minor. The β-thalassemia major is seen in those having the hemoglobin levels of less than 6 g/dl with disease onset before two years old and requiring frequent blood transfusion (two to three times/month). The β-thalassemia intermedia is observed in those who have hemoglobin levels of 7 to 10 g/dl, late disease onset with only occasional blood transfusion requirements. Finally, the β-thalassemia minor is clinically characterized for the β-thalassemia heterozygote[1,2]. At least three genetic factors have been shown to be involved in the phenotypic diversity in the β-thalassemia disease. These factors include the β-thalassemia mutations, β-thalassemia and loci linked to γ-globin gene activation such as the *Xmn*I-^{G}γ polymorphism on ^{G}γ-globin promoter[2-8]. Mild β-thalassemia mutations can lead to mild β-thalassemia, where as severe mutations to severe β-thalassemia. Co-existence of α-thalassemia and γ-globin gene

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activation is also observed in mild β-thalassemia. However, several surveys demonstrated that patterns of interaction of these genetic factors and the clinical phenotypes are ethnically unique[5,9-15]. Thus, information of this phenomenon should be determined for each ethnic group, i.e., to provide information that can be used for predicting the phenotypic outcome of the *in-utero* fetuses at risk of being the β-thalassemia.

Material and Method

The research ethic was approved by the Research Ethics Committee, Faculty of Medicine, Chiang Mai University. Blood samples were collected, after signing informed consents, from 60 patients attending the Pediatric Thalassemia Clinic, Department of Pediatrics, Faculty of Medicine, Chiang Mai University at Maharaj Nakorn Chiang Mai Hospital. Blood samples were collected just before the next blood transfusion. These patients were grouped into β-thalassemia major (32) and β-thalassemia intermedia (28) according to the criterions described by Ho et al[2].

The common β-globin gene mutations including TTCT-deletion at codons 41/42 ($\beta^{41/42\,(-TTCT)}$), A-T substitution at codon 17 ($\beta^{17\,(A-T)}$), adenine addition at codons 71/72 ($\beta^{71/72\,(+A)}$), A-G substitution at nucleotide -28 of β-globin promoter ($\beta^{-28\,(A-G)}$), G-A substitution at codon 26 ($\beta^{26\,(G-A)}$ or β^{E}), C-T substitution at nucleotide 654 within IVS 2 ($\beta^{IVS2:654\,(C-T)}$) were identified by the mutagenically separated polymerase chain reaction (MS-PCR) described previously[16,17]. The rare β-globin gene mutations were detected by the nucleotide sequencing described by Sirichotiyakul et al[18]. The SEA-α thalassemia 1 allele was identified by gap-PCR described elsewhere[19]. Finally, the *Xmn*I-^{G}γ polymorphism was determined by the *Xmn*I-digestion of the amplified products as described by Sampietro et al[20]. Odds ratio (OR) with 95% confidence interval were used to evaluate probability of occurrence of the events of interest.

Results

Demographic data of the subjects

Ages of 32 β-thalassemia major and 28 β-thalassemia intermedia were 19.0±5.1 and 42.9±12.0 months (mean ± SD), respectively. Times, in mean ± SD, of disease onset were 21.5±15.2 months for the β-thalassemia major and 32.8±30.0 months for the β-thalassemia intermedia. Requirements for blood transfusion ranged from 5 to 15 times per year in the β-thalassemia major. One-half of the β-thalassemia inter media patients, however, did not require any blood transfusions, whereas another half only required their first blood transfusions between six and 15 years of age. Hb level was significantly lower in the β-thalassemia major than in the β-thalassemia intermedia (Table 1).

Prevalences of β-globin mutations, SEA-α thalassemia 1 and XmnI-^{G}γ site

Among 120 chromosomes of 60 patients studied, eight β-globin mutations were observed including five β^{O}-producing mutations (42.5% $\beta^{41/42\,(-TTCT)}$, 17.5% $\beta^{17\,(A-T)}$, 5.8% $\beta^{IVS1-nt1\,(G-T)}$, 0.83% $\beta^{27/28\,(+C)}$, 0.83% $\beta^{43\,(G-T)}$ and three β^{+}-producing mutations (25% $\beta^{26\,(G-A)}$ or β^{E}, 6.6% $\beta^{-28\,(A-G)}$, 0.83% $\beta^{-87\,(C-A)}$ (Table 2). Presence of the *Xmn*I-^{G}γ site (*Xmn*I-^{G}γ; +) was found in 35 chromosomes (29.1%) and the SEA-α thalassemia 1 allele in five chromosomes (4.1%).

β-globin mutations, SEA-α thalassemia 1 and XmnI-^{G}γ polymorphism in β-thalassemia major and β-thalassemia intermedia

The homozygotes of β^{O}-producing mutations were mostly observed in the β-thalassemia major (OR = 22.6; 95% CI = 10.3-49.5), while the compound heterozygotes of β^{O} and β^{+} or β^{E} were frequently seen in the β-thalassemia intermedia. Sub-analysis in compound heterozygotes of β^{E} and β^{O} or β^{+} showed that most β^{O}/β^{E} (21 in 28) and all β^{+}/β^{E} (2) had β-thalassemia intermedia symptom. The *Xmn*I-^{G}γ: -/- was frequently seen in the β-thalassemia major, while the *Xmn*I-^{G}γ +/+ and +/- were common in the β-thalassemia intermedia. However, the effect of this

Table 1. Basic data and biomarkers in β-thalassemia major and β-thalassemia intermedia analyzed in the present study

Characteristics	β-thalassemia major (n = 32)	β-thalassemia intermedia (n = 28)
Age of disease onset (months) (mean ± SD)	21.5±15.2	32.8±30.0
Blood transfusions (times/year) (mean ± SD)	9.0±2.8	1.0±1.4
Hb (g/dL) (mean ± SD)	4.5±2.1	7.0±0.7
Presence of hepatomegaly (%)	100.0	82.1
Presence of splenomegaly (%)	87.5	82.1
Splenectomized (%)	12.5	0

Hb = hemoglobin; SD = standard deviation

polymorphism was lower than that of the β-thalassemia mutations (OR = 2.8; 95% CI = 1.6-5.2) (Table 3). Moreover, the ameliorating effect of the SEA-α thalassemia 1 was not evident in this cohort as it was found only in the β-thalassemia major sub-group.

Interaction of β-globin mutations and XmnI-^{G}γ polymorphism in β-thalassemia major and β-thalassemia intermedia

Effects of β-globin mutations combined with the *Xmn*I-^{G}γ polymorphism on clinical symptoms were evaluated. As shown in Table 4, the *Xmn*I-^{G}γ: -/- combined with the β/β mutations was mostly found in the β-thalassemia major (OR = 18.5, 95% CI = 4.9-70.6), whereas combinations of the *Xmn*I-^{G}γ: +/+ or +/- and the β/β were seen equally in both β-thalassemia sub-groups. The β/β^{E} mutations combined with *Xmn*I-^{G}γ: -/- or +/- or +/+ were mostly found in the β-thalassemia intermedia. The impact of the *Xmn*I-^{G}γ polymorphism in the β/β^{E} patients was less marked than that in the β/β patients (OR = 0.3, 95% CI = 0.08-1.09).

Table 2. Frequencies of β-globin mutations, SEA-type α-thalassemia 1 and *Xmn*I-^{G}γ polymorphism in the studied subjects

Genetic factors	Alleles	Number (%)
*Xmn*I-^{G}γ polymorphism	+	35 (29.1)
	-	85 (70.8)
SEA-α thalassemia 1	+	5 (4.1)
	-	115 (95.8)
β-globin mutations	$\beta^{41/42}$	51 (42.5)
	β^{17}	21 (17.5)
	$\beta^{27/28}$	1 (0.83)
	β^{NT-87}	1 (0.83)
	β^{43}	1 (0.83)
	β^{NT-28}	8 (6.6)
	β^{IVS1}	7 (5.8)
	β^{E}	30 (25.0)

SEA = Southeast Asian

Discussion

Mild β-thalassemia mutations, co-existence of β-thalassemia and co-inheritance of the *Xmn*I-^{G}γ; + polymorphism have been shown to ameliorate the clinical severity of β-thalassemia[1]. However, surveys have shown that these modifying factors did not always equally act to modify the illness, hence, making consistent prediction of clinical severity difficult[2]. This has been shown to be due to geographical and ethnical heterogeneity of the occurrence of these genetic factors as revealed by several surveys summarized in Table 5. This report, thus, proposed to identify patterns of associations of these genetic factors and phenotype expression in the β-thalassemia residing in Northern Thailand.

Eight different β-thalassemia mutations, both β^{O} and β^{+}, were observed in this cohort. Homozygote and compound heterozygote of these mutations showed substantial effect in clinical modification of

Table 3. Comparison of frequencies of β-thalassemia and *Xmn*I-^{G}γ genotypes between β-thalassemia major and β-thalassemia intermedia

	β-TM (n = 32), n [%]	β-TI (n = 28), n [%]	Odds ratio (95% CI)	p-value
β^{O}/β^{O}	23 [71.8]	3 [10.7]	22.6 (10.3-49.5)	<0.0001
β^{O}/β^{+}, β^{O}/β^{E}, β^{+}/β^{E}	9 [28.1]	25 [89.3]		
*Xmn*I-^{G}γ (-/-)	20 [51.0]	9 [32.1]	2.8 (1.6-5.2)	0.0005
*Xmn*I-^{G}γ (+/-, +/+)	12 [37.5]	19 [67.8]		

β-TM = β-thalassemia major; β-TI = β-thalassemia intermedia; 95% CI = 95% confidence interval

Table 4. Interaction of *Xmn*I-^{G}γ polymorphism and β-thalassemia mutations and β^{E}-allele in β-thalassemia major and β-thalassemia intermedia

	β-TM (n = 32), n [%]	β-TI (n = 28), n [%]	Odds ratio (95% CI)	p-value
β/β + *Xmn*I-^{G}γ (-/-)	19 [59.3]	1 [3.5]	18.5 (4.9-70.6)	<0.0001
β/β + *Xmn*I-^{G}γ (+/+, +/-)	6 [18.7]	5 [17.8]		
β/β^{E} + *Xmn*I-^{G}γ (-/-)	1 [3.1]	8 [28.5]	0.3 (0.08-1.09)	0.07
β/β^{E} + *Xmn*I-^{G}γ (+/+, +/-)	6 [18.7]	14 [50.0]		

β-TM = β-thalassemia major; β-TI = β-thalassemia intermedia; 95% CI = 95% confidence interval; β/β = β^{O}/β^{O}, β^{O}/β^{+}, β^{+}/β^{+}; β/β^{E} = β^{O}/β^{E}, β^{+}/β^{E}

Table 5. Summary of results from surveys demonstrating heterogeneities of clinical modulating factors in β-thalassemia (✓ indicates significant impact, × indicates less or no impact)

β-globin genotypes	α-thalassemia	*Xmn*I-^{G}γ polymorphism	References
✓	✓	✓	4, 5, 10, 15, 30, 34
✓	×	✓	Present study, 2, 11, 12, 13, 14
✓	×	×	21-23
×	✓	✓	31-32

the patients analyzed in the present study as shown by the average OR value of 22.6. Homozygotes of severe mutations (β^{O}/β^{O}) showed high possibility to generate the β-thalassemia major. However, although most of these homozygotes were seen in the β-thalassemia major, some β-thalassemia intermedia also inherited the β^{O}/β^{O} genotype. Likewise, although the β^{O}/β^{+}, β^{O}/β^{E} and β^{+}/β^{E} were mostly observed in the β-thalassemia intermedia, some β-thalassemia major also inherited these genotypes. This indicated that only types of β-globin gene mutations might not be enough to predict the phenotypes of the β-hemoglobinopathies in this cohort. The same pattern, however, was also shown in southern Thailand where β-globin mutations had some effect and the *Xmn*I-^{G}γ was involved in clinical modification among the patients they analyzed[21-23].

SEA-α thalassemia 1 occurred at the lowest incidence in this cohort and seemed to be the least important marker in modifying clinical symptoms of the patients analyzed in this survey. In fact, this is true for the region of Northern Thailand in which the gene frequency of this type of β-thalassemia has been estimated to be 0.0236-0.0698[24,25]. Most importantly, detailed analysis showed that the SEA-α thalassemia 1 was seen only in the β-thalassemia major. This emphasized that the SEA-α thalassemia had the smallest impact on phenotypic modification of the β-thalassemia in this region.

The *Xmn*I-^{G}γ polymorphism is a C-T substitution at nucleotide position -158 to ^{G}γ-cap site (Fig. 1) and was found to be associated with three to 11 fold increase of ^{G}γ-globin gene expression[3]. This increased γ-globin gene expression leads to mild clinical symptom of the β-hemoglobinopathies as a result of reduced degree of α/non-α globin imbalance, a major pathogenesis of the disease[26,27]. Several studies have collectively demonstrated an association of the *Xmn*I-^{G}γ polymorphism, both heterozygote and homozygote, with mild form of β-thalassemia[7,15,28-33].

In the present report, the *Xmn*I-^{G}γ: +/- and +/+ were frequently found in the β-thalassemia intermedia and *Xmn*I-^{G}γ; -/- in β-thalassemia major. However, slight impact of the *Xmn*I-^{G}γ polymorphism on clinical diversity of the β-hemoglobinopathies in the present studied cohort was also shown as seen by the average OR value of 2.8. This pattern has also been observed in several studies where inconsistent association of the *Xmn*I-^{G}γ polymorphism with phenotype of the β-thalassemia were found[15,21,22,30,31,34].

When the *Xmn*I-^{G}γ polymorphism and the β-thalassemia mutations were simultaneously accounted for (Table 4), the magnitude of the *Xmn*I-^{G}γ polymorphism in alleviating the clinical phenotype was minimal. Most β-thalassemia patients having no *Xmn*I-^{G}γ site (*Xmn*I-^{G}γ: -/-) tended to be the β-thalassemia major. Presence of the *Xmn*I-^{G}γ polymorphism (*Xmn*I-^{G}γ: +/- and +/+) could not improve the clinical appearance of the β-thalassemia patients described in this report. This could be due to the fact that magnitude of the γ-globin chain production should approach 50% of α-globin production in order to reduce degree of globin chain imbalance[35]. The *Xmn*I-^{G}γ: +/+ or +/- might not be powerful enough to induce the γ-globin chain production substantially to

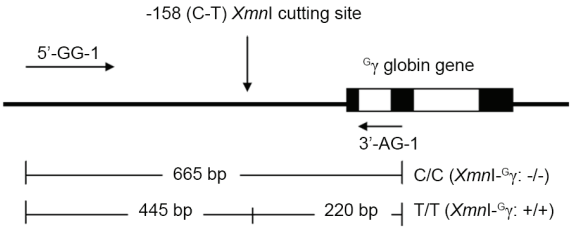

Fig. 1 Graphic demonstration of the *Xmn*I-^{G}γ polymorphism. 5'-GG-1 and 3'-AG-1 are the oligonucleotide primers utilized for amplifying the 665-bp fragments covering the *Xmn*I cutting site at -158 to ^{G}γ-cap site. T/T is the polymorphism creating cutting site of *Xmn*I which, in homozygote, generates a *Xmn*I-^{G}γ: +/+ genotype yielding 445-bp & 220-bp digested products. The *Xmn*I-^{G}γ: -/- is the genotypes indicating absence of *Xmn*I cutting site in a presence of wild-type C/C alleles.

that level. Table 4 also shows that most patients of HbE/β-thalassemia (β/β^E) had β-thalassemia intermedia phenotypes, regardless of types of the β-thalassemia mutations and the *Xmn*I-^{G}γ polymorphism. This could be explained by at least three reasons, 1) mild nature of HbE itself, 2) alternative splice site was less utilized in these patients, and 3) presence of α-thalassemia 2 or Hb Constant Spring[4,36,37].

A recent genome-wide study demonstrated that mild HbE/β-thalassemia was linked to several genetic factors including SNPs in the *BCL11A* gene, *HBS1L-Myb* Intergenic Polymorphism (HMIP) and in the β-globin cluster especially the *Xmn*I-^{G}γ polymorphism[38]. All of these genetic factors are associated with HbF augmentation. Whether the *BCL11A* gene and *HBS1L-Myb* Intergenic Polymorphism (HMIP) were involved in the phenomenon observed in this cohort remained to be clarified. Interestingly, the genome-wide and multicenter studies revealed that the *Xmn*I-^{G}γ polymorphism is in linkage disequilibrium with the HbE allele[7,33,38]. The present report confirmed this observation by the finding that allele frequencies of the *Xmn*I-^{G}γ: + polymorphism and the β^E were almost equal.

In conclusion, the β-thalassemia mutations and *Xmn*I-^{G}γ polymorphism are most likely to influence clinical outcome and should be considered in managing the β-thalassemia in Northern Thailand. Regional and ethnical diversities of the genetic background responsible for phenotypic diversity of the β-hemoglobinopathies were re-confirmed as well as the linkage disequilibrium of the β^E and *Xmn*I-^{G}γ; + polymorphism. However, other genetic modifying factors, particularly those involved in the HbF reactivation, should be further investigated in more sample numbers.

What is already known on this topic?

Clinical diversity of β-thalassemia has already been shown to be caused by at least three genetic factors, including β-globin gene mutations, co-existence of α-thalassemia and of gene(s) involving in increased production of HbF. However, patterns of these genetic factors causing clinical spectrum among different ethnicities are different.

What this study adds?

The present study revealed information about genetic factors associated with the β-thalassemia major/intermedia in β-thalassemia of Northern Thailand, which has never been explored. The results of the present study clearly indicated that types of β-globin gene mutations predominantly accounted for clinical diversity, i.e. mild mutations leads to mild clinical course and *vice-versa*. The *Xmn*I-^{G}γ site was the second genetic factor involved in β-thalassemia intermedia. The *Xmn*I-^{G}γ (+) in combination with HbE mostly leads to the β-thalassemia intermedia. SEA-α thalassemia 1 was not the major determinant of clinical diversity of β-thalassemia in Northern Thailand due to its lowest incidence. Information obtained from the present study would be helpful for those working in field of prenatal diagnosis of fetuses at risk of homozygous β-thalassemia or compound heterozygous Hβ^E and β-thalassemia, i.e. to assist or to guide proper diagnosis of the *in-utero* fetuses.

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Potential conflicts of interest

None.

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ความสัมพันธ์ของ SEA-α thalassemia 1, XmnI-^{G}γ polymorphism และ β-globin gene mutations กับความรุนแรงของอาการทางคลินิกของกลุ่มอาการ β-thalassemia major ในภาคเหนือของ ประเทศไทย

ธนูศักดิ์ ตาตุ, วราทิพย์ ศรีทอง, ต่อพงษ์ สงวนเสริมศรี

ภูมิหลัง: *ปัจจุบันทราบว่ามีปัจจัยระดับโมเลกุลอย่างน้อย 3 ชนิด ประกอบด้วย β-globin gene mutations, SEA-α thalassemia 1 และ XmnI-^{G}γ polymorphism ที่มีผลปรับปรุงลักษณะทางคลินิกของผู้ป่วย β-thalassemia*

วัตถุประสงค์: *เพื่อศึกษาความเกี่ยวพันของ β-globin gene mutations, SEA-α thalassemia 1 และ XmnI-^{G}γ polymorphism กับอาการทางคลินิกของ β-thalassemia ในภาคเหนือของประเทศไทย*

วัสดุและวิธีการ: *ทำการศึกษาในผู้ป่วย β-thalassemia major จำนวน 32 ราย และผู้ป่วย β-thalassemia intermedia จำนวน 28 ราย ที่เข้ารับบริการที่คลินิกธาลัสซีเมีย โรงพยาบาลมหาราชนครเชียงใหม่ ทำการตรวจหา β-globin gene mutations และ SEA-α thalassemia 1 โดยวิธี MS-PCR และ Gap-PCR ตามลำดับ ทำการตรวจหา XmnI-^{G}γ polymorphism โดยวิธี RFLP analysis และทำการคำนวน odds ratio เพื่อวิเคราะห์ความเกี่ยวพันของปัจจัยทั้งสามกับอาการทางคลินิก*

ผลการศึกษา: *พบ β-globin gene mutations (ทั้ง β^{O} และ β^{+}) ทั้งหมด 8 ชนิด ผู้ป่วย 29.1% มี XmnI-^{G}γ site อย่างน้อย 1 อัลลีล (XmnI-^{G}γ: +) และพบพาหะ SEA-α thalassemia 1 ใน 4.1% ของผู้ป่วยที่ศึกษา ความรุนแรงของอาการทางคลินิกได้รับผลจาก β-globin gene mutations มากที่สุดแต่ได้รับผลจาก XmnI-^{G}γ site เพียงเล็กน้อย ส่วน SEA-α thalassemia 1 ไม่มีผลเนื่องจากมีอุบัติการณ์น้อยที่สุด*

สรุป: *แม้ว่า β-globin gene mutations, SEA-α thalassemia 1 และ XmnI-^{G}γ polymorphism มีส่วนในการปรับปรุงอาการทางคลินิกของผู้ป่วย β-thalassemia การดูแลและตรวจวินิจฉัยก่อนคลอดผู้ป่วย β-thalassemia ในชาวไทยภาคเหนือควรพิจารณา β-globin gene mutations เป็นอันดับแรก ตามด้วย XmnI-^{G}γ polymorphism และ SEA-α thalassemia 1 ตามลำดับ*