

## Molecular characterisation and frequency of $G\gamma$ *Xmn* I polymorphism in Chinese and Malay $\beta$ -thalassaemia patients in Malaysia

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### Abstract

The molecular basis of variable phenotypes in  $\beta$ -thalassaemia patients with identical genotypes has been associated with co-inheritance of  $\alpha$ -thalassaemia and persistence of HbF production in adult life. The *Xmn* I restriction site at -158 position of the  $G\gamma$ -gene is associated with increased expression of the  $G\gamma$ -globin gene and higher production of HbF. This study aims to determine the frequency of the different genotypes of the  $G\gamma$  *Xmn* I polymorphism in  $\beta$ -thalassaemia patients in two ethnic groups in Malaysia. Molecular characterisation and frequency of the  $G\gamma$  *Xmn* I polymorphism were studied in fifty-eight Chinese and forty-nine  $\beta$ -thalassaemia Malay patients by *Xmn* I digestion after DNA amplification of a 650 bp sequence. The in-house developed technique did not require further purification or concentration of amplified DNA before restriction enzyme digestion. The cheaper Seakem® LE agarose was used instead of Nusieve agarose and distinct well separated bands were observed. Genotyping showed that the most frequent genotype observed in the Malaysian Chinese was homozygosity for the absence of the *Xmn* I site (-/-) (89.7%). In the Malays, heterozygosity of the *Xmn* I site (+/-) was most common (63.3%). Homozygosity for the *Xmn* I site (+/+) was absent in the Chinese, but was confirmed in 8.2% of the Malays. The ratio of the (+) allele (presence of the *Xmn* I site) to the (-) allele (absence of the *Xmn* I site) was higher in the Malays (0.66) compared to the Chinese (0.05). The (+/-) and (+/+) genotypes are more commonly observed in the Malays than the Chinese in Malaysia.

**Keywords:**  $\beta$ -Thalassaemia;  $G\gamma$  *Xmn* I polymorphism; DNA amplification; Malays; Chinese; Malaysia

### INTRODUCTION

$\beta$ -thalassaemia is an inherited disorder, characterised by a deficit ( $\beta^+$ ) or complete absence ( $\beta^0$ ) of  $\beta$ -globin chain production.<sup>1</sup> Deficiency of  $\beta$ -globin chains which leads to an excess in  $\alpha$ -globin chains can cause intramedullary destruction of the red cell precursors, ineffective erythropoiesis and anaemia.<sup>2,3</sup> Anaemia can trigger major complications like bone deformities, hepatosplenomegaly, impaired growth and failure to thrive. Anaemia when treated with adequate blood transfusions will allow normal growth and physical activity for affected individuals.<sup>4</sup> However, multiple blood transfusions often result in iron overload which can cause organ failure and death if transfused individuals are not treated with regular chelation therapy using

desferrioxamine.<sup>5</sup>

Beta-thalassaemia can clinically be classified into  $\beta$ -thalassaemia major, intermedia and minor.  $\beta$ -thalassaemia major is the most severe form and affected patients require regular blood transfusions for survival. Patients with  $\beta$ -thalassaemia intermedia show a milder phenotype while  $\beta$ -thalassaemia minor is usually a symptom-free carrier state characterised by a mild degree of anaemia.<sup>2,6</sup> Both  $\beta$ -thalassaemia major and intermedia result from homozygous or compound heterozygous states for  $\beta$ -globin gene mutations<sup>7</sup> and the clinical severity of the disease depends on the types of  $\beta$ -gene mutations involved. However, clinical severity can also be influenced by other genetic factors like concomitant  $\alpha$ -thalassaemia and increased  $\gamma$ -chain production.<sup>7</sup>

Alpha-thalassaemia causes a reduction in  $\alpha$ -globin chain synthesis and co-inheritance of the  $\alpha$ -thalassaemia gene can ameliorate the severity of  $\beta$ -thalassaemia.<sup>2,3</sup> Factors which increase  $\gamma$ -globin gene expression lead to a persistence of synthesis of  $\gamma$ -globin chains into adult life. Persistence of foetal haemoglobin (HbF) in adult life is another factor that greatly influences the severity of  $\beta$ -thalassaemia. Several loci, within and outside the  $\beta$ -globin gene region, have been implicated in higher production of HbF. Among the loci involved are large deletions in the  $\beta$ -cluster or specific point mutations in the promoter regions of the  $^G\gamma$  and  $^A\gamma$ -genes. The *Xmn* I restriction site at the -158 position of the  $^G\gamma$ -gene ( $^G\gamma$  *Xmn* I polymorphism) has been strongly correlated with increased HbF production in adults under haematopoietic stress.<sup>7,8,9</sup>

The  $^G\gamma$  *Xmn* I polymorphism causes a C to T base pair substitution at the -158 position in the promoter region of the  $^G\gamma$ -globin gene. The C to T base pair substitution creates a digestion site for the restriction enzyme *Xmn* I. Presence of the  $^G\gamma$  *Xmn* I polymorphism has been reported to be associated with a three to 11-fold elevation of  $^G\gamma$ -globin chain production, however, this has been reported mainly in conditions characterised by erythropoietic stress.<sup>8,9</sup> The  $^G\gamma$  *Xmn* I polymorphic site has been reported to be near a DNase I hypersensitive site located 50 to 150 bp 5' of the  $\gamma$ -gene Cap sites. This polymorphic site has been suggested to be responsible for the chromatin of the region to possess an "open" structure, thus increasing the accessibility of DNase *in vitro* and components of the transcription apparatus.<sup>9,10</sup> The -158 (C-T) mutation exerts its effect on the transcription rate of the gene with which it is associated with and may, therefore, allow a higher level of the  $^G\gamma$ -globin gene to be transcribed.<sup>11</sup>

In Malaysia,  $\beta$ -thalassaemia is common among the Malays and Chinese with a heterozygous carrier rate of 4-6%.<sup>6</sup> The molecular defects involved in  $\beta$ -thalassaemia in the three main ethnic groups – Malays, Chinese and Indians – have been extensively studied.<sup>12,13,14</sup> To date, there has been no reported studies on the molecular characterisation and frequency of the  $^G\gamma$  *Xmn* I polymorphism in the Malaysian population. Increased expression of the  $^G\gamma$ -globin gene and higher production of Hb F is associated with homozygosity for the *Xmn* I cleavage site (+/+) and thus, with less severe anaemia, while the effect is not observed in heterozygotes for the *Xmn* I site (+/-).<sup>15</sup> This study aims to determine

the frequency of the  $^G\gamma$  *Xmn* I polymorphism in  $\beta$ -thalassaemia major patients in the Malay and Chinese ethnic groups in Malaysia.

## MATERIALS AND METHODS

### *Sample collection*

DNA from 107  $\beta$ -thalassaemia major patients registered with the Department of Paediatrics, University of Malaya Medical Centre (UMMC) was analysed at the  $^G\gamma$  *Xmn* I polymorphic site. The ethnic make-up of the patients consisted of 58 Chinese and 49 Malays. Blood samples were collected in EDTA as anticoagulant and stored at -70°C. Verbal and written consent were obtained from the patients prior to blood sample collection. This study was approved by the Ethics Committee of the UMMC in accordance with the Declaration of Helsinki.

### *DNA amplification and Xmn I digestion*

DNA was extracted in TRIS-EDTA (pH 8) using sodium dodecyl sulphate and proteinase K, and digested overnight at 37°C. DNA was purified using phenol-chloroform-isoamyl alcohol and precipitated in 4 M sodium acetate and ethanol.

The *Xmn* I polymorphism at -158 position of the  $^G\gamma$ -gene was confirmed by *Xmn* I restriction enzyme digestion of a 650 bp amplified DNA sequence from the promoter region of the  $^G\gamma$ -gene.

The 650 bp target DNA sequence was first amplified using the oligonucleotide primers *Xmn*1: 5' - AAC TGT TGC TTT ATA GGA TTT T - 3' and *Xmn*2: 5' - AGG AGC TTA TTG ATA ACT CAG AC - 3' at a final concentration of 20 pmol each.<sup>16</sup> The PCR conditions were initial denaturation at 95°C for 5 minutes followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute and elongation at 72°C for 1.5 minutes. A final extension at 72°C for 3 minutes was included.

PCR products (15  $\mu$ l) were used directly for restriction enzyme digestion with *Xmn* I. DNA from a patient homozygous for the  $^G\gamma$  *Xmn* I polymorphism was included in every digestion as a control to show complete restriction enzyme digestion. Digested fragments were electrophoresed on 2% Seakem® LE agarose and visualised using ultraviolet light illumination.

## RESULTS

DNA amplification of the 650 bp sequence from the promoter region in the  $^G\gamma$ -globin gene

using the primers and PCR protocol developed in-house produced very distinct PCR products with absence of non-specific bands (Fig 1, lane 3). Digestion of PCR products with *Xmn* I produced bands of 450 bp and 200 bp when both chromosomes possessed the *Xmn* I polymorphic site (+/+) (lanes 4 and 9). Bands of 650 bp, 450 bp and 200 bp were observed when only one chromosome possessed the site (+/-) (lanes 6, 7 and 8). Absence of the *Xmn* I site in both chromosomes (-/-) showed only the original undigested 650 bp fragment (lane 5). The DNA blank (lane 2) did not show any amplified DNA as it served as a control where no DNA was added to the PCR mixture.

*Frequency of the  $\text{G}\gamma$  Xmn I polymorphism in the Malays and Chinese*

The sample population of 107  $\beta$ -thalassaemia major patients consisted of 58 Chinese and 49 Malays. The  $\text{G}\gamma$ *Xmn* I polymorphism genotyping in the Chinese  $\beta$ -thalassaemia patients (n=58) showed that the most frequent genotype observed was the absence of the *Xmn* I cleavage site in both chromosomes (-/-) (Table 1). Homozygosity for the *Xmn* I (-/-) genotype in the Chinese studied was 89.7% (52/58). The presence of both the *Xmn* I (+) and (-) sites in the Chinese was confirmed in only six patients (10.3%) while homozygosity for the *Xmn* I cleavage site (+/+) in both chromosomes was not detected (0/58) in any of the Chinese studied. Of the 116 chromosomes studied in the Chinese, only six possessed the *Xmn* I restriction while 110 did not, thus, the ratio of the (+) allele to the (-) allele in the Chinese was 0.05.

In contrast, the most common genotype of the  $\text{G}\gamma$  *Xmn* I polymorphism observed in the Malaysian Malays was the presence of both the *Xmn* I (+) and (-) sites and this was confirmed at a frequency of 63.3% (31/49) (Table 1).

Homozygosity for the *Xmn* I (-/-) genotype was observed in 28.6% (14/49) while the presence of the *Xmn* I cleavage site in both chromosomes (+/+) was observed in 8.2% (4/49) of the Malays. Of the 98 chromosomes studied in the Malays, 39 possessed the *Xmn* I restriction while 59 did not, thus, the ratio of the (+) allele to the (-) allele was 0.66.

**DISCUSSION**

DNA amplification of the 650 bp sequence from the promoter region in the  $\text{G}\gamma$ -globin gene using PCR protocol developed in-house produced very distinct PCR products (Fig 1). The DNA amplification protocol did not result in any non-specific amplifications, thus, the amplified products did not contain any non-specific bands. The amplified DNA was digested with *Xmn* I without further purification of the PCR products as mineral oil was not used during PCR. The amplified DNA was not concentrated further as 15  $\mu$ l of the PCR product was sufficient to produce distinct digested fragments of 450bp and 200bp. This is in contrast to the DNA amplification protocols used by other investigators<sup>16,17</sup> which required the PCR products to be purified and concentrated by chloroform extractions or isopropanol precipitation before digestion with *Xmn* I. The in-house method also used the cheaper Seakem® LE agarose (2%) instead of the more expensive Nusieve agarose at 3% gel concentration,<sup>16,17</sup> and distinct well separated bands were observed after gel electrophoresis. A 3% gel concentration is more difficult to handle in terms of boiling the agarose and clarity of bands is also reduced as a 3% gel is more opaque in appearance. Thus, the concentration of primer sequences, DNA amplification conditions and gel separation protocol established in this study were rapid, sensitive and specific for the genotyping of the  $\text{G}\gamma$  *Xmn* I polymorphism.

**TABLE 1: The frequency of the *Xmn* I-5' $\text{G}\gamma$  polymorphism genotypes in the Chinese and Malays in Malaysia.**

	Frequency of <i>Xmn</i> I-5' $\text{G}\gamma$ polymorphism genotypes, % (n)		
	-/-	+/-	+/+
Chinese (n=58)	89.7 (52)	10.3 (6)	0
Malay (n=49)	28.6 (14)	63.3 (31)	8.2 (4)
Total (n=107)	61.7 (66)	34.6 (37)	3.7 (4)

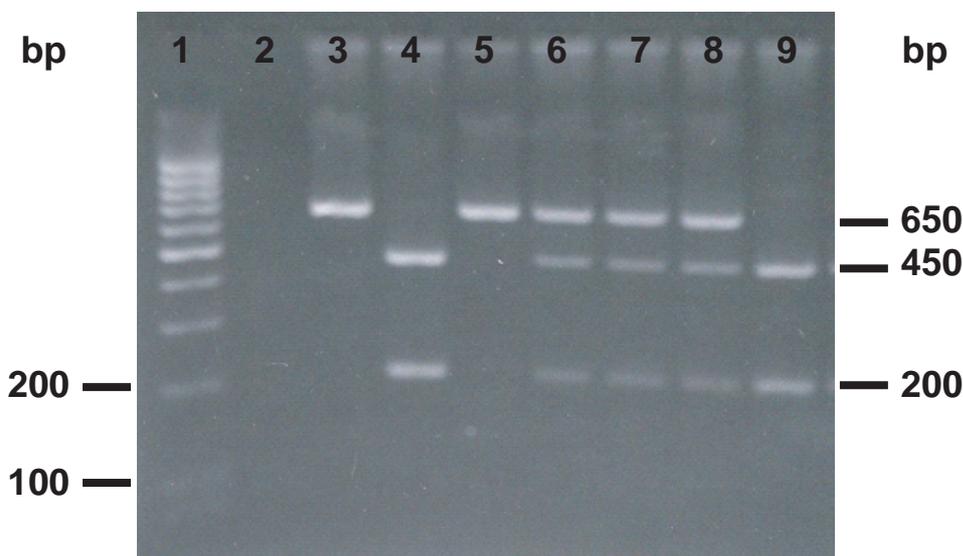


FIG. 1: Agarose gel electrophoresis of amplified DNA after digestion with *Xmn* I for the detection of the  $^{\text{G}}\gamma$  *Xmn* I polymorphism genotypes.

Lane 1: 100 bp DNA ladder;

Lane 2: DNA blank where no amplified DNA bands are observed;

Lane 3: Undigested 650 bp amplified sequence;

Lanes 4, 9: 450 bp and 200 bp digested fragments from a patient with *Xmn* I (+/+) genotype;

Lane 5: 650 bp fragment from a patient with *Xmn* I (-/-) genotype;

Lanes 6, 7, 8: 650 bp, 450 bp and 200 bp fragments from patients heterozygous for the *Xmn* I (+/-) genotype

The *Xmn* I cleavage site is located at 158 bases upstream from the transcription start site of the  $^{\text{G}}\gamma$ -gene in the  $\beta$ -globin gene cluster.<sup>8</sup> In this study, homozygosity for the *Xmn* I (-/-) genotype was much higher in the Chinese  $\beta$ -thalassaemia major patients (89.7%) studied when compared with the Malays (28.6%). The most common genotype of the  $^{\text{G}}\gamma$  *Xmn* I polymorphism confirmed in the Malays studied was the presence of both the *Xmn* I (+) and (-) sites in 63.3% of the  $\beta$ -thalassaemia major patients. Heterozygosity for the *Xmn* I polymorphic site (+/-) was found to be much lower in the Chinese, at a frequency of only 10.3%. In the Chinese studied, it was very conclusive that homozygosity for the presence of the *Xmn* I cleavage site (+/+) in both chromosomes was absent while 8.2% of the Malaysian Malays showed homozygosity for the *Xmn* I (+/+) site. The ratio of the (+) allele (presence of *Xmn* I site) to the (-) allele (absence of site) was found to be much higher in the Malay  $\beta$ -thalassaemia patients at 0.66 compared to the Chinese at 0.05.

The  $^{\text{G}}\gamma$  *Xmn* I polymorphism studied in 12 Chinese patients with  $\beta$ -thalassaemia intermedia in Hong Kong also showed that the most common

genotype encountered was homozygosity for the *Xmn* I polymorphic site (-/-) (91.7%) followed by heterozygosity for the *Xmn* I site (+/-) at 8.3%.<sup>17</sup> The results of Antonarakis *et al* (1988) were similar to this study as homozygosity for the *Xmn* I (+/+) site was not detected, and the (-) allele was the predominant allele in both the Malaysian (94.8%) and Hong Kong (95.8%) Chinese.<sup>17</sup>

In a  $^{\text{G}}\gamma$  *Xmn* I polymorphism study carried out on 64  $\beta$ -thalassaemia patients in Calcutta and West Bengal, the investigators reported the (+/-) genotype to be the most common genotype (76.6%), followed by the (-/-) genotype (14.1%) and the (+/+) genotype (9.4%).<sup>18</sup> The majority of the patients (90.6%) in the study had HbE- $\beta$ -thalassaemia and the results reported by the above investigators<sup>18</sup> were similar to the frequency of the *Xmn* I polymorphism in our Malaysian Malays studied, where the majority of patients also had HbE- $\beta$ -thalassaemia (79.6%). Bandyopadhyay *et al* (2001) reported that homozygosity of the *Xmn* I site (+/+) was strongly correlated with a mild  $\beta$ -thalassaemia phenotype and its absence (-/-) with a severe phenotype.<sup>18</sup> Hb E- $\beta$ -thalassaemia in Thailand was reported

to show a clinical severity equivalent to that of  $\beta$ -thalassaemia major and this was associated with the low frequency (8.9%) of the *Xmn* I (+/+) genotype in the HbE- $\beta$  patients.<sup>15</sup> The absence of the *Xmn* I site in both chromosomes was reported in 20% while heterozygosity of the site was found in 71% of the Hb E- $\beta$  thalassaemia patients in Thailand. The investigators concluded that two copies of the *Xmn* I site were necessary in ameliorating the severity of  $\beta$ -thalassaemia major while patients with a single copy of the allele appeared to have a variable clinical course and a wide range of haemoglobin levels.

In conclusion, this preliminary study of the *Xmn* I restriction site at the -158 position of the  $\text{G}\gamma$ -gene is the first such study in  $\beta$ -thalassaemia patients in Malaysia. This study is also the first reported study of  $\text{G}\gamma$  *Xmn* I polymorphism in Malay and Chinese  $\beta$ -thalassaemia major patients in the same population. Further studies on the association of the different genotypes of the  $\text{G}\gamma$  *Xmn* I polymorphism with clinical severity of the disease will be carried out as clinical data of the patients is being collected. In addition, further research on  $\text{G}\gamma$  *Xmn* I polymorphism in the Malaysian Malays and Chinese will involve a more detailed study with co-inheritance of  $\alpha$ -thalassaemia and the inheritance of mild  $\beta$ -thalassaemia mutations. This will allow a more comprehensive evaluation of different genetic factors involved in the amelioration of the clinical severity of  $\beta$ -thalassaemia major in Malaysia.

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