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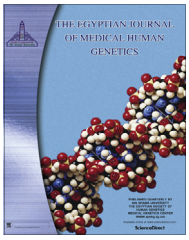

ORIGINAL ARTICLE

XmnI polymorphism: Relation to *β-thalassemia* phenotype and genotype in Egyptian Children

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KEYWORDS

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Abstract *Background:* β-Globin mutations with Xmn1 site might be associated with elevated HbF expression which may in turn ameliorate the severity of β-thalassemia phenotype.

Aim of the study: To investigate the frequency of −158 (C > T) XmnI polymorphism among Egyptian Children and young adults with β-thalassemia, to examine the relationship between XmnI polymorphism and β-thalassemia genotypes and phenotypes and to assess the possible relation of XmnI polymorphism and response to hydroxyurea (Hu) therapy.

Patients and methods: Seventy-two β-thalassemia patients (37 females; M/F ratio 0.95) with a mean age of 7.53 ± 6.99 were included. Laboratory investigations included Complete blood count (CBC), Hb electrophoresis by high performance liquid chromatography (HPLC), β-thalassemia mutation identification by the reverse dot blot hybridization technique (RDB) and detection of XmnlGg polymorphism by RFLP.

Results: The frequency of positive heterozygote XmnI gene polymorphism was 8.3%. Eighty-three percent of XmnIG$\gamma^{+/-}$ patients were never transfused ($p = 0.001$) and had higher total hemoglobin compared to XmnIG$\gamma^{-/-}$ ($p = 0.01$); while mean HbF was higher among XmnIG$\gamma^{+/-}$ patients compared to the other group but the difference was marginally insignificant ($p = 0.06$). β-Thalassemia mutation IVS II-1 showed relatively higher XmnI polymorphism frequency (50%) and followed by its frequency among 10 undefined β-thalassemia mutations which was 20%. The frequency of positive heterozygote XmnI gene polymorphism was 11.6% among the TI group vs. 3.5% among the TM group ($p = 0.4$). Among 20 cases who received HU; 5/14 responders vs. 1/6 none responder had positive heterozygote XmnI gene polymorphism ($p = 1.0$).

Conclusions and recommendations: In conclusion, molecular determination of genetic markers in childhood will help to identify phenotypes of our patients and to avoid over or under treatment

Abbreviations: Hu, hydroxyurea; TI, thalassemia intermedia; TM, thalassemia major; CBC, complete blood count; HPLC, high performance liquid chromatography; RDB, reverse dot blot hybridization

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strategies. Further prospective studies concerning the genetic markers that could predict the response to hemoglobin F inducers like hydroxyurea are highly recommended.

1. Introduction

β-Thalassemia syndromes are the most common form of chronic hemolytic anemia due to impaired globin chain synthesis [1]. The clinical presentation of β-thalassemia varies in severity, ranging from severe transfusion-dependent anemia to milder conditions [2]. One of the clinical challenges in the management of β-thalassemia is to clearly identify the phenotype of patients as early as possible. This will help in avoiding mismanagement with subsequent transformation of milder syndromes to a severe one [3,4]. However, this early clear identification of the phenotype is not that simple; especially in those who lie in the gray zone between the transfusion-dependant thalassemia major and the non transfusion dependant thalassemia intermedia.

A lot of progress has been made in understanding the molecular basis of β-thalassemia and to help in predicting phenotype from genotype [5–7]. It was found that the variable phenotypes may occur from the nature of β-globin gene mutations, α-thalassemia gene interaction or differences in the amount of fetal hemoglobin (HbF) production [8]. In the literature, there is a large body of evidence that increased HbF production has an ameliorating effect in patients who were mildly affected despite being homozygotes or compound heterozygotes for β0 or β+ thalassemia [9–11]. This increase of HbF production is genetically determined and partially associated with β-haplotypes which show particular microsatellite sequences and/or the XmnI polymorphism [12–14]. The association of some β-globin mutations with Xmn1 site with elevated HbF expression has been previously published [9,15,13,16,17].

In this study we aimed to investigate the overall prevalence of XmnI polymorphism among Egyptian Children and young adults with β-thalassemia, to examine the relationship between XmnI polymorphism and β-thalassemia genotypes and phenotypes and to assess the possible relation of XmnI polymorphism and the response to hydroxyurea (Hu) therapy.

2. Patients and methods

This was an observational prospective study that included seventy-two β-thalassemia patients; 37 (51.4%) females (male/female ratio 0.95) with a mean age of 7.53 ± 6.99 (range: 0.6–29 years). Forty-three patients were diagnosed as thalassemia intermedia (TI) based on conventional clinical (late presentation and/or transfusion independency) and hematologic criteria [3]. All patients were regularly followed up at the Pediatric Hematology Clinic of New Children Hospital, Faculty of medicine, Cairo University. The study protocol was approved by the Ethical Committee of Cairo University & the Ethical Committee of National Research Center, Cairo, Egypt, according to the Institutional Committee for the Protection of Human Subjects and adopted by the 18th World Medical Assembly, Helsinki, Finland.

All patients underwent medical history clinical examination. Fourteen patients were treated with hydroxyurea (HU) in a dose ranging from 10 to 20 mg/kg/day orally once a day for at least 3 months.

Laboratory investigations included a complete blood count using (Ceii-Dyn 3700 hematology analyzer), Hb electrophoresis by high performance liquid chromatography (HPLC) using the VARIANT II β-thalassemia Short Program, Bio-Rad Laboratories [18]. β-Thalassemia mutation identification of samples was performed by the reverse dot blot hybridization technique (RDB). For RDB, a panel of primers and probes ($n = 22$) using the beta globin strip assay was used (β-Globin Strip Assay MED kit, VIENNA LAB) [19].

Detection of GγXmnI polymorphism of C to T base pair substitution at the −158 position in the promoter region of the Gγ-globin gene (−158 (C > T) XmnI polymorphism): Blood samples were collected from patients into EDTA vacutainers for genomic DNA analysis by polymerase chain reaction–restriction fragment-length polymorphism (PCR–RFLP). DNA was extracted using the QI Amp DNA Mini Kit; Blood Mini Kit (Catalog No: 51104). The primer set used for DNA amplification was the 5′-AAC TGT TGC TTT ATA GGA TTT T-3′ and 5′-AGG AGC TTA TTG ATA ACT CAG AC-3′ [20]. PCR was performed using a Perkin Elmer Thermal Cycler Gen Amp 9700 (Applied Biosystems, UK). A total volume of 25 μl PCR reaction contained 12.5 μl of ready-to-use PCR Master Mix, 5, 5 μl of nuclease-free water, 1 μl (20 pmol) of primer F, 1 μl (20 pmol) of primer R and 5 μl of genomic extracted DNA.

The cycling reaction was performed under the following conditions: Denaturation at 95 °C for 10 min, 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. The PCR products were digested with the XMN 1 restriction enzyme. Digestion products were electrophoresed on a 3% agarose gel. Amplification with the primers produced a 650 bp fragment in the wild genotype, the heterozygous genotype gives 2 bands at 400 bp and 250 bp [20].

2.1. Statistical analysis

SigmaStat program; version 3.5 (Systat Software, Inc., USA) was used for Data management and analysis. Numerical data were presented as mean ± SD or median and interquartile range (IQR). Comparisons between numerical variables between two groups were done by Student's t test for parametric data or Mann–Whitney Rank Sum test for non-parametric data. Comparing of categorical variables was done by Fisher exact test. $P < 0.05$ was considered significant for all statistical tests.

3. Results

Seventy-two patients were included; their baseline demographic and clinical characteristics are illustrated in Table 1.

Table 1 Demographic and clinical data of the studied cases ($n = 72$).

Variables	All patients ($N = 72$)	
	Mean ± SD (range)	Median (IQR)
Age at 1st transfusion (mo) ($n = 57$)	34.25 ± 56.40 (2.0–324.0)	14 (7–36)
Age at diagnosis (mo)	26.82 ± 22.35 (2–96)	24 (8.25–40.5)
Total hemoglobin (g/dl)	7.26 ± 1.45 (4.5–10.7)	7.35 (6.15–8.45)
Hemoglobin F (%)	43.46 ± 26.23 (0–100)	41.00 (23.0–59.0)
TR* (times/year) ($n = 57$)	12.38 ± 4.52 (4–24)	12 (10.5–12)

* TR: transfusion rate.

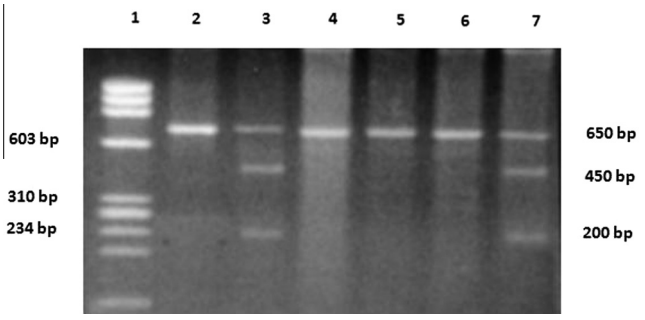

Figure 1 PCR Polymorphisms of the Gγ-globin gene (−158 (C > T) XMN1 polymorphism in β-thalassemia patients: Lane 1:ΦX174 DNA/BsuRI (HaeIII) Marker. Lane 2,4–6: 650 bp fragment from patients with XmnI (−/−) genotype. Lane 3 and 7:650 bp, 450 bp and 200 bp fragment from a patient heterozygous for the XmnI (+/−) genotype.

The overall frequency of positive heterozygote XmnI gene polymorphism among the studied patients was 8.3%. Eighty-three percent of XmnIGγ$^{+/-}$ patients were never transfused ($p = 0.001$) and had significantly higher total hemoglobin compared to XmnIGγ$^{-/-}$ ($p = 0.01$); meanwhile mean HbF was higher among XmnIGγ$^{+/-}$ patients compared to the other group but the difference was marginally insignificant ($p = 0.06$) (Fig. 1 and Table 2).

3.1. XmnIGγ polymorphism and β-thalassemia mutations

Among the 72 patients, 26 different genotypes were identified. There were 37 homozygous patients, while the rest were compound heterozygous. Twenty-five of the 37 (67.6%) homozygous patients were the products of consanguineous marriages. The most frequent mutation among homozygous patients was IVS-I-6 followed by IVS-I-110. The most frequent compound heterozygous state was IVS 1–110 (G > A)/IVS 1.6 T > C. β-Thalassemia mutation IVS II-1 showed relatively higher XmnI polymorphism frequency (50%) regarding other β-thalassemia mutations observed in our patients and it was followed by the frequency of positive heterozygote XmnI gene polymorphism among 10 undefined β-thalassemia mutations in 72 patients which was 20% (Table 3).

3.2. XmnIGγ polymorphism and β-thalassemia phenotype and response to hydroxyurea (HU) therapy

The frequency of positive heterozygote XmnI gene polymorphism was 11.6% among the TI group vs. 3.5% among the TM group ($p = 0.4$). Among 20 cases who received HU; 5/

Table 2 Comparison of XmnIGγ$^{-/-}$ and XmnIGγ$^{+/-}$ patients.

Variables	XmnIGγ$^{-/-}$ ($n = 66$)	XmnIGγ$^{+/-}$ ($n = 6$)	p-value
Phenotype			
BT-major	28 (42.4%)	1 (16.7%)	0.391
BT-intermedia	38 (57.6%)	5 (83.3%)	
Age at diagnosis (mo)			
Mean ± SD (range)	25.694 ± 22.045	40.80 ± 23.774	0.161
Median (IQR)	20.0 (8.0–36.0)	42.0(28.5–54.0)	
Hb (g/dl)			
Mean ± SD (range)	7.123 ± 1.420	8.683 ± 1.025	0.011*
Median (IQR)	7.1 (6.1–7.9)	8.9 (7.5–9.6)	
Hb F (%)			
Mean ± SD (range)	41.502 ± 26.042	62.367 ± 26.042	0.063
Median (IQR)	37.55 (20.20–57.0)	58.5 (47.0–69.2)	
Transfusion			
Yes	56 (84.8%)	1 (16.7%)	0.001*
No	10 (15.2%)	5 (83.3%)	

* Statistically significant.

Table 3 Genotype study of patients with positive XmnI polymorphism ($n = 6$).

Genotype	β/β	No of cases	Clinical syndrome
IVS 1–110 (G > A)/IVS 1.6 (T > C)	+/+	1	TI
IVS 1.1 (G > A)/IVS 1.6 (T > C)	0/+	1	TM
IVS 2.1(G > A)/not characterized	0/?	1	TI
IVS 2.1(G > A)/IVS 2.745(C > G)	0/+	1	TI
Not characterized	−/−	2	TI

Table 4 XmnIGγ polymorphism of the studied patients in relation to clinical phenotype and response to hydroxycarbamide (Hu).

	Phenotype		*p*-value	HU response		*p*-value
	TI ($n = 43$)	TM ($n = 29$)		Yes ($n = 14$)	No ($n = 6$)	
XmnIG$\gamma^{-/-}$	38 (88.37%)	28 (96.55%)	0.391	10 (71.4%)	5 (83.3%)	1.000
XmnIG$\gamma^{-/+}$	5 (11.63%)	1 (3.45%)		4 (28.6%)	1 (16.6%)	

14 responders vs. 1/6 none responder had positive heterozygote XmnI gene polymorphism (1.0) (Table 4).

4. Discussion

Positive XmnI gene polymorphism was reported to be one of the main phenotype modifying factors of β-thalassemia [6,13,14,20,22,23]. In this study we aimed to investigate the overall prevalence of XmnI polymorphism among Egyptian Children and young adults with β-thalassemia, to examine the relationship between XmnI polymorphism and β-thalassemia genotypes and phenotypes and to assess the possible relation of XmnI polymorphism and the response to hydroxyurea (Hu) therapy.

We found that the overall frequency of positive heterozygote XmnI gene polymorphism was 8.3% in our β-thalassemia patients. Our data showed significant differences in transfusion dependency as most of XmnI positive patients were transfusion independent, and total hemoglobin level in XmnIG$\gamma^{+/-}$ patients, and also HbF was higher among XmnIG$\gamma^{+/-}$ patients compared to the other group but the difference was marginally insignificant ($p = 0.06$). This was in line with earlier studies that confirmed that the presence of the Xmnl polymorphism might contribute to overproduction of HbF, causing mild features of β-thalassemia [16,24,25]. In addition, patients with Xmnl polymorphism were usually transfusion independent or had an older age at first transfusions [26].

To our knowledge, few previous studies compared the frequency of positive heterozygote XmnI gene polymorphism in thalassemia major and intermedia and found no statistically significant difference [23,27], and two previous studies carried out to assess it among Egyptian β-thalassemia patients and reported a frequency 9% in β-thalassemia intermedia patients [16] and 4% in β-thalassemia major patients [17]. Our data were in line with the previous reports; the frequency of positive heterozygote XmnI gene polymorphism was 11.6 % among the TI group vs. 3.5% among the TM group ($p = 0.4$).

Also, we observed that 2/4 chromosomes with IVS II-1(G > A), 2/45 chromosomes with IVS I-6 (T > C) mutation and 2 out of 10 chromosomes with non characterized mutations were positive for the XmnI [+] polymorphism. This means that β-thalassemia mutation IVS II-1 showed the highest XmnI polymorphism frequency (50%) when compared to other β-thalassemia mutations observed in our patients. Previous studies reported that several β-globin gene mutations including codon 44 (−C), IVS I-5 (G > C), IVS II-1 (G > A) − 87 (C > G) and FSC 8 (−AA) were positive for the XmnI [+] polymorphism in different countries [6,7,13,21–23,28].

Our data showed that XmnI polymorphism had no impact on the response to hydroxyurea. This was in concordance with previous studies that reported no correlation between the presences of XmnI polymorphism and response to HU therapy in β-thalassemia patients [7,29].

However, other researchers reported that the response to HU was significantly correlated with the presence of XmnI polymorphism [28,30,31].

Our study is one of few studies that compared the frequency of globin gene promoter XmnI polymorphism in thalassemia major and thalassemia intermedia and is the first to report the frequency of XmnI polymorphism in Egyptian β-thalassemia patients in relation to β-thalassemia chromosomes.

In conclusion, molecular determination of genetic markers in early childhood will help to identify phenotypes of our patients and to avoid over or under treatment strategies. Further prospective studies concerning the genetic markers that could predict the response to hemoglobin F inducers like hydroxyurea (HU) are highly recommended.

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