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Clinical and molecular aspects of haemoglobinopathies in Tunisia

Amel Haj Khelil[a], Sandrine Laradi[a,b], Abdelhedi Miled[b], Ghazi Omar Tadmouri[c], Jemni Ben Chibani[a], Pascale Perrin[d,*]

[a] *Département de Biologie Clinique «A», Laboratoire de Biochimie et de Biologie Moléculaire, Faculté de Pharmacie, 5019 Monastir, Tunisia*
[b] *Laboratoire de Biochimie et de Toxicologie, CHU Farhat Hached, 4000, Sousse, Tunisia*
[c] *Department of Biology, Fatih University, 34500 Buyukcekmece, Istanbul, Turkey*
[d] *Centre de Génétique Moléculaire et Cellulaire, UMR 5534, Bât. Gregor Mendel, Université Claude Bernard, Lyon1-16, rue Raphaël Dubois, 69622 Villeurbanne cedex, France*

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Abstract

Background: For the last two decades, studies on the population genetics of Tunisians have focused on variations of protein and genetic markers. Results confirmed the genetic heterogeneity of Tunisians caused by the admixtures with migratory human groups arriving mainly from Africa, Europe, and Asia. These studies also allowed the screening of rare mutants and many haemoglobin variants. *Methods*: The present study delineates the incidence of the different haemoglobinopathies in Tunisia. Previously collected data and results obtained from epidemiological and clinical studies of 1238 blood donors and 276 patients were compared. The chromosomal backgrounds of different haemoglobinopathies were explored by molecular techniques [denaturing gradient gel electrophoresis (DGGE), amplification refractory mutation system (ARMS) polymerase chain reaction (PCR), and sequencing]. *Results*: This study indicates that appropriate DNA methodologies required for a nationwide preventive program in Tunisia are available and that prenatal diagnosis is feasible. Additionally, analysis of sequence polymorphisms allowed a better understanding of the gene recombination events and their application for tracing back the origin and the diffusion of the mutations. *Conclusions*: Molecular analysis techniques such as DGGE and ARMS PCR are socially and economically the most suitable techniques to be used in Tunisia for the detection and the identification of haemoglobin abnormalities. At present, their use is essential to conduct a clear and efficient screening program.

Keywords: Thalassaemias; Sickle cell disease; Haemoglobin; Haplotypes; Prenatal diagnosis

1. Introduction

Haemoglobinopathies are the world's most widespread genetic diseases and may cause a life-long blood transfusion-dependent anaemia. Their high frequencies constitute a real public health problem in Tunisia from the economical and human aspects. Previous epidemiological studies showed a great incidence of these diseases in populations originating from tropical and Mediterranean countries. The prevalence of haemoglobinopathies in malaria-infested regions is suggestive for possible mechanisms of

* Corresponding author. Tel.: +33-4-72-13-26-48; fax: +33-4-72-44-05-55.
E-mail address: pperrin@univ-lyon1.fr (P. Perrin).

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protection against *Plasmodium falciparum* malaria in heterozygotes carrying the trait of such disorders [1,2].

In 1930, the first study on the population genetics of Tunisians using data of the ABO blood system was reported [3]. Since the 1980s, many studies using serum markers were conducted in Tunisia (namely, immunoglobulins, transferrin, protein Gc and complement factors, HLA markers) [4–10]. All of these studies revealed an important heterogeneity in the genetic structure of the Tunisian population. This heterogeneity is the result of the mixing with diverse human groups that succeeded in Tunisia. The advent of molecular analyses has favoured a great progress in research on haemoglobinopathies. Further advances in the methodologies for analysing the β-globin gene have made possible the identification of a large spectrum of mutations causing haemoglobinopathies especially those leading to β-thalassaemia and sickle cell disease (SCD). The admixture with African blacks is confirmed by the studies of haemoglobinopathies, especially the sickle cell disease (S haemoglobinosis), which reaches its highest frequencies in Black populations (20–30% in some ethnic African groups). Its presence in the Tunisian population may be linked either to a selective effect associated with malaria or to the admixture with human groups of black African origins. Endogamy, as yet frequent (reaching 32.28% in the North of Tunisia [11]), has maintained rare genotypes and particular features.

Research on haemoblobinopathies in the Maghreb began in the 1950s [12] with studies on thalassaemia [13] and on sickle cell disease [14]. In Tunisia, the first studies focused on the epidemiology of haemoglobinopathies [15,16] and presented the first attempts to perform genetic counselling for these disorders [17]. The screening of β-thalassaemia mutations was conducted first by haematological and biochemical studies, and then by molecular analyses including restriction endonuclease, hybridization on specific oligonucleotide probes, amplification, and sequencing [18,19]. The same methods were used in the examination of sickle cell disease haplotypes [19,20]. Few analyses were done on α-thalassaemia in Tunisia [16,20–22].

The present study offers a twofold contribution to a better knowledge of haemoglobinopathies in Tunisia since it (1) utilizes haemoglobin as a genetic marker to describe the genetic structure of the Tunisian population and (2) its result is essential for the proper biological diagnosis of abnormalities encountered in haematology clinical services. These clinical investigations have been complemented by molecular studies, which have led to the successful application of prenatal diagnosis in our laboratories. Prenatal diagnosis is the only alternative to families at risk when bone marrow transplantation cannot be carried out because of restrictions imposed by HLA incompatibility and the high cost of intervention. Hence, the aim of this paper is to present a complete synthetic study of haemoglobinopathies in Tunisians and to reveal the population heterogeneity caused by historic migrations.

2. Material and methods

2.1. Epidemiological and clinical analyses

To study the prevalence of haemoglobinopathies, two samples from the Tunisian population were studied. Samples from 1238 blood donors were collected during an inquiry carried out at the haematological services of CHU in the central region of Tunisia (Kairouan, Mahdia, Monastir, and Sousse). Samples were also collected from 276 patients who were admitted to the haematological and paediatric services of the same hospitals between 1994 and 1998.

Clinical investigations were made on blood samples collected in EDTA tubes from the index cases expected to have a haemoglobinopathy in their families. Clinical diagnosis was performed by haematological and biochemical investigations. Blood counts were done by routine methods using automated cell counter (Coulter type S). The RBC was used as a diagnostic adjunct since thalassaemias produce a microcytic anaemia associated with increased RBC count.

Biochemical investigations allow the complete determination of the different haemoglobin fractions. We used alkaline electrophoresis (pH 8–6) on cellulose acetate gel [23] to separate each haemoglobin fraction and to detect Hb variants. Isoelectric focusing (IEF) [24] on polyacrylamide gel in presence of Ampholine pH 6–9 using a GD 2200 generator (Sebia) was sometimes performed for a best resolution

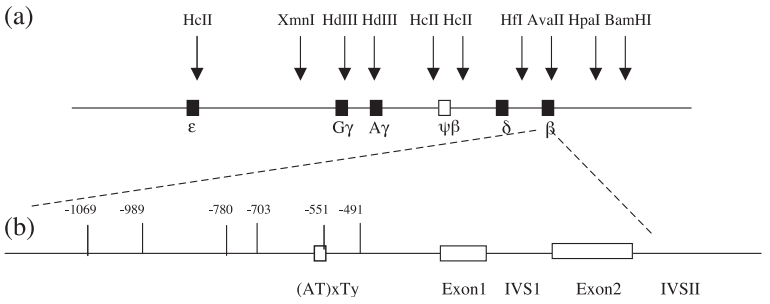

Fig. 1. Map of the β-globin gene cluster and the polymorphic sites analysed (a) for RFLP haplotypes and (b) for sequence haplotypes.

of haemoglobin fractions. Microchromatography on anion exchange column type DEAE 52 (Whatman) was used in some cases for a precise quantification of HbA_2. The method favours a differential elution system [25].

2.2. Molecular analysis

DNA extracted by the phenol/chloroform standard method from white blood cells, amniocytes or chorionic tissues was used for molecular analyses of various haemoglobinopathies [26].

Of the 276 patients clinically investigated, samples from 193 individuals were subjected to molecular analyses for the identification of their mutations. We performed the allele specific oligonucleotide (ASO) hybridization assay and the amplification refractory mutation system PCR (ARMS PCR) using allele specific primers [27] to identify the most common β-globin gene mutations as yet described in Tunisia. These mutations include the HbS mutation and the three most frequent β-thalassaemia mutations in the central Tunisia: stop codon 39 (C → T), IVS-I-110 (G → A), and IVS-I-2 (T → G). Denaturing gradient gel electrophoresis (DGGE) [28] was used to detect known and unknown mutations. DNA samples, in which a mutation was not detected by the above methods, were subjected to genomic sequencing by an automatic procedure on the Perkin-Elmer 373 apparatus and using Perkin-Elmer ABI PRISM Dye Terminator and Amersham Dyenamic and Terminator kits. This latter technique allows the simultaneous analysis of the nucleotide sequence of the β-globin gene with its polymorphism. The 5'-flanking region of the β-globin gene includes nine single nucleotide polymorphisms (SNPs) and an (AT)xTy microsatellite [29]. The haplotypes observed were used to trace back the origin of the corresponding β-globin gene mutations by comparison with other populations or ethnic groups (Fig. 1).

3. Results and discussion

3.1. Clinical aspects

Table 1 shows the data collected in our epidemiological study. Results showed that SCD and β-thalassaemia are the most frequent molecular defects in donors and patients sampled in the framework of this study. Of the 276 patients studied, 193 are clinically diagnosed to carry haemoglobinopathies. Among

Table 1
Haemoglobinopathies detected in the donors and patients investigated in this study

	Sample of donors ($N=1238$)		Sample of patients ($N=276$)	
	Nb of cases	Frequency %	Nb of cases	Frequency %
Homozygous SCD	–	–	21	7.6
Heterozygous SCD	31	2.50	33	11.95
Homozygous Haemoglobinosis C	–	–	4	1.45
Heterozygous Haemoglobinosis C	4	0.32	–	–
Homozygous β^0 Thal.	–	–	21	7.6
Heterozygous β^0 Thal.	29	2.34	69	25
Homozygous β^+ Thal.	–	–	6	2.17
Heterozygous β^+ Thal.	16	1.3	31	11.23
Compound thalassaemia/ sickle cell disease	–	–	8	2.89
Total	80	6.45	193	69.89

them, 54 have SCD, 90 have β^0-thalassaemia, and 37 have β^+-thalassaemia.

Our results on SCD patients do not favour the hypothesis of a correlation between the clinical severity of the sickle cell symptoms and the polymorphism of the upstream haplotype [30]. Our findings suggest that more probably, the polymorphic region upstream of the G_γ foetal globin gene is involved in the modulation of foetal haemoglobin (HbF) expression [31].

3.1.1. β-Thalassaemias

Haematological data demonstrate a wide spectrum of variation among homozygous and heterozygous β-thalassaemia individuals (Table 2). Homozygous β^+ thalassaemia subjects show a slight decrease in RBC and MCV values and an incomplete synthesis of HbA compensated by intermediate levels of HbF (10–35%). Familial investigations have confirmed the absence of hereditary persistence of HbF and δ/β-thalassaemia. Heterozygous carriers of β^0 or β^+ thalassaemia are usually asymptomatic at clinical level. Their haematology is characterized by a slight to moderate anaemia, a low MCV value (59–68 fl) and a raised level of HbA_2 (3.5–4.3). The increase of HbA_2 level is enough to detect heterozygotes in both β^0 and β^+ thalassaemia.

Four therapeutic measures were implemented on β-thalassaemia patients in Tunisia: blood transfusion, iron chelation by Desferal® (Desferioxamine®) administration, splenectomy or bone marrow transplantation. However, the insufficiency of donors for blood transfusion because of the constantly increasing number of patients, the complications observed in the iron chelation (namely, lack of Desferal pumps, toxicity, etc...), the infection risk relative of splenectomy, and the limited number of bone marrow transplantation with the high cost of this operation might limit the efficiency of the treatment and the follow-up of β-thalassaemia patients. To overcome these problems, geneticists and clinicians should double their efforts and focus on new and more suitable methods of prevention and genetic counselling.

3.1.2. Sickle cell disease

Haematological data in sickle cell disease have shown a constant anaemia in homozygous and heterozygous HbS patients (Table 2). HbA_2 is normal in both homozygous and heterozygous HbS but is slightly increased in HbS/β-thalassaemia patients. HbF rate is highly variable in homozygous HbS and only appears in 9 cases out of our 21 homozygous patients. In these cases, values range from 3.3% to 28%. The haematological results and the heterogeneous rate of HbF are compatible with those observed in two other Tunisian samples: 14 patients originating from the region of Kebili in the South of Tunisia [32] and 35 patients mostly originating from Tunis in the north [33].

3.2. Molecular analysis

3.2.1. β-Thalassaemia

In Tunisia, 12 haemoglobin variants have been described so far: Hb F Clamart [34], Hb F Ouled

Table 2
Haematological data for β^+ (IVSI nt 110) and β^0 (codon 39) thalassaemia, HbS, and compound heterozygote β thal/HbS (first consultation)

RBC (10^{12}/l)	MCV (fl)	Hb (g/dl)	HbA (%)	HbA2 (%)	HbF (%)	HbS (%)	Patients number	Mutation type
(6.93 ± 0.25)	(87.7 ± 6.5)	(13.5 ± 1.5)	(97.82 ± 0.17)	(2.18 ± 0.17)	0	–	–	none (usual values)
2.87 ± 0.8	72.48 ± 2.97	6.53 ± 2.27	0	2.13 ± 0.19	98.12 ± 0.21	–	21	homozygous β^0 thal
4.79 ± 5.3	65.54 ± 7.9	9.56 ± 2.74	93.38 ± 2.17	5.05 ± 1.53	3.25 ± 5.09	–	69	heterozygous β^0 thal
3.31 ± 0.41	74.56 ± 7.35	6.7 ± 2.62	57 ± 35	3 ± O	40 ± 35	–	6	homozygous β^+ thal
5.55 ± 0.41	63.77 ± 4.24	11.44 ± 1.33	94.55 ± 1.55	4.69 ± 0.86	1 ± 1.13	–	31	heterozygous β^+ thal
							Total 127	
2.59 ± 0.79	81.15 ± 8.77	6.95 ± 1.81	0	2.97 ± 0.95	5.16 ± 8.56	91.87 ± 8.72	21	homozygous HbS
4.33 ± 0.82	80.22 ± 7.6	12.5 ± 1.54	60.1 ± 7.94	3.03 ± 0.57	0	37.07 ± 6.98	33	heterozygous HbS
							Total 54	
2.45 ± 0.92	73.2 ± 3.71	6.15 ± 1.63	0	3.77 ± 1.72	36.77 ± 3.65	54.09 ± 1.93	3	comp. het. HbS/βthal

Average values with standard deviations are given.
RBC: red blood cells; MC: mean corpuscular volume; Hb: total haemoglobin; HbA and HbA_2: adult haemoglobin; HbF: foetal haemoglobin; HbS: sickle cell haemoglobin.

Rabeh [34], Hb D Iran [35], Hb Bab Saadoun [36], Hb Athens-Georgia [37], Hb Kairouan [38], Hb O Arab [39], Hb Tunis [40], Hb Montgomery [40], Hb Punjab [41], Hb Camperdown [42] and Agamma75 Thr [43]. Some of these variants were first detected in Tunisian patients. Of these new variants is the Hb Kairouan caused by a mutation at codon 30 (G → C) [38]. Other new variants were described elsewhere. On the other hand, 19 β-thalassaemia mutations have been described up to now in Tunisia (Table 3). The combined data [18,19,28] show that stop codon 39 (C → T) and IVS-I-110 (G → A) mutations are the most common β-thalassaemia defects in Tunisia (about 36% and 14%, respectively; Table 4). They are followed, in decreasing order, by IVS-I-2 (T → G), codon 6 (−A), IVS-I-6 (T → C), codon 44 (−C), IVS-II-745 (C → G), codon 30 (G → C), −30 (T → G), IVS-I-5 (G → A), codon 5 (−CT), IVS-I-1 (G → A), IVS-II-848 (C → A), IVS-I-2 (T → C), codons 25/26 (+T), codon 8 (−AA), IVS-I-1 (G → T), codon 37 (G → A), and IVS-II-849 (A → C). The latter two mutations were recently observed (Chouk et al., personal communication). A comparison of the mutation frequencies in different regions of Tunisia demonstrates that the distribution of β-thalassaemia alleles differs significantly within each area (Table 3). Northern Tunisia displays the greatest heterogeneity. On the other hand, although less heterogeneous, the central part of Tunisia seems to have its own spectrum of mutations, e.g., IVS-I-6 (T → C), IVS-II-745 (C → G), IVS-I-1 (G → A), and IVS-I-2 (T → G), which have a significantly higher occurrence. The IVS-I-2 (T → G) mutation was detected in the region of Essouassi–Eldjem in central Tunisia [18] and had not been found elsewhere, suggesting a local Tunisian origin. Some of the patients carrying this mutation do not show the usual symptoms of β^0-thalassaemia and do not suffer from anaemia. The variability in their clinical phenotypes needs further investigations.

Table 3
The 19 β-thalassaemia mutations described in the Tunisian population

Mutation	Type	Maximum recorded frequency (%)	Chr Nb	Reference	Region
Codon 39 (C → T)	β^0	48.76	121	[28]	Central Tunisia
IVS-I-110 (G → A)	β^+	20.5	44	[19]	North
IVS-I-2 (T → G)	β^0	19.83	121	[28]	Central Tunisia
IVS-I-6 (T → C)	β^+	10.5	68	[18]	Central Tunisia
Codon 44 (−C)	β^0	9.1	44	[19]	North
IVS-II-745 (C → G)	β^+	7.5	68	[18]	Central Tunisia
−30 (T → G)	β^+	6.8	44	[19]	North
Codon 5 (−CT)	β^0	4.5	44	[19]	North
Codon 30 (G → C)	β^+	4.5	44	[19]	North
IVS-I-1 (G → A)	β^0	3.31	121	[28]	Central Tunisia
IVS-I-5 (G → A)	β^+	3.31	121	[28]	Central Tunisia
IVS-II-849 (A → C)	β^0	2.5	80	PC	North
Codon 37 (G → A)	β^0	2.5	80	PC	North
Codon 6 (−A)	β^0	2.3	44	[19]	North
IVS-I-2 (T → C)	β^0	2.3	44	[19]	North
Codon 8 (−AA)	β^0	2.3	44	[19]	North
Codons 25/26 (+T)	β^0	2.3	44	[19]	North
IVS-II-848 (C → A)	β^+	2.3	44	[19]	North
IVS-I-1 (G → T)	β^0	1.5	68	[18]	Central Tunisia

The last column indicates the region from where patients originated. The third column indicates the highest frequency recorded for each mutation in Tunisia [18,19,28].
Chr Nb: chromosome number, PC: Chouk et al., personal communication (2002).

The alleles causing β-thalassaemia in Tunisia and in some other Mediterranean countries (namely, Algeria [44–46], Turkey [47,48], Syria [49], and Lebanon [50]) are reported in Table 4. Although we note a considerable heterogeneity of β-thalassaemia mutations in Tunisia, we also estimate that more mutations could be potentially present in the population. The same level of heterogeneity is observed in Algeria, Syria, and Lebanon. A higher level of variability is observed in Turkey (45 different mutations and variants), which is by far a much larger country. The most frequent mutation in Tunisia and Algeria is the codon 39 (C → T) followed by the IVS-I-110 (G → A), which is the most prevalent β-thalassaemia allele in eastern Mediterranean countries. Tunisia and Algeria show the same pattern of frequencies, which are very different from those observed in the East. In fact, these two neighbouring countries have shown very similar history with the same pattern of invasions and migrations.

The screening of a homozygous patient carrying the codon 44 (−A) mutation, detected for the first time in a southern Iranian patient [51], revealed the eastern introduction of genes in the Tunisian population. In

Table 4
Spectrum of the β-thalassaemia mutations in Tunisia and in other Mediterranean countries

Mutation	Type	Tunisia				Average	Algeria			Average	Turkey		Average
Codon 39 (C → T)	β^0	19	40.8	48.76		**36.18**	33.9	26	27.6	**29.2**		3.8	3.8
Codon 6 (−A)	β^0	16	2.3	1.65		**6.65**	11.3	17.9	17.1	**15.4**		0.4	0.4
IVS-I-6 (T → C)	β^+	10.5		2.48		**4.32**	3.2	3.5	3.3	**3.3**		10.1	10.1
IVS-I-110 (G → A)	β^+	7.5	20.5	15.7		**14.56**	19.3	25.4	24.7	**23.1**		39.3	39.3
IVS-I-116 (T → G)	β^0											0.2	0.2
IVS-I-130 (G → C)	β^0										×		×
IVS-I-130 (G → A)	β^0											0.1	0.1
Cd 29 (C → T)	β^+												
Cd 30 (G → A)	β^0												
IVS-II-1 (G → A)	β^0											4.7	4.7
IVS-II-745 (C → G)	β^+	7.5				**2.5**			0.9	**0.3**		5	5
IVS-II-654 (C → T)	β^+										×		×
IVS-II-848 (C → A)	β^+		2.3	0.83		**1.04**	1.6		0.4	**0.7**		0.4	0.4
IVS-II-849 (A → C)	β^0				2.5								
IVS-I-2 (T → G)	β^0	3		19.83		**7.66**							
IVS-I-2 (T → A)	β^0						1.6		1.3	**1.0**			
IVS-I-2 (T → C)	β^0		2.3			**0.76**	12.9		3.3	**5.4**			
IVS-I-1 (G → T)	β^0	1.5				**0.5**		14.5		**4.8**	×		×
IVS-I-1 (G → C)											×		×
IVS-I-1 (G → A)	β^0			3.31		**1.1**	8.1		11.7	**9.9**		5	5
Codon 44 (−C)	β^0		9.1	1.65		**3.58**						1.3	1.3
− 28 (A → C)	β^+											0.1	0.1
− 29 (A → G)	β^+						6.5			**2.2**			
− 29 (A → C)									3.8	**1.3**			
− 30 (T → G)	β^+		6.8			**2.26**							
− 30 (T → A)	β^+						1.6		0.4	**0.7**		3.1	3.1
− 87 (C → G)	β^+											0.8	0.8
− 88 (C → T)	β^+												
− 101 (C → T)	β^+											0.1	0.1
Codon 5 (−CT)	β^0		4.5			**1.5**						2.2	2.2
Codon 74/75 (−C)	β^0											0.1	0.1
Codon 15 (TGG → TAG)	β^0											0.1	0.1
Codon 22/23/24 (−AAGTTGG)	β^0											0.1	0.1
Codon 26 (G → A) Hb E											×		×
Codon 27 (G → T)	β^+											0.1	0.1
Codon 30 (G → C)	β^+		4.5	2.48		**2.32**			0.9	**0.3**	×		×
Codon 37 (G → A)	β^0				2.5						×		×
Codon 8 (−AA)	β^0		2.3			**0.76**						5.5	5.5
Codon 8/9 (+G)	β^0											1.3	1.3
IVS-I-5 (G → A)	β^+		2.3	3.31		**1.87**		1.5	0.9	**0.8**	×		×
IVS-I-5 (G → C)	β^+								0.4	**0.1**		1.1	1.1
IVS-I-5 (G → T)	β^+										×		×
IVS-II-843 (T → G)									0.4	**0.1**			
Codons 25/26 (+T)	β^0		2.3			**0.76**							
Codons 36/37 (−T)	β^0											0.1	0.1
Codons 37/38/39 (− 7 bp)	β^0										×		×
5′ splice IVSII	β^0												
Co27 (G → T)	β^+								0.4	**0.1**	×		×
Hb Lepore Boston									0.4	**0.1**			
HbD Los Angeles												0.1	0.1
HbE Saskatoon												0.1	0.1
5′UTR +22 (G → A)	β^+										×		×

Table 4 (*continued*)

Mutation	Type	Tunisia					Algeria				Turkey		
						Average				Average			Average
3′UTR +1565 to +1577 (−13 bp)	€											0.1	0.1
25 bp deletion	β^0												
290 bp deletion	β^0											0.1	0.1
7.6 Kb deletion											×		×
12 Kb deletion	β^0										×		×
30 Kb deletion											×		×
Poly A, (AATAAA → AATAAG)	β^+										×		×
Poly A, (AATAAA → AACAAA)	β^+										×		×
Poly A, (AATAAA → AATGAA)	β^+											0.5	0.5
Total number of chromosomes		**68**	**44**	**121**	**80**	**313**	**62**	**172**	**239**	**473**		**683**	**683**
References		**[18]**	**[19]**	**[28]**	**PC**		**[44]**	**[45]**	**[46]**		**[48]**	**[47]**	

PC: Chouk et al., personal communication (2002).
×: Mutations cited in Ref. [49]; no frequencies were indicated by the authors.

fact, Tunisia had constituted since the 16th century and over three centuries, one of the Ottoman Empire provinces. We note the same eastern origin for the HbO Arab variant [16,39], which could have been introduced in Tunisia with Arab groups.

The RFLP method constitutes a powerful tool either for biomedical research or for the comprehension of the structure and the origin of human populations. In Tunisia, the RFLP technique was first used to analyse the polymorphisms of the immunoglobulin gene [52]. Among the haplotypes found in Tunisians, some are observed in the European populations while others, absent in Europeans, are encountered only in the sub-Saharan African populations. This confirms the fact that Maghrebine ethnic groups are genetically localized between the European and the African populations. Haplotype analyses are also used in tracing back the origin of mutations. Similar to the results deduced from combined data from reports on β-thalassaemia in Tunisia [18,19,28], the non-sense codon 39 (C → T) and IVS-I-110 (G → A) are the two most common alleles encountered in the 193 patients analysed in the framework of these study, since they exist in 56 (29%) and 33 (17.1%) individuals, respectively.

RFLP polymorphisms analyses [18] have demonstrated that the codon 39 mutation is associated with six haplotypes in Tunisia: I, II, A, 5a, Nc, and Nd (Table 5a), while only one haplotype is found to be associated with this mutation in the eastern Mediterranean [53]. On the contrary, the IVS-I-110 mutation is associated to a single RFLP haplotype in Tunisia: I (Table 5a) and to two haplotypes in Lebanon [54]. The above data are consistent with the hypothesis of the old age of the stop codon 39 mutation in the Maghreb and a more recent introduction of the IVS-I-110 mutation in this region.

Molecular analyses of haemoglobinopathies have contributed not only to the elucidation of the population genetics of Tunisians, but also to the development of new methods for the prevention of these diseases. Although there are excellent treatment modalities today, such as hypertransfusion therapy and administration of iron chelating agents, these practices are usually expensive. Hence, emphasis has to be given to prevention programs such as carrier screening and prenatal diagnosis. In our laboratory, we have implemented the DGGE method and the ARMS PCR technique for the prenatal diagnosis of haemoglobinopathies. These PCR-based methods are reliable, rapid, have a reasonable cost (using psoralen as

Table 5a
Tunisian β-globin gene RFLP and sequence haplotype data: RFLP haplotypes observed in Tunisian chromosomes carrying β-thalassaemia mutations according to Ben Chibani et al. [18]

	HcII	HdIII	HdIII	HcII	HcII	AvaII	BamHI	Chr Nb
I	+	−	−	−	−	+	+	2
II	−	+	+	−	+	+	+	6
A	−	−	−	−	−	+	+	1
5a	−	+	+	−	−	+	+	2
Nc	+	+	+	−	−	+	+	1
Nd	−	−	−	−	+	+	+	2

chemical clamps), and do not require the use of radioisotopes. These advantages make these techniques more adapted to our economic and working conditions and to the molecular heterogeneity of the Tunisian β-thalassaemia. Then, prenatal diagnosis is feasible when early methods of foetal sampling are combined with DGGE and the amplification refractory mutation system. In fact, this constitutes actually the main option for couples at risk, especially for economically disadvantaged families. In addition to the high incidence of thalassaemia, Tunisia shows an elevated rate of consanguineous marriages within specific regions (32%) [11]. Consequently, the expected number of infants born annually with β-thalassaemia has been estimated to be around 50 [28]. A high proportion of pregnant women should seek prenatal diagnosis each year. This indicates the urgent need for implementing a comprehensive genetic preventive program including community education and informed genetic counselling.

3.2.2. Sickle cell disease

Sickle cell disease is a common hereditary anaemia. It is characterized by the presence of HbS in the blood cells of the patient. The highest gene frequencies are found in Equatorial Africa where it can exceed 20%. Elsewhere, and especially on the coast of North Africa, it occurs generally at a frequency of less than 5%. Worldwide, the β^S gene responsible for SCD has been found to be associated with five different haplotypes namely Benin, Bantu, Senegal, Cameroun, and Arab–Indian named after the region or ethnic groups in which the designated β^S was common [29,55]. In Tunisia, previous studies [20,32] using RFLP haplotype analysis have shown that the HbS mutation is generally associated with the Benin haplotype (− − − − + − + − +; Table 5b). In the sample of Abbes, an atypical haplotype was described in two patients. The clinical features of this disease are marked by a large variability among affected persons in the extent of anaemia, the amount of Hb F and other haematological characteristics (Table 2). In our sample, three patients have high values of HbF (15.5%, 27% and 28%). Even if the average value is in concert with the data of Öner et al. [33] and Schroeder et al. [56], these high values are very intriguing. Analysis of the sequence haplotype polymorphism of all the HbS alleles reveals that all (including the above patients) but two are found to carry the typical Benin haplotype (haplotype #19): AGATTC(8-4) (Table 5b). This allele could have been introduced in Tunisia by trans-Saharian population migrations and selected by malaria in the NW of Tunisia, which represents the major location of sickle cell disease [16]. The atypical haplotype; GCATTC (8-4) (Table 5b), found in two of our patients may be the result of a recombination event that occurred between the Benin haplotype and one of the wild-type chromosomes in a heterozygote sample: GCTTTC(8-4). These results confirm the unicentric origin of the HbS allele in Tunisia and its arrival from the west of Africa.

Table 5b
Tunisian β-globin gene RFLP and sequence haplotype data: RFLP and sequence haplotypes observed in Tunisian chromosomes carrying sickle cell disease. Sum up of previously published data from references [20] and [32] and the present study

	RFLP Haplotype									Sequence Haplotype							Reference	Chrom. Nb
	XmnI	HdIII	HdIII	HcII	HcII	HfI	AvaII	HpaI	BamHI	− 1069	− 989	− 780	− 703	− 551	− 491	(AT)xTy		
Benin										A	G	A	T	T	C	8-4	Present study	75
	−	−	−	−	+	−	+	−	+								[20]	31
	−	−	−	−	+	−	+	−	+								[32]	26
																	Total	**132**
Atypical										G	C	A	T	T	C	8-4	Present study	2
	+	−	−	−	−	+	+	+	+								[20]	2
	+	−	−	−	−	+	+	+	+								[32]	0
																	Total	**4**

3.2.3. α-Thalassaemia

Limited research has been done on α-thalassaemia in Tunisia. The only study [20] shows that four patients among the Benin β^S/β^S group (33 patients) carry a deletional α-thal ($-\alpha/\alpha\alpha$). In Mediterranean populations, α^+-thalassaemia is prevalent and usually results from the 3.7-kb rightward deletion called ($-\alpha^{3.7}$) haplotype, especially in Algeria [57]. That is probably the case for the α-thalassaemia described in the Abbes study. No more information is available except the reports of one case [21] and then three cases [22] of Hb H. A more exhaustive survey should be conducted on Tunisian α-thalassaemias even if the incidence of this defect seems to be lower than in Algerians (4.26% versus 9%) [16,57]. In the study of Fattoum and Abbes, the 28 cases of α-thal trait, found over a total number of 656 carrying haemoglobin abnormalities, have been documented by biosynthetic studies after measuring the serum iron. Our small survey on some patients with haematological abnormalities has been conducted using the PCR multiplex technique [58]: no new case has been detected. Some intriguing clinical and haematological data need an exhaustive survey on common and uncommon α-thalassaemias.

4. Conclusion

In conclusion, the exploration of the β-globin gene, since the 1980s (by RFLP haplotypes) and until the present (by sequence haplotypes), has led to the identification of several mutations and Hb variants. Subsequently, a better understanding of gene recombination mechanisms and mutation diffusion patterns was obtained. Globin genes can be used as genetic markers to describe the high genetic heterogeneity in the Tunisian population. The identification of a large battery of mutations and the possibility of their early detection represent a cornerstone for the establishment of a β-thalassaemia prevention program in Tunisia based on prenatal diagnosis. On the contrary, little is known about α-thalassaemia in Tunisia and detailed investigations in the α-globin gene should be conducted. The results of such studies will surely shed light on the correlation between the α-globin gene and β-thalassaemia phenotype. Ultimately, these will complement the available knowledge and constitute important tools for prenatal diagnosis of both α and β-thalassaemia in Tunisia.

List of abbreviations

ARMS	amplification refractory mutation system
ASO	allele specific oligonucleotide
CBC	complete blood count
CHU	Complexe Hospitalier Universitaire
DGGE	denaturing gradient gel electrophoresis
EDTA	ethylene diamine tetraacetic acid
Hb	Haemoglobin
HbS	sickle cell haemoglobin
IEF	Isoelectrofocalisation
IVS	intervening site
MCV	mean corpuscular volume
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
SCD	sickle cell disease
SNP	single nucleotide polymorphism

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