Hemoglobin, 34(4):333–342, (2010)

ISSN: 0363-0269 print/1532-432X online
DOI: 10.3109/03630269.2010.486354

informa
healthcare

ORIGINAL ARTICLE

REGION-SPECIFIC GENETIC HETEROGENEITY OF *HBB* MUTATION DISTRIBUTION IN SOUTH-WESTERN GREECE

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□ *β-Thalassemia (β-thal), is caused by reduced or absent synthesis of β-globin chains resulting in impaired erythropoiesis. It is the most common single gene defect disease in Greece, with heterozygous rates reaching, on average, 8% in the general population. Here, we performed molecular analyses on 199 unrelated β-thal and compound β-thal/sickle cell disease patients, of whom 157 originated from three prefectures of South-Western Greece, namely Achaia, Ilia and Etoloakarnania.*

Our results indicate that the frequency of specific HBB *gene mutations, namely the* HBB*:c.118C>T (codon 39, C>T),* HBB*:c.92+6T>C (IVS-I-6, T>C), and HBB:c.20A>T [Hb S, β6(A3)Glu→Val, GAG>GTG], present distinct distribution patterns in the Achaia and Ilia prefectures* ($p < 0.001$, $p < 0.003$ *and* $p < 0.002$*, respectively). This detailed analysis of the distribution of the HBB gene mutations is useful for genetic counseling in the region, and illustrates that the identification of the HBB gene mutation spectrum in this region is necessary for population carrier screening and for efficient provision of prenatal diagnosis.*

Keywords β-Thalassemia (β-thal), Mutation frequencies, Greek population, Globin genes

Received 6 February 2010; Accepted 21 February 2010.

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INTRODUCTION

Hemoglobinopathies are among the most common genetic diseases worldwide with 4.83% of world populations being carriers of globin variants. Also, 1.67% of the world's population are carriers of a thalassemia mutation and 1.92% are carriers of the mutation leading to sickle cell disease (1,2). The thalassemias are a group of devastating, autosomal recessive, hematological disorders that affect hemoglobin (Hb) synthesis, characterized by the absence or reduction of one or more globin chains having a direct effect in the resulting amounts of adult Hb A. The thalassemia syndromes are characterized by dyserythropoiesis, transfusion dependence and iron overload. A *HBB* genetic defect in homozygosity (or compound heterozygosity) leads to pronounced anemia, poor growth, bone deformities, hepatomegaly and splenomegaly (3). Current treatment for these patients consists of regular, life-long blood transfusions combined with iron chelation, transplantation of healthy bone marrow, or fetal Hb (Hb F) pharmacological reactivation (4–6). Also, a point mutation at codon 6 of the *HBB* gene (c.20A>T) [Hb S, β6(A3)Glu→Val, G*A*G>G*T*G], leads to sickle cell disease and results in Hb S production that changes the shape of the erythrocytes and leads, most of the time, to painful vascular occlusion episodes and organ infarctions.

β-Thalassemia affects populations in the Mediterranean Basin, Africa, Middle East and Southeast Asia, while sickle cell disease affects mostly the Black populations (2). Due to migration, patients suffering from these diseases are present in almost every country in the world and their incidence peaks in the Mediterranean, African and Southeast Asian countries (3). Approximately 80 million people worldwide are carriers of β-thal trait. Over 200 point mutations and short insertion/deletions are responsible for β-thal (7,8). This heterogeneity at the molecular level of the disease reflects, also, to the high heterogeneity to the clinical level of the disease, that presents a range of severe-to-moderate and rarely to mild clinical phenotypes, from β-thal major (β-TM) to β-thal intermedia (β-TI), (9,10). Sickle cell disease also presents a range of severe-to-mild clinical phenotypes.

In Greece, the β-thal syndromes present at a rather high frequency, with 8% average frequency of heterozygotes. For that purpose, national screening programs have been initiated, aiming at reducing the overall number of affected neonates. The determination of *HBB* allele frequencies allows for optimized provision of prenatal diagnosis and genetic services in this area. The *HBB* gene mutation frequencies have been previously characterized for the Hellenic population (11–13). These studies have calculated the overall *HBB* gene mutation spectrum as it is recorded in the Hellenic National Genetic database (14). Few studies, however, that have followed a region-specific approach, such as in Central (15) and North-Western Greece (16),

demonstrated that the allelic frequencies of certain *HBB* gene mutations can vary significantly compared to the *HBB* gene frequencies, that are deposited in the Hellenic National Genetic database.

In this paper, we determined the *HBB* gene mutation frequencies in each of the three big prefectures, Achaia, Ilia and Etoloakarnania in South-Western Greece. Our data indicate, that, overall, there is no substantial deviation from the mutation distribution pattern of mutation frequencies registered in the Hellenic National Genetic database. However, statistically significant differences exist among Achaia and Ilia prefectures, when compared to each other, regarding the *HBB*:c118C>T and *HBB*:c.92+1G>A (IVS-I-1, G>A), mutations leading to β-thal ($p < 0.001$ and $p < 0.003$, respectively) and the *HBB*:c.20A>T, leading to sickle cell disease ($p < 0.002$). These data underline the need to comprehensively and systematically document the frequencies of the mutant alleles in specific regions of the country, to facilitate screening programs and downstream healthcare initiatives.

MATERIALS AND METHODS

Patient Recruitment and Hematological Studies

Blood samples from 199 patients (men 47.7%, women: 52.3%), of Hellenic descent, mainly originating from three major prefectures of South-Western Greece (Achaia, Ilia and Etoloacarnania), were collected at the Patras University General Hospital, and analyzed at the Molecular Genetics Unit, Patras, Greece. Written informed consent was given by all participants. Hematological indices were obtained with an automated cell counter (Abbott Cell-DYN3700; Abbott Laboratories, Abbott Park, IL, USA). Hb A_2 and Hb F levels were measured by high performance liquid chromatography (HPLC) (VARIANT^{TM}, Bio-Rad Laboratories, Hercules, CA, USA).

DNA Studies

Genomic DNA was isolated from peripheral blood using the QIAamp DNA Blood Midi kit (Qiagen, Hilden, Germany). The *HBB* gene amplification conditions and primer sequences are available upon request. *HBB* gene mutation screening was done by real-time polymerase chain reaction (PCR) (Light Cycler^{TM} 2.0; Roche, Basel, Switzerland) using the QuantiFast Probe PCR (Qiagen) and specific primers and probes for each of the known *HBB* gene mutations in the Hellenic population, as described (17). Where necessary, DNA resequencing was performed to ascertain the presence of rare *HBB* gene mutations. DNA sequence alignment was carried out by the use of BLAST engine for local alignment (18). *HBD* gene mutation and δβ-thal screening was done as previously described (19).

Statistical Analysis

The statistical evaluation of the resulting *HBB* gene mutation frequencies in South-Western Greece, as well as their comparison with *HBB* gene mutation frequencies, documented in the Hellenic National Genetic Database was performed using SPSS 16.0. Only chromosomes from individuals of Hellenic descent born in any of the three regions were included in the statistical analysis. Also, a mapping of the five most common mutations was performed using GIS technology (ArcMap 9.2, USA).

RESULTS

In this study, a total of 199 β-thal patients were analyzed at the molecular level, 157 of whom originated from the three prefectures, Achaia, Ilia and Etoloakarnania, in South-Western Greece. The remaining patients were excluded because their ethnic ancestry was either unknown (23 patients) or different (19 patients). Furthermore, in 14 chromosomes from the 157 patients analyzed, the underlying HBB gene mutation was not found (Table 1).

Fourteen *HBB* gene mutations were identified, which led to either β^0 or to β^+ phenotypes. The frequencies of *HBB* gene mutations were calculated cumulatively for South-Western Greece and separately for each one of the three prefectures, and compared with the mutation frequencies documented in the Hellenic National Genetic Database (Figure 1). Our data indicate that the overall distribution of *HBB* gene mutation frequencies is in agreement with those registered in the Hellenic National Genetic database, except the *HBB*:c.20A>T mutation, leading to sickle cell disease (Figure 1, Table 1). However, more differences were observed, when each of the three aforementioned prefectures was separately compared to each other, or to the data recorded in the Hellenic National Genetic Database (Table 1). In particular, the distribution of specific *HBB* gene mutations, namely *HBB*:c.118C>T (codon 39, C>T), *HBB*:c.92+6T>C (IVS-I-6, T>C) and *HBB*:c.20A>T, present statistically significant differences in Achaia and Ilia prefectures ($p = 0.001$, $p = 0.003$ and $p = 0.002$ respectively). Also, the *HBB*:c.118C>T mutation presents similar frequency to the one found in the Achaia prefecture (Figure 2).

Additionally, comparison between the frequencies of *HBB* gene mutations in prefectures of South-Western Greece and the Hellenic National Genetic Database frequencies by correlation analysis depicts a statistically significant difference ($p < 0.05$) between the frequencies of common *HBB* gene mutations in Etoloakarnania and those deposited at the Hellenic National Genetic database (Table 2).

Notably, differences were also observed in frequencies of the other common *HBB* gene mutations. In particular, the *HBB*:c.93-21G>A (IVS-I-110, G>A), the most common *HBB* gene mutation in Greece, is the most

TABLE 1 Percentages of *HBB* Gene Mutations in Three Prefectures of South-Western Greece and Their Comparison with the Hellenic National Genetic Database (*: Calculated on the Basis of 975 Alleles Bearing a Mutant *HBB* Gene)

Genetic defects *HBB* gene mutations		Achaia		Etoloakarnania		Ilia		Greece
Conventional nomenclature	HGVS nomenclature	Number of chromosomes	%	Number of chromosomes	%	Number of chromosomes	%	%
IVS-I-110, G>A	c.93-21G>A	62	34.07	3	9.38	34	34	42.97
Codon 39, C>T	c.118C>T	43	23.63	8	25	10	10	19.51
IVS-I-1, G>A	c.92+1G>A	15	8.24	5	15.63	13	14	13.65
IVS-I-6, T>C	c.92+6T>C	28	15.38	4	12.5	2	2	8.19
IVS-II-745, C>G	c316-106C>G	6	3.3	4	12.5	3	3	4.35
FSC[a] 6, –A	c.20delA	1	0.55	0	0	4	4	2.83
FSC[a] 8, –AA	c.25_25delAA	6	3.3	0	0	2	2	0.1
IVS-II-1, G>A	c.315+1G>A	6	3.3	1	3.13	2	2	0.91
FSC[a] 5, (–CT)	c.17_18delCT	1	0.55	0	0	0	0	0.61
IVS-I-5, G>A	c.92+5G>A	0	0	1	3.13	1	1	0.3
–101, C>T	c.-151C>T	1	0.55	0	0	0	0	0.3
5'UTR +10, –T	c.-41delT	1	0.55	0	0	0	0	NA
Codon 6, A>T (Hb S)[b]	c.20A>T	8	4.4	4	12.5	18	18	2.83
Sicilian δβ-thal	NG_0000073:g.64336 _77738del13403	0	0	0	0	1	1	NA
+1480, C>G	HBB:c.*+6C>G	1	0.55	0	0	0	0	NA
Not characterized	–	3	1.65	2	6.25	9	9	NA
Total	–	182	100	32	100	100	100	100

5'UTR: 5' untranslated region; NA: not available.
[a]FSC: frameshift codon.
[b]Hb S [β6(A3)Glu→Val, G*A*G>G*T*G].

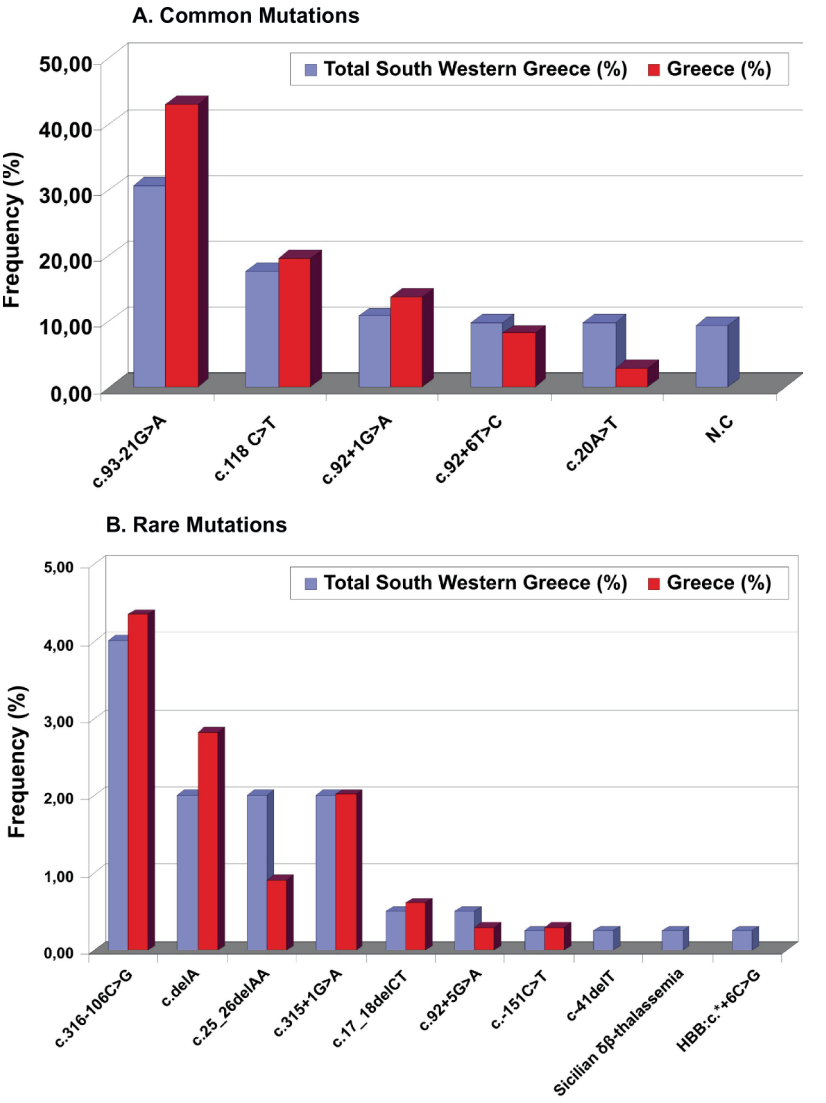

FIGURE 1 Frequencies of common (A) and rare (B) *HBB* gene mutations in South-Western Greece compared with those of the Hellenic National Genetic database (14). N.C.: not characterized. Sicilian δβ-thal: NG_000007.3:g.64336_77738del13403.

frequent *HBB* gene mutation in Achaia and Ilia but not in Etoloakarnania, accounting for only 9.38% of the β-thal chromosomes. Also, the *HBB*:c.92+1G>A anomaly accounts for 15.63% of the chromosomes in Ilia and in Etoloakarnania but only for 8.24% in the Achaia prefecture. However, these differences were not found to be statistically significant in this patient group. The regional distribution of the five most common *HBB*

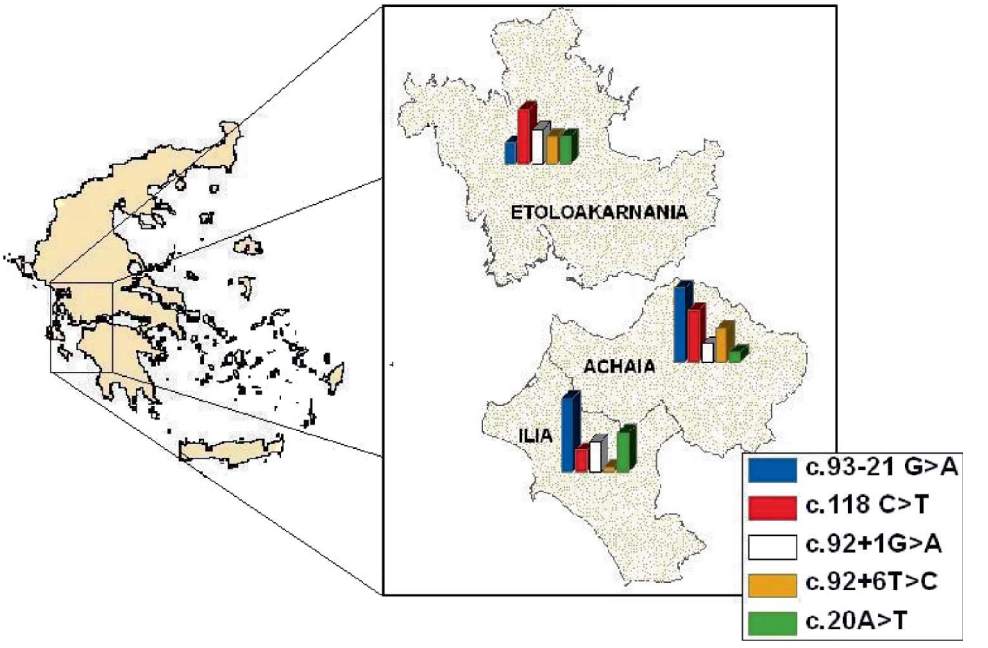

FIGURE 2 Distribution of the five most common *HBB* gene mutation frequencies in the three prefectures of South-Western Greece involved in the study.

TABLE 2 Paired Sample Correlations

Prefecture in Greece	Standard deviation	Standard error mean	Correlation	Significance
Achaia	4.170306	1.203863	0.945	0
Etoloakarnania	11.110259	3.207256	0.483	0.112
Ilia	6.31608	1.8233	0.864	0

gene mutations in the three prefectures is presented in Figure 2, while a detailed analysis of the distribution of the frequencies of each of the 14 β-globin gene mutations in each prefecture is given in Table 1. A heterozygous individual for the Sicilian δβ-thal was also identified.

DISCUSSION

β-Thalassemia is common in the Hellenic population as in many other Mediterranean countries, resulting from over 200 point mutations and short insertion/deletions (8). These mutations result in reduced or absent synthesis of β-globin chains, causing pronounced anemia and leading to an excess of α-globin chains, which precipitate as inclusion bodies in the red blood cells of the patients. The β-globin chain(s) production of the affected

β-globin genes can range from near normal to completely absent, reflecting the wide spectrum of heterogeneity of the disease (7,9,10).

In countries where β-thal occurs at high frequencies, the populations seem to carry a few common mutations that are characteristic for a particular region of the country, together with some rare ones. In general, the mutation spectrum of certain genetic diseases varies within populations, not necessarily among ethnic groups that reside in different countries but also in different parts of the same country (e.g., Israel) (20), even in populations that are considered to be rather homogeneous. In Greece, previous experimental evidence suggests that the frequency of certain *HBB* mutant alleles in Central (15) and North-Western Greece (16) is different, compared to those recorded in the Hellenic National Genetic database (14). This information can be particularly useful for prenatal and postnatal diagnosis at the molecular level and for carrier screening programs.

In the present study, the β-globin gene mutations were determined, as well as their distribution in the three prefectures of South-Western Greece (Achaia, Ilia and Etoloakarnania). Overall, there is no substantial deviation from the mutation distribution pattern of mutation frequencies registered in the Hellenic National Genetic database, with the exception of the *HBB*:c.20A>T mutation (Figure 1). However, statistically significant differences exist in the Achaia and Ilia prefectures, when compared to each other, regarding the *HBB*:c.118C>T and *HBB*:c.92+1G>A mutations ($p < 0.001$ and $p < 0.003$, respectively). Interestingly, the *HBB*:c.118C>T is the most frequent *HBB* gene mutation in Italy and its higher frequency in Achaia might be attributed to population movement, and in particular of the Italian revolutionaries who took refuge in Patras at the end of the 19th century. At the same time, the port of Patras was a major commercial center of Greece, also with close commercial ties to Etoloakarnania's port (http://www.e-patras.gr/portal/web/common/130). This fact might also explain the high frequency of the *HBB*:c.118C>T mutation in this region (Figure 2).

Regarding the genotype of the sickle cell disease patients originating from South-Western Greece, they are all compound heterozygotes for β-thal and sickle cell disease. Our findings indicate that the sickle cell mutation (*HBB*:c.20A>T) in Ilia is more frequent that in Achaia ($p < 0.002$), although the two prefectures are adjacent to each other, and no geographical barriers exist between them (Figure 2). This finding may be explained by the fact that in past decades, when endemic malaria was high, people in Ilia were farmers and they were living in rather isolated communities, compared to the Achaia prefecture, where genetic isolation was less profound (http://www.e-patras.gr/portal/web/common/130).

Region-specific differences in *HBB* gene mutation distribution was also observed in all prefectures. However, these differences were not found to be statistically significant, most likely due to the limited number of mutant

HBB alleles that were analyzed from the Etoloakarnania prefecture. The *HBB*:c.93-21G>A mutation was the most frequent in South-Western Greece, following the nation-wide frequency pattern, although it shows a significant decrease by approximately 11%, particularly in Etoloakarnania (9.38%; Figure 2). Notably, the *HBB*:c.25_26delAA [frameshift codon (FSC) 8, –AA] mutation, third in frequency in Turkey (21), is over represented in the Achaia (3.30%) and Ilia (2.0%) prefectures, compared to the frequencies reported for the country as a whole (0.1%). Also, δβ-thal presents with substantially low frequencies in South-Western Greece, where only one Sicilian δβ-thal chromosome was detected. This finding contrasts with the high prevalence of δβ-thal in other regions, such as Central and North-Western Greece, where the Sicilian and Turkish type of δβ-thal occur at rather high frequencies (15).

We could not identify the underlying *HBB* gene mutation in 14 alleles (4.46%). The fact that the entire coding and regulatory region of the *HBB* gene was fully resequenced suggests that the β-thal causing genetic defect most likely lies outside the human β-globin gene cluster. Mutations in genes unrelated to the human β-globin gene cluster but yielding a β-thal-like phenotype have been described in the past, the most characteristic of those being located on the *XPD* gene (22).

Overall, our data indicate that there are statistically significant differences in the distribution patterns of three common *HBB* gene mutations in the Hellenic population, namely *HBB*:c.118C>T, *HBB*:c.92+6T>C and *HBB*:c.20A>T, between the Achaia and Ilia prefectures in South-Western Greece, although the overall *HBB* mutation frequency spectrum in South-Western Greece is similar to the nation-wide distribution pattern. This, hitherto undescribed β-thal situation dictates precise identification of the genetic heterogeneity in a region-specific manner for improving public health services, i.e., population carrier screening, prenatal and postnatal diagnosis and genetic counseling not only for the β type hemoglobinopathies but also for other inherited disorders.

ACKNOWLEDGMENTS

This study was partially funded by the European Commission and by a Research Promotion Foundation grant ΠΔΕ46_002 (to GPP).

Declaration of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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