Hb A2 Episkopi – a novel δ-globin chain variant [HBD:c.428C>T] in a family of mixed Cypriot–Lebanese descent

Carsten W. Lederer, Eleni Pavlou, George A. Tanteles, Paola Evangelidou, Carolina Sismani, Annita Kolnagoud, Maria Sitarou, Soteroulla Christou, Michael Hadjigavriel and Marina Kleanthous

Department of Molecular Genetics Thalassaemia, The Cyprus Institute of Neurology and Genetics, Cyprus & Cyprus School of Molecular Medicine, Nicosia, Cyprus; Thalassaemia Centre, Ministry of Health, Cyprus.

ABSTRACT

Objective: Thalassaemia is a potentially lethal inherited anaemia, caused by reduced or absent synthesis of globin chains. Measurement of the minor adult haemoglobin Hb A2, combining α- with δ-globin, is critical for the routine diagnosis of carrier status for α- or β-thalassaemia. Here, we aim to characterize a novel δ-globin variant, Hb A2 Episkopi, in a single family of mixed Lebanese and Cypriot ancestry with mild hypochromic anaemia and otherwise normal globin genotype, which also presents with a coincidental 0.78-Mb sequence duplication on chromosome 1 (1q44) and developmental abnormalities.

Methods: Analyses included comprehensive haematological analyses, cation-exchange high-performance liquid chromatography (CE-HPLC), cellulose acetate electrophoresis (CAE), Sanger sequencing and structure-based stability predictions for Hb A2 Episkopi.

Results: The GCT > GTT missense mutation, underlying Hb A2 Episkopi, HBD:c.428C>T, introduces a cd142 codon change in the mature protein, resulting in reduced normal Hb A2 amounts and a novel, less abundant Hb A2 variant (HGVS: HBD:p.A143V), detectable as a delayed peak by CE-HPLC. The latter was in line with structure-based stability predictions, which indicated that the substitution of a marginal, non-helical and non-interface residue, five amino acids from the δ-globin chain carboxy-terminus, was moderately destabilizing.

Discussion: Detection of the new variant depends on the diagnostic set-up and had failed by CAE and on an independent CE-HPLC system, which, in unfavourable circumstances, may lead to misdiagnoses of β-thalassaemia as α-thalassaemia. Given the mixed background of the affected family, the ethnic origin of the mutation is unclear, and this study thus suggests awareness for possible detection of Hb A2 Episkopi in both the Cypriot and the Lebanese populations.

Introduction

The potentially lethal monogenic anaemia β-thalassaemia requires lifelong and costly disease management [1]; therefore, national screening and prevention programmes are crucial to countries with a high proportion of disease carriers. The latter present haematologically with characteristically low mean cell volume and, through depletion of β-globin and thus the main adult haemoglobin Hb A (α2β2), show an increase in the minor adult haemoglobin Hb A2 (α2δ2) from 2.5–3.0% to above 3.5% of total haemoglobin [2]. In consequence, δ-globin expression is central to the haematological diagnosis of thalassaemias. This is exemplified by the situation in which double heterozygotes for δ- and β-thalassaemia present with an atypical phenotype characterized by microcytosis and hypochromia, but with normal Hb A2, similar to certain α-thalassaemia heterozygotes. In countries such as Cyprus, where the occurrence of α-, β- and δ-thalassaemia mutations is common [3], the presence of δ-thalassaemia masks the typical β-thalassaemia phenotype and thus interferes with population screening for β-thalassaemia carriers [4]. Cases with borderline or abnormal haematological findings are, therefore, routinely undergoing molecular analysis of the δ-globin gene as well. This allows a definitive diagnosis and safeguards disease prevention and national disease control for thalassaemia on the one hand, while on the other hand continuously accumulating and updating a comprehensive catalogue of mutations in the respective population [5]. Here, we report a novel δ-globin gene variant, Hb A2[δA142V] or Hb A2 Episkopi, unresolved by cellulose acetate electrophoresis (CAE) but detectable by cation-exchange high-performance liquid chromatography (CE-HPLC), presenting in a normal genetic background of a family with mixed Lebanese–Cypriot descent.

CONTACT Carsten W. Lederer lederer@cing.ac.cy

Supplemental data for this article can be accessed at http://dx.doi.org/10.1080/10245332.2016.1265043.

© 2016 Informa UK Limited, trading as Taylor & Francis Group
Materials and methods

Subjects

Study subjects were analyzed between 2011 and 2013 as part of the national thalassaemia prevention programme of the Republic of Cyprus and have given full informed consent for their inclusion in this study.

Haematological analyses

Haematological studies were performed using routine methods. Haemoglobin analysis and detection of Hb A2 Episkopi were achieved by CE-HPLC on the β-thalassaemia VARIANT™ program (Bio-Rad Life Sciences), while employment of an alternative HPLC system (Shimadzu) (see online supplementary material Fig. S1) and of standard CAE procedures and equipment (Helena Laboratories) had failed to do so. Additional post-analysis HPLC peak quantification was performed using Fiji ImageJ 1.49 [6].

Molecular analyses

Nucleotide and amino-acid numbers are given with RefSeq NG_000007 as reference for genomic DNA positions and NM_000519.3 as reference for human HBD cDNA and predicted amino-acid sequences. By convention in the haemoglobin field and contrary to guidelines of the Human Genome Variation Society (HGVS), amino-acid references do not count the initiating methionine, absent in the mature protein, and therefore differ by −1 from corresponding HGVS position references.

In addition to the analyses in the δ-globin gene HBD, the specific study subjects were tested for −a3.7, aα, aα−mt, aAg, δαPolyA1α and δαPolyA2α mutations of the α-globin locus [7] and for any mutations in the β-globin locus between 288 nt upstream of the HBB 5′ end codon and 240 nt downstream of the HBB stop codon [3]. Mutations in HBD were detected by sequencing of two overlapping PCR fragments. The first fragment of 1161 bp was amplified with primers δF1: 5′-TACATCCACTATATTAGC-3′ and δ6R: 5′-CAG- TATTCTATGGCCTCTCAT-3′ and spanned the 5′ UTR, exon 1 and exon 2, extending from −328 to +814 relative to the HBD start codon. The second fragment of 947 bp, amplified with primers δ5F: 5′-TGCTACA- CAGCTCTCCAGT-3′ and δ8R: 5′-CAGGAACCTTCTTA- CACACC-3′, extended from +707 to +1653 relative to the HBD start codon. Sequencing reactions (Invitrogen, BigDye Terminator v. 1.1 cycle sequencing kit) used primers δ1F, δ15S (5′-AACCAACCTGCTCAGGAG-3′) and δ17S (5′-ATTATTGCTGATGGGAAATAAC-3′) and were analyzed on a 3130xl Genetic Analyzer (Applied Biosystems Hitachi). Hb Episkopi has been deposited in the IthaGenes database with IthaiID 2560 (www.ithanet.eu) [8].

Bioinformatics and statistical analyses

In silico protein-structure and stability analyses were based on RCSB PDB entry 1si4 of the relaxed (R2) state of normal Hb A2 [9]. Modelling of normal and variant Hb A2 was performed by FoldX3.0 in command-line mode [10] for the creation and energy-optimization of variants, and using YASARA [11] and the POV-Ray rendering tool (www povray.org) for rendering of images. Structure-dependent comparative energy calculations for tetramers by FoldX assumed default conditions, including a temperature of 37°C and a pH of 7, and used five iterations to calculate averages and standard deviations of energy differences (kcal mol⁻¹) between variant and normal Hb A2. The significance of changes in Hb A2 was assessed for each test case against a reference population of individuals matched for gender and α- and β-globin genotype, using the Microsoft Excel NORM.DIST function. The isoelectric point (pI) for all δ-globin variants and pI differences for variants compared to normal δ-globin are based on the protein pI estimate of the Isoelectric Point Calculator (IPC) [12].

Results

An index case presenting with anatomical and haematological abnormalities

The index case (M1) from Episkopi of the Limassol district of Cyprus was first seen at the age of 1 month for evaluation regarding cryptorchidism and a U-shaped cleft palate. The proband had, in addition, deep-set, hypoplastic toe-nails, but otherwise his evaluation was unremarkable. Review of the family history revealed that the child was born to healthy, non-consanguineous parents of mixed Cypriot-Lebanese descent, with a family history non-contributory for clefting but contributory on the maternal side for cryptorchidism.

These findings indicated more comprehensive analyses, which revealed an apparently normal male karyotype, normal biochemistry, ophthalmology, cardiology and ultrasound scan of the abdomen, while array-CGH revealed a maternally inherited duplication of 0.78 Mb, on chromosome 1 on chromosomal band 1q44, location: 244,935,100-245,724,786 [build GRCh37 (hg19)] [arr 1q44(244,935,100-245,724,786)x3 mat], the clinical significance of which remains unclear. The father was unavailable for testing.

Subsequent analyses of blood smears and a duplicate complete blood count (CBC) for the index case at 10 months of age and for his mother (F1), aged 33, in both cases revealed mild hypochromic, microcytic anaemia. Mean cell volume (73.3 fl and 78.5 fl) and mean cell haemoglobin (24.6 pg and 26.7 pg) for M1 and F1, respectively, were slightly below the reference
Table 1. Study subjects.

| Subject | Place of residence | Zpp (mole haeme) | RBC (10^6/μl) | Hb (g/dl) | MCV (fl) | MCH (pg) | Hb A2 (CAE) (%) | α-globin gene | β-globin gene |
|---------|--------------------|-----------------|---------------|-----------|----------|-----------|----------------|----------------|---------------|
| M1      | Episkopi           | 74              | 4.4           | 11.1      | 72.7     | 25.0      | 2.6            | αα/αα         | N/N           |
| Reference |                    | <90             | 3.7-5.3       | 10.5-13.5 | 70-86    | 23-31     | 1.6-3.3        | αα/αα         | N/N           |
| F1      | Episkopi           | 102             | 4.84          | 12.9      | 78.5     | 26.7      | 1.9            | αα/αα         | N/N           |
| Reference |                    | <105            | 4.0-5.2       | 12.0-16.0 | 80-100   | 26-34     | 2.2-3.5        | αα/αα         | N/N           |

Two individuals from the Cypriot family of Cypriot-Lebanese ancestry are heterozygous for Hb A2 Episkopi. Reference ranges are normal ranges for children of 0.5-2 years and for adult women, respectively [20]. αα/αα, N/N – diagnosed homozygous normal according to haematological sample parameters, respectively, for the α-globin locus and for the β-globin locus after the detection assays specified in the section ‘Materials and methods’.

In agreement with literature references [20], Hb A2 levels for F1 are slightly but significantly below gender-matched control levels of the respective reference population in Cyprus (p = 0.033), in contrast to those for M1 (p = 0.495). The underlying cause is unknown.

range, while other CBC parameters and indices for iron deficiency (zinc protoporphyrin and RDW-CV) were in the normal range (Table 1).

CAE analysis showed reduced Hb A2 for F1 but normal levels for M1 (Table 1), each compared to the age-matched (and for the mother gender-matched) reference range. However, CE-HPLC analysis revealed a split peak for Hb A2, indicating a novel stable Hb A2 variant (with 87% the peak area of normal Hb A2) and prompting molecular analyses.

Confirmation of the novel δ-globin variant by sequence analysis

Comprehensive globin sequence analysis and gap-PCR [13] of the index case M1 revealed heterozygosity for a hitherto unreported δ-globin mutation (Figure 1(a)), in the absence of any of the other α- or β-globin mutations tested, with identical findings for F1. Mutations in the globin loci are, therefore, not responsible for the slightly abnormal or borderline haematology of M1 and F1. The novel δ-globin missense mutation introduces a codon change GCT > GTT (alanine to valine) at codon 143 of the HBD gene and (thus at amino acid 142 of the mature δ-globin protein). For the same nucleotide position, an alternative GCT > GAT mutation has been characterized before and brings about an amino acid change of alanine to aspartate, giving rise to HbA2 Fitzroy instead [14].

Predicted and observed consequences of the amino-acid substitution

HBD:c.428C > T is the mutation closest to the 3’ end of the δ-globin gene yet reported in Cyprus [15], positioning the affected amino-acid at the transition of the final α-helix of δ-globin towards the flexible carboxy-terminus. The predicted A > V amino-acid change from one non-polar, uncharged aliphatic side chain to another maintains the predicted isoelectric point (pI) of 7.42 for the resultant variant δ-globin (see Table 2) [12], in contrast to the HBD:c.428C > A mutation underlying HbA2 Fitzroy [14], which results in an A > D amino-acid change and a predicted pI of 6.87. This is in line with an inability to detect the new Hb A2[δA142V] variant by the charge-dependent method of CAE in the basic heterozygote state, necessitating detection of a split peak by CE-HPLC (Figure 1(b)) to avoid possible misdiagnosis, while the more acidic HbA2 Fitzroy is readily detectable by CAE [14]. The properties of both alternative same-residue variants are compared in Table 3. Of note, elevated oxygen affinity was inferred for Hb Fitzroy from the equivalent Hb A variant Hb Ohio (Hb A[βA142D]) [16], and we likewise infer for Hb Episkopi that it will have elevated oxygen affinity, as was shown for its paralogous β-globin variant Hb Waterland (Hb A[βA142V]) [17]. For the Hb A2 Episkopi heterotetramer, structure-based stability prediction gives only subtle changes in the predicted protein backbone of the heterotetramer but indicates a moderately destabilizing effect of the mutation (see Figure 1(c) and Table 2), which agrees with the observed steady-state abundance of the new variant being slightly below that of normal Hb A2 in the heterozygote state.

Discussion

Here, we have described and sequenced a novel δ-globin variant in a family of mixed Cypriot-Lebanese descent and named after the family’s place of residence, Hb A2 Episkopi. Experimental CE-HPLC data and stability predictions suggest that the variant is slightly destabilized compared to normal Hb A2 and is readily detectable as an additional peak (albeit without baseline separation) on suitable HPLC systems.

This description of Hb A2 Episkopi increases the heterogeneity of the δ-globin gene in Cyprus from 15 to 16 alleles [3], including 11 variants. Of routine laboratory methods, CE-HPLC is able to discern Hb A2[δA142V] from normal Hb A2 where a suitable CE-HPLC system is available, owing to the variant’s stability and its overall charge, while CAE fails to detect the variant. This is in contrast to HbA2 Fitzroy (Hb A2[δA142D]), which results from an alternative missense mutation of the same codon and is readily detectable by CAE [14]. Our observations once more emphasize the need to employ multiple analysis methods for the routine detection of haemoglobin variants, which also applies to environments without
access to HPLC equipment, where complementary electrophoresis methods might be used instead. For instance, the combination of methods detecting changes in pI or in charge at a given pH, such as isoelectric focussing (IEF) or CAE [18,19], could be combined with methods detecting changes in molecular weight, such as acid-urea-Trition X-100 polyacrylamide gel electrophoresis (AUT-PAGE) [19], to minimize false-negative variant detection and thus the risk of misdiagnosis of haemoglobinopathies.

**Figure 1.** Characterization of the Hb A2 Episkopi mutation (a) Sequencing revealed a GCT > GTT codon change (A143V, indicated by an arrow) for δ-globin underlying the observation of a novel variant. (b) CE-HPLC analysis reveals a split peak at the Hb A2 position (expected elution time 2.9 minutes), with a trailing peak (elution time 3.07 minutes) of comparable height to the normal Hb A2, indicating a variant of similar stability. The inlayed cake diagram shows the relative abundance of each Hb A2 species. (c) The known Hb A2 tetramer structure (PDB 1si4 [9], left panel) has been energy-optimized using FoldX to accommodate the A143V amino-acid change (right panel), which is located five amino acids from the carboxy-terminus of δ-globin. For a comparison of the most common Cypriot variants, see online supplementary material Fig. S2. The α- and δ-globin chains (α1, α2 and δ1, δ2) are displayed as flat ribbon diagrams in grey turquoise and yellow, respectively, with residue V142 in each mature δ-globin chain highlighted in mauve and displayed as a ball-and-stick diagram. Numbers indicate the δ-globin C and N termini and the variant amino-acid. Haeme groups are shown as stick diagrams in red.
Table 2. Stability of common Hb A2 variants detected in Cyprus.

| δ-globin variant | Codon change   | Common name        | HGVS protein name | ΔΔG     | ΔpI     | dbsNP reference | IthaGenes reference | Variant origin and reference |
|------------------|---------------|-------------------|-------------------|---------|---------|-----------------|----------------------|-----------------------------|
| Normal           | n/a           | n/a               | HBD               | 0       | 0       | n/a             | GeneID 9              | n/a                        |
| T45              | ACT > ATT     | CD4 (ACT > ATT)   | HBD.p.731         | 1.13 ± 0.53 | 0       | rs35406175      | 1331 Greek, Cypriot     |
| N198             | AAC > AAA     | Hb A2 Famagusta   | HBD.p.N20K        | −1.53 ± 0.07 | −0.41 | n/a             | 2299 Japanese, Cypriot  |
| G25D             | GGT > GAT     | Hb A2 Yokoshina   | HBD.p.G66D        | 8.13 ± 1.19  | −0.553   | rs34839944      | 1360 Greek, Cypriot     |
| A27S             | GCC > TCC     | Hb A2 Yialousa    | HBD.p.A285        | 1.40 ± 0.01  | 0       | rs35152987      | 1332 Greek, Cypriot     |
| V98M             | GTG > ATG     | Hb A2 Wrens       | HBD.p.V99M        | −0.94 ± 0.07 | 0       | rs28933076      | 1369 Black              |
| R116C            | CCG > TGC     | Hb A2 Troodos     | HBD.p.R117C       | 3.80 ± 0.03  | −0.635   | rs33971270      | 1384 Cypriot, Greek     |
| L141P            | CTG > CCG     | Hb A2 Pelendri    | HBD.p.L142P       | 17.33 ± 0.42 | 0       | rs33956485      | 1392 Cypriot, Greek     |
| A142V            | GCT > GTT     | Hb A2 Episkopi    | HBD.p.A143V       | 3.50 ± 0.38  | 0       | rs2560 Cypriot-Lebanese [this report] |

Predicted free energy changes (ΔΔG) for variant vs. normal Hb A2 tetramers were calculated using FoldX 3.0 and five modelling iterations per variant, with negative scores indicating a stabilizing mutation [21]. The change in isoelectric point (ΔpI) is based on predictions using the Isoelectric Point Calculator (IPC) [12].

Table 3. Comparison of properties for Hb A2 Episkopi and Hb A2 Fitzroy.

| Hb A2 | Hb A2 Episkopi (HBD.p.A143V) | Hb A2 Fitzroy (HBD.p.A143D) |
|-------|-----------------------------|-----------------------------|
| Ratio Hb A2/Hb A2 in heterozygotes | 0.87 (CE-HPLC) | 1 (CAE) |
| Paralogous Hb A (β-globin) variant | Hb Waterland | Hb Ohio |
| Inferred change in oxygen affinity | elevated | elevated |
| Codon change in variant δ-globin | GCT > GTT | GCT > GAT |
| pl of variant δ-globin [IPC] | 7.422 | 6.869[25] |
| pl difference compared to normal δ-globin [IPC] | 0 | −0.553 |
| Charge of variant δ-globin at pH 7.4 (cytosol) [IPC] | 0 | −1 |
| Molecular mass [Da] of variant δ-globin [IPC] | 16083.53 | 16099.49 |
| Mass difference (Da) compared to normal δ-globin [IPC] | +28.05 | +44.01 |
| dbSNP reference | n/a | rs35848600 |
| IthaGenes reference | 2560 | 1393 |
| Variant origin | Cypriot-Lebanese | Greek |

Known and predicted properties of the same-residue δ-globin variants Hb A2 Episkopi (HBD.p.A143V) and Hb A2 Fitzroy (HBD.p.A143D) are summarized as given in this study for the former and in the original publication for the latter [14], and as calculated by the Isoelectric Point Calculator (IPC) [12]. No experimental data exist for the oxygen affinity of either variant.

With this paper, we have established the detection and identity of the novel δ-globin variant Hb A2 Episkopi, which may lead to the targeted discovery of the same sequence variant in other, and in particular Lebanese, populations.

Acknowledgements

The authors thank all study subjects for their participation, the Population Thalassaemia Screening Laboratory of the Thalassaemia Centre of Cyprus for its services and Eleni Karitzi for technical assistance.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was, in part, funded by the European Union Seventh Framework Programme THALAMOSS project [Project ID: 306201].

Notes on contributors

Dr Carsten W. Lederer is a group leader in the Department of Molecular Genetics Thalassaemia at the Cyprus Institute of Neurology and Genetics and a Lecturer at the Cyprus School of Molecular Medicine, Nicosia, Cyprus. He is an expert in the field of haemoglobinopathies and in the gene therapy of the haematopoietic system.

Ms Eleni Pavlou is a laboratory scientific officer in the Department of Molecular Genetics Thalassaemia at the Cyprus Institute of Neurology and Genetics. She is an expert in the molecular diagnosis of thalassaemias.

Dr George A. Tanteles MD DM is Head of the Clinical Genetics Clinic and a Consultant Clinical Geneticist at the Cyprus Institute of Neurology and Genetics.

Dr Paola Evangelidou is a scientist in the Department of Cytogenetics and Genomics at the Cyprus Institute of Neurology and Genetics. She is an expert in the field of cytogenetic diagnosis.

Dr Carolina Sismani is Head of the Department of Cytogenetics and Genomics at the Cyprus Institute of Neurology and Genetics and Assistant Professor at the Cyprus School of Molecular Medicine, Nicosia, Cyprus. She is an expert in the field of cytogenetic diagnosis.

Dr Anna Kolnagou is a Consultant Physician, Head of the Thalassaemia Clinic Paphos, Cyprus.

Dr Maria Sitarou MD, MSc in Haemoglobinopathy, is Head of the Thalassaemia Clinic General Hospital Larnaca, Cyprus.

Dr Soteroulla Christou is Head of the Thalassaemia Clinic Nikosia, Cyprus.

Dr Michael Hadjiaviel is Head of the Thalassaemia Clinic Limassol, Cyprus. He is a specialist in internal medicine, and an associate of the course MSc in Haemoglobinopathy.

Dr Marina Kleanthous is Head of the Department of Molecular Genetics Thalassaemia at the Cyprus Institute of Neurology and Genetics and an Associate Professor at the Cyprus School of Molecular Medicine, Nicosia, Cyprus. She is an expert in the
field of haemoglobinopathies and of thalassaemia in particular.

**ORCID**

Carsten W. Lederer https://orcid.org/0000-0003-3920-9584

George A. Tanteles https://orcid.org/0000-0002-8117-9852

**References**

[1] Higgs DR, Engel JD, Stamatoyannopoulos G. Thalassaemia. Lancet [Internet]. 2011/09/13. 2011 Jan 28 [cited 2016 May 26];379(9813):373–383. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21908035

[2] Old JM. Screening and genetic diagnosis of haemoglobin disorders. Blood Rev. Scotland; 2003 Mar;17(1):43–53.

[3] Kountouris P, Kousiappa I, Papasavva T, et al. The molecular spectrum and distribution of haemoglobinopathies in Cyprus: a 20-year retrospective study. Sci Rep [Internet]. 2016 [cited 2016 May 26];6:26237. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27199182

[4] Trifillis P, Ioannou P, Schwartz E, et al. Identification of four novel delta-globin gene mutations in Greek Cypriots using polymerase chain reaction and automated fluorescence-based DNA sequence analysis. Blood [Internet]. 1991 Dec 15 [cited 2016 May 26];78(12):3298–3305. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1742490

[5] Mosca A, Paleari R, Ivaldi G, et al. The role of haemoglobin A(2) testing in the diagnosis of thalassaemias and related haemoglobinopathies. J Clin Pathol [Internet]. 2009 Jan [cited 2016 May 26];62(1):13–17. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19103851

[6] Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods [Internet]. 2012 Jul [cited 2016 May 28];9(7):671–675. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22930834

[7] Baysal E, Kleinhousen M, Bozkurt G, et al. Alpha-thalassemia in the population of Cyprus [Internet]. Vol. 89, Br J Haematol. 1995. p. 496–499. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=7734346

[8] Kountouris P, Lederer CW, Fanis P, et al. IthaGenes: an interactive database for haemoglobin variations and epidemiology. PLoS One. 2014;9(7) Available from: https://www.ncbi.nlm.nih.gov/pubmed/25058394

[9] Sen U, Dasgupta J, Choudhury D, et al. Crystal structures of HbA2 and HbE and modeling of hemoglobin delta 4: interpretation of the thermal stability and the antitickling effect of HbA2 and identification of the ferrocyanide binding site in Hb. Biochemistry [Internet]. 2004 Oct 5 [cited 2016 May 26];43(39):12477–12488. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15449937

[10] Van Durme J, Delgado J, Stricher F, et al. A graphical interface for the FoldX forcefield. Bioinformatics [Internet]. 2011 Jun 15 [cited 2016 May 26];27(12):1711–1712. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21505037

[11] Krieger E, Koraimann G, Friedl G. Increasing the precision of comparative models with YASARA NOVA – a self-parameterizing force field. Proteins [Internet]. 2002 May 15 [cited 2016 May 26];47(3):293–402. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11948792

[12] Kozlowski LP. IPC – Isoelectric Point Calculator. Biol Direct [Internet]. BioMed Central; 2016 Oct 21 [cited 2016 Nov 10];11(1):55. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27769290

[13] Chong SS, Boehm CD, Higgs DR, et al. Single-tube multiplex-PCR screen for common deletional determinants of alpha-thalassaemia. Blood [Internet]. 2000 Jan 1 [cited 2016 May 26];95(1):360–362. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10607725

[14] Williamson D, Brennan SO, Strosberg H, et al. Hemoglobin A2 Fitzroy delta 142 Ala–Asp: a new delta-chain variant. Hemoglobin [Internet]. 1984 [cited 2016 Nov 10];8(4):325–332. Available from: http://www.ncbi.nlm.nih.gov/pubmed/6548205

[15] Lederer CW, Pavlou E, Makariou G, et al. Hb Famagusta – analysis of a novel δ-globin chain variant [HδBx.60C>A] in four families with diverse globin genotypes. Ann Hematol [Internet]. 2014 Sep [cited 2016 May 26];93(9):1625–1627. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24452365

[16] Moo-Penn WF, Schneider RG, Shih T, et al. Hemoglobin Ohio (beta 142 Ala replaced by): a new abnormal hemoglobin with high oxygen affinity and erythrocytosis. Blood [Internet]. 1980 Aug [cited 2016 Nov 11];56(2):246–250. Available from: http://www.ncbi.nlm.nih.gov/pubmed/7397380

[17] van Zwieten R, Veldhuis M, Delzenne B, et al. Hemoglobin analyses in The Netherlands reveal more than 80 different variants including six novel ones. Hemoglobin [Internet]. 2014 Feb 7 [cited 2016 Nov 13];38(1):1–7. Available from: http://www.tandfonline.com/doi/full/10.1080/03630269.2013.849608

[18] Khosa SM, Usman M, Moinuddin M, et al. Comparative analysis of cellulose acetate hemoglobin electrophoresis and high performance liquid chromatography for quantitative determination of hemoglobin A2. Blood Res [Internet]. 2015 [cited 2016 Nov 10];50(1):1–6. Available from: http://synapse.koreamed.org/DOIx.php?id=10.5045/brr.2015.50.1.46

[19] Manca L, Cherchi L, De Rosa MC, et al. A new, electrophoretically silent, fetal hemoglobin variant: Hb F-Calabria [Gγα118(GH1)Phe-->Leu]. Hemoglobin [Internet]. 2000 Feb [cited 2016 Nov 20];24(1):37–44. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10722114

[20] Wintrobe MM, Adamko DJ, Agarwal N, et al. Wintrobe's clinical hematology. 12th ed. Philadelphia (PA): Lippincott Williams & Wilkins; 2009.

[21] Guerois R, Nielsen JE, Serrano L. Predicting changes in the stability of proteins and protein complexes: a study of more than 1000 mutations. J Mol Biol.2002/89. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12098438

[22] Pavlou E, Phylactides M, Kyrris A, et al. Delta-thalassemia in Cyprus: a 20-year retrospective study. Sci Rep [Internet]. 2016 [cited 2016 Nov 10];6:26371. Available from:http://www.ncbi.nlm.nih.gov/pubmed/27769290

[23] Codrington JF, Kutlar F, Harris HF, et al. Hb A2-Wrens or alpha 2 delta 2 98(FG5) Val–Met, an unstable delta chain variant identified by sequence analysis of amplified DNA. Biochim Biophys Acta [Internet]. 1989 Sep 21 [cited 2016 Jul 11];1009(1):87–89. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2477064

[24] Trifillis P, Adachi K, Yamauchi T, et al. Expression studies of delta-globin gene alleles associated with reduced hemoglobin A2 levels in Greek Cypriots. J Biol Chem [Internet]. 1996 Oct 25 [cited 2016 Jun 6];271(43):26931–26938. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8900178