Does absolute excess of alpha chains compromise the benefit of splenectomy in patients with thalassemia intermedia?

β thalassemia intermedia (TI) is a clinical definition referring to a heterogeneous group of hereditary anemias resulting from defective β chain production, an α/β globin chain imbalance, and anemia that lie in severity between that of thalassemia minor and the transfusion-dependent thalassemia major (TM).[1] The phenotype of TI may also result from the increased production of α globin chains by a triplicated or quadruplicated α genotype associated with β heterozygosity, also leading to α/β globin chain imbalance, and a presumably mild clinical phenotype.[2-5] We report here 2 siblings with transfusion-independent TI who had immediate worsening of their clinical course and became transfusion-dependent after splenectomy, and discuss how the absolute excess of α globin chains in these 2 patients may explain this development.

The father was diagnosed with TI at the age of 16 years (baseline hemoglobin level 80-85 g/L) and the mother had a normal hematologic profile. The 2 parents are not consanguineous at least as far back as four generations, although both are from a small village in the North East of Italy. The 2 children were diagnosed with TI at the ages of two and four years. The 2 children, who had never been transfused before (baseline hemoglobin levels 75-80 g/L), underwent splenectomy at the ages of ten and 12 years because of splenomegaly and secondary anemia. Five months following splenectomy, the clinical course of both patients worsened and anemia (hemoglobin level <70 g/L) necessitated initiating a transfusion regimen. Chelation therapy was initiated at the ages of 14 and 19 years. The younger child had thrombophlebitis at 17 years. The father, now aged 51 years, shows a well tolerated chronic hemolytic anemia without the need for transfusion (hemoglobin level maintained at 80-85 g/L), mild jaundice, splenomegaly and leg ulcers. The mother, still has a completely normal hematological profile and hemoglobin pattern.

The father and the 2 children were initially considered to have unusual TI because at molecular analysis they were carriers of a single β^+ mutation (IVS1-110 G>A). After the unusual clinical outcome following splenectomy, all family members were referred to our center for molecular reevaluation. As according to our center's ethical committee guidelines, all family members signed written informed consent to molecular studies for both diagnostic and research purposes. DNA analysis of β and α globin genes was performed. Mutation analysis of the β globin gene was established by direct DNA sequencing on the ABI Prism 310 genetic analyzer (PE Biosystems Foster City CA, USA). The α globin gene cluster was analyzed by Multiplex Ligation-dependent Probe Amplification (MLPA) according to Harteveld *et al.*[4,6] RNA analysis was performed in triplicate on TaqMan 7500 (PE Biosystems Foster City, CA, USA) after DNAse treatment using specific gene assay (HBA1/2: Hs00361191_g1). The relative quantification (RQ) of alpha genes expression was

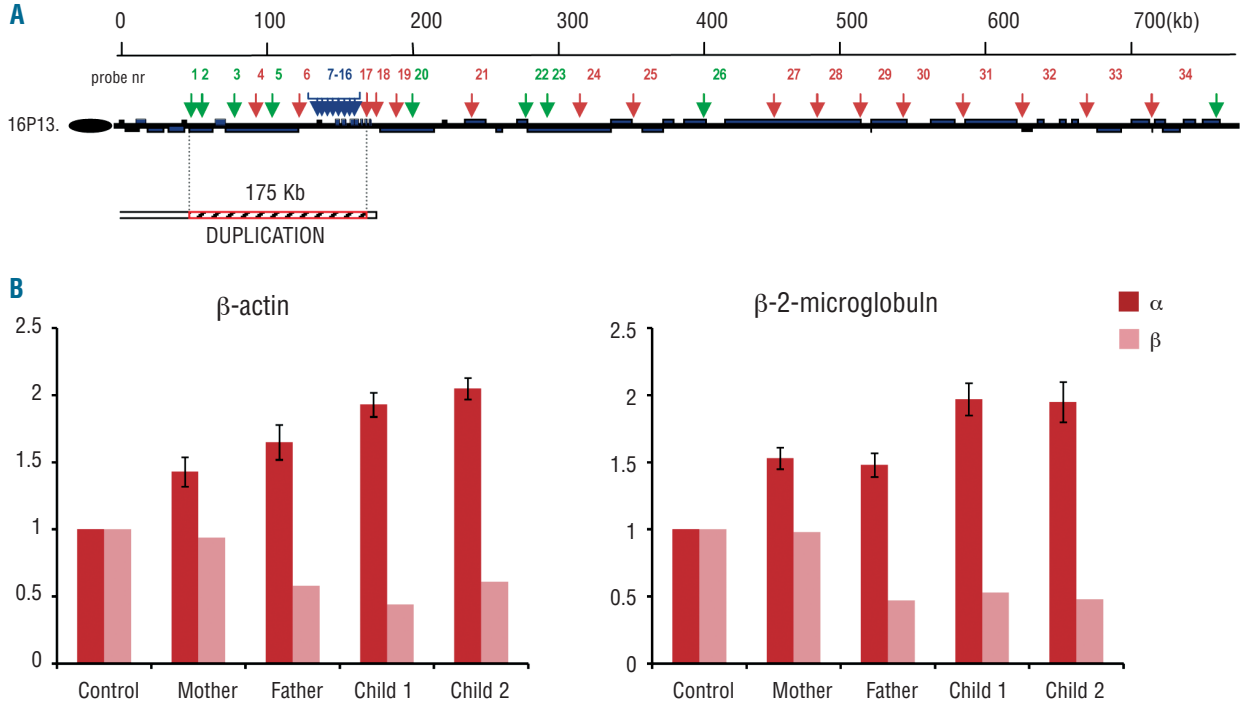

Figure 1. (A) Schematic representation of the 750 Kb region from the telomere of the short arm of chromosome 16 analyzed by Multiplex Ligation-dependent Probe Amplification (MLPA). The position of the 35 MLPA-probe pairs along the region is indicated by green, red and blue arrows, referring to HEX, ROX and FAM labeling, respectively. The duplicated region is indicated as arched red bars and spans at least 175 Kb. (B) Real-time polymerase chain reaction experiments: the graphs show the mean and standard deviation of three different experiments. The expression of α and β globin genes is compared to β-actin and β-2 microglobulin house-keeping genes.

Table 1. Hematologic and molecular data of the family at the time of study.

	Age/ gender	Hb g/ L	RBC $\times 10^{12}$/L	MCV fL	MCH pg	Hb A2 %	Hb F %	RBC_n %	Spleen	LDH U/L	Transf	Chelation	α genes	β genes	α/β genes
Mother	46/F	128	4.87	81.7	26.3	2.5	0.9	0	normal	245	no	no	αα,αα/αα	β^N/β^N	1.5
Father	51/M	85[a]	4.47	64.0	19.0	5.0	3.7	13	20 cm	356	no	no	αα,αα/αα	β^T/β^N	3
Child 1	19/M	60[b]	3.80	60.0	16.5	4.3[c]	7.5[d]	40[e]	splenectomy	642	yes	yes	αα,αα/αα,αα	β^T/β^N	4
Child 2	14/F	59[b]	3.69	59.9	16.0	4.0[c]	7.7[d]	45[e]	splenectomy	689	yes	yes	αα,αα/αα,αα	β^T/β^N	4

[a]Baseline 80-85 g/L; [b]Baseline 75-80 g/L; [c]Baseline 4%; [d]Baseline 6%; [e]Baseline <15%. F: female; M: male; Hb: hemoglobin; RBC: red blood cells; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; RBC_n: nucleated red blood cells (orthochromatic erythroblasts); LDH: lactate dehydrogenase; Transf: transfusion-dependence.

achieved by normalization against two different housekeeping genes Beta-actine (ACTB:Hs99999903_m) and β2 microglobuline (β2M:Hs00187842-m1) using the $2^{-\Delta\Delta ct}$ formula.

Molecular analysis confirmed that the father and the 2 children were heterozygotes for the β+ mutation IVSI-110 G>A. MLPA analysis disclosed a full duplication of the α globin locus spanning at least 175 kb, from the telomere to the 3'HVR downstream of the α globin gene, including the upstream regulatory element HS-40 (Figure 1A).[4] This rearrangement increases the number of functional α globin genes *in cis* from 2 to 4 and was found in heterozygosis in both parents and in homozygosis in both children. The overproduction of alpha chains was confirmed by Q-PCR experiments with RQ increased in all members of the family (Figure 1B). As expected, the parents showed RQ values of about 1.5 while the children showed values of about 2. Hematologic and molecular data of the family are summarized in Table 1.

In the father, coinheritance of a β globin mutation with 6 α globin genes led to a moderate-severe, well tolerated TI phenotype. The 2 children had 8 functional α globin genes (since the regulatory element HS-40 was also duplicated) producing a large amount of α chains (twice than normal) that, associated with reduced β chain production, causes a severe α/β globin imbalance.

In transfusion-independent TI, erythropoietic stress, ineffective erythropoiesis, and chronic intravascular hemolysis are highly variable. An increased number of erythroblasts, reticulocytes, and damaged erythrocytes are seen in the peripheral blood. The excess free α chains precipitate within the erythroid precursors, form hemichromes, and alter the membrane cytoskeletal proteins by clustering band 3 and enhancing deposition of opsonin autologous immunogobulins and C3 fragments.[7-8] About 80% of senescent or altered erythrocytes are removed extravascularly by macrophages present mainly in the spleen.[7] Thus, removal of the spleen in patients with an absolute excess of α chains, as in our patients, may lead to more severe hemolysis and persistence of damaged erythroblasts and erythrocytes in the blood stream compared to patients with defective β chain production and a relative excess of α chains. These persistent abnormal erythroid cells may justify the high rate of thromboembolism in splenectomized TI patients,[9-10] including the one reported herein. They acquire procoagulant properties from increased exposure of phosphatidylserine instead of phosphatidyletanolamine on their outer surface, by an altered flip flop mechanism mediated by band 3 clusters.[11] Thus, in the 2 siblings, splenectomy was associated with an increase in the number of nucleated red blood cells (as documented in the blood smear), increased peripheral hemolysis (worsening anemia), and increased risk of thrombotic events (in one of the children).

In the light of several reports linking splenectomy to adverse long-term outcomes, it was recently recommended that the decision to perform a splenectomy in TI patients should be taken with caution and the procedure should be avoided or delayed unless absolutely necessary.[1] Given our experience,we agree with this recommendation, especially in TI patients characterized by a severe imbalance between α/non-α globin chains due to an absolute excess of α globin chains. We also recommend including α globin gene testing in the molecular workup of patients with TI where the β gene defect does not completely explain the observed phenotype.

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Key words: thalassemia intermedia, alpha chains, excess, splenectomy, adverse outcome.

Citation: Graziadei G, Refaldi C, Barcellini W, Cesaretti C, Cassinero E, Musallam KM, and Cappellini MD. Does absolute excess of alpha chains compromise the benefit of splenectomy in patients with thalassemia intermedia? Haematologica 2012; 97(1):151-153. doi:10.3324/haematol.2011.046730

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Analysis of *NOTCH1* mutations in monoclonal B-cell lymphocytosis

Monoclonal B-cell lymphocytosis (MBL) represents asymptomatic monoclonal B-cell expansions characterized by a chronic lymphocytic leukemia (CLL) phenotype, but with less than 5.0×10^9/L circulating cells.[1-3] Clinical MBL (cMBL) is recognized during the diagnostic workup of an asymptomatic lymphocytosis.[4-6] Although the molecular pathogenesis of MBL is little known, the biological indolence of this condition is documented by the rare occurrence of genetic lesions predicting poor prognosis in CLL, such as *TP53* and *ATM* disruption.[4-6]

Recently, two independent investigations of the CLL coding genome have revealed that activating mutations of the *NOTCH1* proto-oncogene occur in approximately 10% CLL at diagnosis and their frequency increases in advanced disease phases, exemplified by the case of Richter syndrome.[7,8] Initial evidence suggests that *NOTCH1* alterations might predict an unfavorable clinical outcome in CLL. The prevalence of *NOTCH1* mutations in MBL is currently unknown.[7-11]

Here we investigated the occurrence of *NOTCH1* mutations in 63 consecutive cMBL presenting at our clinic for the initial evaluation of an asymptomatic lymphocytosis. The cMBL cohort was provided with prospectively collected peripheral blood mononuclear cell samples drawn at presentation, and with a prospectively maintained clinical database. All cMBL were analyzed for *NOTCH1*, *TP53* and *IGHV* mutations by DNA Sanger sequencing, and for FISH karyotype using the LSI13 and LSID13S319, CEP12, LSIp53 and LSIATM probes (Abbott, Rome, Italy).[5,7] A *NOTCH1* mutation (c.7544_7545delCT) that is known to be highly recurrent in CLL was also independently investigated by amplification refractory mutation system (ARMS) PCR. Patients provided informed consent in accordance with local IRB requirements and the Declaration of Helsinki. The study was approved by the Ethical Committee of the Ospedale Maggiore della Carità of Novara, Northern Italy, associated with the Amedeo Avogadro University of Eastern Piedmont (Protocol Code 59/CE; Study Number CE 8/11).

The clinical profile of the cMBL cohort was representative of this condition. Median age was 68 years (range 40-

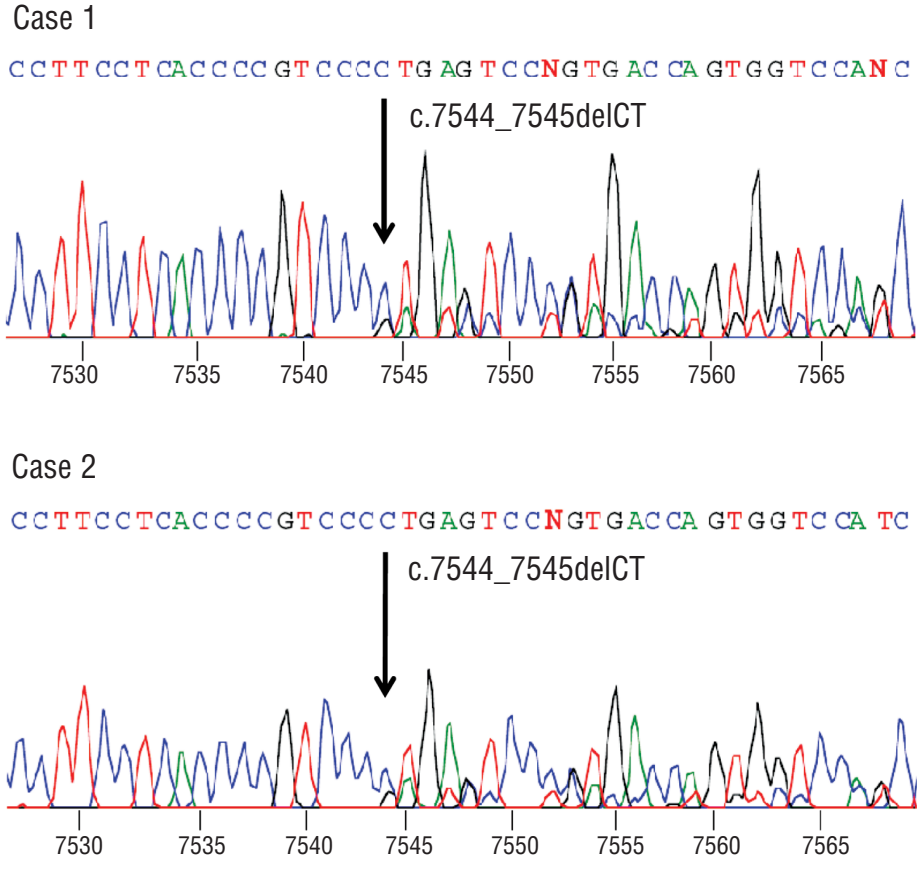

Figure 1. ***NOTCH1*** **mutations in clinical monoclonal B-cell lymphocytosis. Sequencing traces of the two clinical monoclonal B-cell lymphocytosis tumor samples (case 1 and case 2) harboring the *NOTCH1* c.7544_7545delCT mutation (RefSeq NM_017617.2); arrows point to the position of the nucleotide change.**