bjh research paper

β+45 G → C: a novel silent β-thalassaemia mutation, the first in the Kozak sequence

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 British Journal of Haematology, **124,** 224–231

Received 6 June 2003; accepted for publication 14 October 2003

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Summary

A family from the Southeast of Italy was found to have a novel β-globin mutant, β+45 G → C, with the features of a silent β-thalassaemia mutation. It was asymptomatic in two heterozygotes, but its interaction with the severe thalassaemia mutation β-IVS-II-654 C → T worsened the haematological and biosynthetic phenotype in two compound heterozygotes; moreover, another compound heterozygote, who was also heterozygote for the $\alpha\alpha\alpha^{anti3.7}$, suffered from thalassaemia intermedia. The mutation was found associated in *cis* with the IVS-II-754 T → C substitution, which did not lead to abnormally spliced mRNA. Furthermore, the amount of β+45 mRNA was the same as the βA mRNA in the reticulocytes of the carriers. *In vitro* transcription/translation experiments demonstrated that the β+45 G → C decreased the efficiency of translation of the β-globin chain by about 30%: this slight impairment was consistent with the observed clinical phenotype. The β+45 G → C is the first mutation found in the Kozak sequence (GACACCATGG) of the β-globin gene and the first one at the position -6 upstream the ATG. The Kozak consensus sequence plays a major role in the initiation of translation process. The present finding supports the hypothesis that the G in position -6 is important in this process.

Keyword: silent β-thalassaemia, Kozak consensus sequence, leader sequence, β-thalassaemia modifier.

β-Thalassaemia is a hereditary disease caused by mutations in the β-globin gene that result in the decrease or abolition of β-globin chain synthesis. The majority of them are point mutations or small deletions localized along the β-globin gene (Huisman & Carver, 1998). Only five mutations have been reported in the leader sequence between the cap site and the initiation codon (50 bp). They are: β+10-T (Athanassiadou *et al*, 1994); β+40/43-AAAC (Huang *et al*, 1991); β+22 G → A (Oner *et al*, 1991); β+33 C → G (Ho *et al*, 1996). In addition, a β+20 C → G substitution has been reported *in cis* to the β-IVS-II-745 C → G mutation, but its contribution to the β-thalassaemia phenotype has not been clarified (Oner *et al*, 1991).

The β+10-T and the β+33 C → G mutations are 'silent mutations' because the heterozygotes showed no changes in the red blood cells (RBC) indices, whereas compound heterozygotes with classical β-thalassaemia mutations showed the mild 'thalassaemia intermedia' phenotype. The β+22 G → A can be included in the group of 'mild mutants' because carriers showed decreased mean cell volume and mean cell haemoglobin (MCV, MCH) levels and increased Hb A2, both of modest entity. Compound heterozygotes exhibited a variable clinical picture from thalassaemia intermedia to Cooley's anaemia. The only heterozygote for β+40/43-AAAC had a β-thalassaemia carrier phenotype, but molecular studies *in vitro* failed to demonstrate the effective correlation of the four nucleotide deletion and phenotype (Frances *et al*, 1993).

These mutations show different molecular mechanisms. Two mutations (+22 and +33), located in the downstream core element (DCE), interfere with the transcription (Lewis *et al*, 2000), whereas the +10 allele should interfere with the translation (Ho *et al*, 1999).

Here, we report a new mutation β+45 G → C (β+45) that is the sixth substitution in the 5′UTR and the first one in the Kozak sequence (GACACCATGG) of β-globin gene. It was found in a family from Puglia (Southeast of Italy) in two heterozygotes, two compound heterozygotes with the β-IVS-II-654 C → T (β-IVS-II-654) and one compound heterozygote who was also heterozygous for the $\alpha\alpha\alpha^{anti3.7}$ and homozygous

for the $(TA)_7$ allele of the uridine diphosphate-glucuronyl transferase (*UGT1A1*) gene. The mRNA analysis did not reveal transcriptional alterations. Experiments *in vitro* demonstrated that the mutation impairs translation by about 30%. This finding confirms the role of the G at position -6 of the Kozak consensus sequence in the translation process of the β-globin gene.

Material and methods

Haematological data and Hb analyses

Blood samples from the patients were obtained after informed consent. Haematological data were determined by standard methods. Hb analyses were carried out by cation exchange high-performance liquid chromatography (HPLC), using the Diamat System (Bio-Rad Laboratories, Richmond, CA, USA). Biosynthesis *in vitro* and separations of the globin chains were performed as previously reported (Pagano *et al*, 1991).

DNA analysis

The sequences and positions of the primers used for the DNA analysis are reported in Fig 1. The β-globin gene was analysed by sequencing DNA fragments (primers from A to F) amplified by polymerase chain reaction (PCR) as previously described (De Angioletti *et al*, 2002). Molecular screens for the β+45 and β-IVS-II-754 T → C (β-IVS-II-754) substitutions were carried out by amplification refractory mutation system (ARMS) analysis (Old *et al*, 1990). The β+45 was revealed by the synthesis of a 552 bp fragment using the primers +45 and M. The β-IVS-II-754 was revealed by the synthesis of a 729 bp fragment using the primers 745 and O. In both cases a control fragment of 861 bp was amplified using the primers N and O.

α-Globin gene rearrangements were detected by the digestion of genomic DNA with restriction enzymes, Southern blot and hybridization with an α-globin gene probe as previously reported (Lacerra *et al*, 1991).

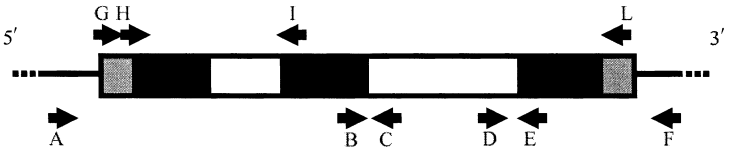
Screening of the TA insertion in the A(TA)nTAA motif in the promoter of the *UGT1A1* gene was performed by PCR using the conditions and primers described by Monaghan *et al* (1996). The analysis of the PCR products was performed on 6% polyacrylamide gels and the bands were revealed with silver staining.

Haplotype and β-globin gene framework

Restriction fragment length polymorphisms (RFLPs) *Hin*dIII/$^{G}\gamma$, *Hin*dIII/$^{A}\gamma$, *Hin*cII/ψβ, *Hin*cII/3′ψβ, *Ava*II/β and *Bam*HI/3′β were analysed on genomic DNA by Southern blot as already reported (Carestia *et al*, 1987). Sequence polymorphisms of the β-globin gene framework were determined by DNA sequencing. Haplotypes and frameworks associated with the mutations were determined through family linkage analysis and classified according to Orkin *et al* (1982).

Ratio of mRNA synthesized by β-globin alleles

The ratio of mRNA synthesized by the two β-globin alleles was measured in subjects that were heterozygous for the



Primers	Position on the β-globin gene	DNA strand	Sequence 5′→3′
A	- 158 / - 138	Sense	TAAGCCAGTGCCAGAAGAGCC
B	+432 / +453	Sense	CACCTTTGCCACACTGAGTGAG
C	+621 / +602	Antisense	CACTGATGCAATCATTCGTC
D	+1055 / +1074	Sense	CCCTAATCTCTTTCTTTCAG
E	+1394 / +1372	Antisense	CTTTGCCAAAGTGATGGGCCAGC
F	+1744 / +1723	Antisense	CCTTCTTTCATGGAGTTTAAGA
G	- 4 / +24	Sense	GCTTACATTTGCTTCTGACACAACTGTG
H	+15 / +25	Sense	CACAACTGTGTTCACTAGCA
I	+297 / +277	Antisense	GGTCCAAGGGTAGACCACC AG
L	+1573 / +1553	Antisense	TCCAGATGCTCAAGGCCCTTC
M	+567 / +544	Antisense	CCCCTTCCTATGACATGAACTTAA
N	+1088 / +1111	Sense	CAATGTATCATGCCTCTTTGCACC
O	+1948 / +1925	Antisense	GAGTCAAGGCTGAGAGATGCAGGA
+45	+16 / +45	Sense	ACAACTGTGTTCACTAGCAACCTCAAAgAc
754	+1220 / +1249	Sense	TAATAGCAGCTACAATCCAGCTACCATaCc

Fig 1. Schema of the β-globin gene. The primers used for the DNA sequencing and the reverse trasncription-polymerase chain reaction (RT-PCR) analyses are indicated by arrows below and above the gene respectively. Primer E was used for both DNA sequencing and RT-PCR. The sequence and position from the cap site of the primers are reported. Small letters indicate nucleotide substitutions introduced for the amplification refractory mutation system (ARMS-PCR). Black areas, exons; white areas, introns; grey areas: 5′UTR, 3′UTR.

polymorphism codon 2 C → T, taking advantage of the fact that the β-codon 2 'C' (βC) and β-codon 2 'T' (βT) alleles are associated respectively, with the presence/absence of the *Alw*44I restriction site. Total RNA was isolated from reticulocyte-enriched peripheral blood cells (Pagano *et al*, 1991) with Triazol (Life Technologies, New York, NY, USA). The cDNA was synthesized from the RNA by using the Moloney murine leukaemia virus reverse transcriptase and the Oligo d(T) primer (GeneAmp, Perkin-Elmer, Foster City, CA, USA). cDNA was amplified using dCTP (α-^{32}P) and the primers G and I. To reduce the production of the heterodimers we carried out a first PCR round of 15 cycles and a second round of two cycles after increasing 50 times the volume of reaction. The DNA samples were digested with *Alw*44I restriction enzyme and run on a 7·5% polyacrylamide gel at 150 V for 1·5 h. The intensity of each band was quantified by using a phospho-imager with ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). The ratio of βT and βC mRNA was calculated as the ratio between the intensity of the undigested 301 bp band and the sum of the two 58 and 243 bp bands obtained with the *Alw*44I digestion.

Detection of abnormally spliced mRNA

The cDNA was amplified by radioactive nested-PCR, using the primers G and L for the first PCR and H and E for the nested-PCR. DNA samples were run on a polyacrylamide gel and DNA bands were detected with autoradiography with RX film and with a phospho-imager, as reported above.

Experiments of transcription/translation in a cell-free system

The cDNA from a β+45 heterozygote was amplified using the primers G and L (from the cap site to the 3′UTR at 99 nucleotides downstream from the termination codon). The PCR product was introduced into the plasmid vector pCRII by using TA Cloning Kit (Invitrogen, San Diego, CA, USA) under conditions recommended by manufacturer. The plasmids were prepared with the methods of Sambrook *et al* (1982), tested for the presence of the insert in the right orientation with restriction analysis and then sequenced. Plasmids with the normal (βA) and mutated (β+45) sequence were selected and used in transcription and translation experiments co-expressing each of them with the control plasmid, a pGEM 3Z (Promega, Madison, WI, USA) carrying the pepsinogen (Pep) cDNA from *Trematomus bernacchii*, an Antarctic fish. In each reaction we used 0·5 μg of βA or β+45 plasmid and part of a master-mix containing 0·25 μg of the control plasmid for each reaction, the rabbit reticulocyte lysates and T7 RNA polymerase (TNT coupled transcription/translation system; Promega), and ^{35}S-methionine. The synthesis reactions were performed for 60 min. The synthesized proteins were electrophoresed on a 16% sodium dodecyl sulphate polyacrylamide gel. The radioactive bands were detected and quantified by a phospho-imager with ImageQuant software (Molecular Dynamics). The ratio of the globin synthesized by mutated and normal alleles was calculated by dividing the radioactivity values of β+45 and βA bands after their normalization with that of the Pep band from the same lane. The background values had been subtracted from all experimental values.

Results

Haematological and clinical data

The family under study originated from Puglia, a region of South-eastern Italy. Current haematological data are reported in Table I. The 27-year-old male proband (II-3) was affected with thalassaemia intermedia and did not require blood transfusions. He had been referred to the hospital when aged 9 years for anaemia (Hb = 9·0 g/dl) and severe icterus (unconjugated bilirubin = 78·7 μmol/l). Since then, his Hb level had ranged between 8·1 and 9·6 g/dl. He had hepatosplenomegaly that increased with the age until it was 4 cm below the costal margins. At the age of 23, he underwent a cholecystectomy because he had gallstones. At the time of the present study, the patient showed normal physical and sexual development, although he was underweight (height 1·69 m, weight 49 kg). A blood smear showed severe microcytosis, anisocytosis, hypochromia, poikilocytosis and a large number of tear drop and target erythrocytes were present. *In vitro* biosynthesis of the globin chains showed a severe imbalance in globin synthesis (β/α = 0·27). His father and two brothers had normal blood counts, a normal blood film and balanced β/α ratio (Table I). The proband mother and two sisters had a classical β-thalassaemia heterozygote phenotype, but the sisters showed more severe RBC morphological alterations and a more unbalanced β/α biosynthetic ratio than their mother (0·39 and 0·38 in the sisters, 0·48 in the mother).

Genetic analysis

The proband and the two sisters were compound heterozygotes for the mutation β-IVS-II-654 C → T (β-IVS-II-654), inherited from the mother, and for a novel allele, inherited from the father, with two substitutions: G → C at 45 nucleotides downstream the cap site (β+45) and T → C at the nucleotide 754 of the IVS-II (β-IVS-II-754) (Fig 2; Table I). One brother (II-5) was a carrier of the new allele. No other sequence alteration was detected in the β-globin gene.

The proband, his brother (II-4) and mother were heterozygous for the triplicated $\alpha\alpha\alpha^{anti3{\cdot}7}$ haplotype (Table I).

Considering the high-bilirubin level of the proband, we analysed the A(TA)nTAA motif of the promoter of the *UGT1A1* gene, which normally consists of six TA repeats. The proband was homozygous for the $(TA)_7$ allele; his parents and two sisters were heterozygotes.

The β-IVS-II-654 allele was associated with haplotype V (−−−−/+−) and β-globin gene framework 2 (Orkin *et al*,

Table I. Haematological and molecular data of family members.

Patient	Father (I-1)	Mother (I-2)	Sister (II-1)	Sister (II-2)	Proband (II-3)	Brother (II-4)	Brother (II-5)
Sex/age (years)	M/56	F/53	F/30	F/28	M/27	M/24	M/20
Hb (g/dl)	15·4	11·5	10·0	11·1	9·1	14·5	15·7
RBC ($\times 10^{12}$/l)	4·9	6·4	5·1	5·9	5·0	5·1	5·2
MCV (fl)	88	56	63	58	56	83	84
MCH (pg)	32	18	20	19	18	29	30
Hb A2 (%)	2·8	4·8	5·9	4·9	5·9	2·8	2·7
Hb F (%)	<1	<1	1·3	1·4	2·9	<1	<1
Reticulocytes (%)	1·1	2·4	1·9	1·4	2·9	0·8	0·9
Indirect bilirubin (μmol/l)	10·26	11·97	11·97	11·97	153·9	6·84	10·26
Total bilirubin (μmol/l)	13·17	13·34	12·48	15·22	167·92	8·21	13·17
Iron (μmol/l)	15·93	12·53	9·66	12·71	16·11	9·49	14·86
Ferritin (μg/l)	531	216	227	52	175	83	99
β/α	0·95	0·48	0·39	0·38	0·27	0·81	0·98
β-Genotype	+45/A	IVS-II-654/A	+45/IVS-II-654	+45/IVS-II-654	+45/IVS-II-654	A/A	+45/A
β-Haplotype *	IV/V	IIR/V	IV/V	IV/V	IV/V	IIR/V	IIR/IV
α-Genotype	αα/αα	$\alpha\alpha\alpha^{anti3.7}$/αα	αα/αα	αα/αα	$\alpha\alpha\alpha^{anti3.7}$/αα	$\alpha\alpha\alpha^{anti3.7}$/αα	αα/αα

*The haplotype IIR was ++−+/−+ (the restriction fragment length polymorphism (RFLP) analysed were: *Hin*dIII/$^{G}\gamma$; *Hin*dIII/$^{A}\gamma$; *Hin*cII/ψβ and 3′ψβ; *Ava*II/β; *Bam*HI/3′β).

M, male; F, female; Hb, haemoglobin; RBC, red blood cell; MCV, mean cell volume; MCH, mean cell haemoglobin.

1982) (C, T, C, T at codon 2, IVS-II nucleotide 74, 81, 666 respectively). The new allele was found to be associated with the rare haplotype IV (+−++/−+) and with the Asian framework (T, T, C, C at codon 2, IVS-II nucleotide 74, 81, 666 respectively) (Antonarakis *et al*, 1982).

The frequency of the β+45 and β-IVS-II-754 substitutions in our population was investigated by ARMS-PCR in 69 healthy subjects, of whom 19 had the IV haplotype. None of them showed any of the two substitutions. Therefore, we concluded that, in our population, the β+45 and β-IVS-II-754, if present, were rare.

Transcription analysis

The effect of the substitution β+45 and of the β-IVS-II-754 on the transcription was tested by measuring the ratio of mRNA synthesized from the βA and from the new allele with the two substitutions. The analysis was carried out in the father who was heterozygous for the new allele and for the codon 2 C/T polymorphism. The new allele was associated with the codon 2 'T' (βT), the βA was associated with the codon 2 'C' (βC). The βT/βC cDNA ratio was 0·92 ± 0·12 in the β+45 heterozygote father, 1·15 ± 0·05 in the unaffected son (II-4) and 0·91 ± 0·09 in a healthy unrelated subject. In the mother, the proband and his sisters, who had the β-IVS-II-654 allele associated with the βC, the levels of mRNA synthesized from the βC were much lower than those from the βT (Fig 3B).

Abnormally spliced mRNA

Analysis using the software for predictions of splice site [http://searchlauncer.bcm.tmc.edu Baylor College of Medicine (Houston, TX)] did not find any putative splicing site in the DNA sequence with the substitution β-IVS-II-754, even using a cut-off score of 0·1. Moreover, experimental analysis did not reveal any abnormal length mRNA in the carriers (Fig 4). mRNA that was 75 bp longer than normal was detected in the heterozygotes or compound heterozygotes for the β-IVS-II-654.

Translation efficiency analysis

The cDNA from the βA and β+45 alleles was cloned from the carrier (II-5). The βA and β+45 plasmids obtained from three different preparations were used in nine transcription/translation experiments, three for each pair of plasmids. The efficiency of translation of the β+45 allele was lower than the βA in all the experiments (Fig 5). The mean ratio (β+45/control)/(βA/control) was similar for the three pairs of plasmids; 0·67 ± 0·10, 0·67 ± 0·05 and 0·66 ± 0·09 respectively (Fig 5). These data strongly suggest that, at least *in vitro*, the β+45 mutation reduced the translation efficiency.

Discussion

We have detected, in a family from Southern Italy, a new β-globin allele that is characterized by two substitutions *in cis*: the +45 G → C in the Kozak consensus sequence (GACACC-AUGG → CACACCAUGG) and the β-IVS-II-754 T → C. The proband was a compound heterozygote for the new allele and for the β-IVS-II-654, heterozygous for the α-globin gene triplication and homozygous for the $(TA)_7$ allele of the *UGT1A1* gene. He had been affected since the childhood with thalassaemia intermedia not requiring blood transfusion and

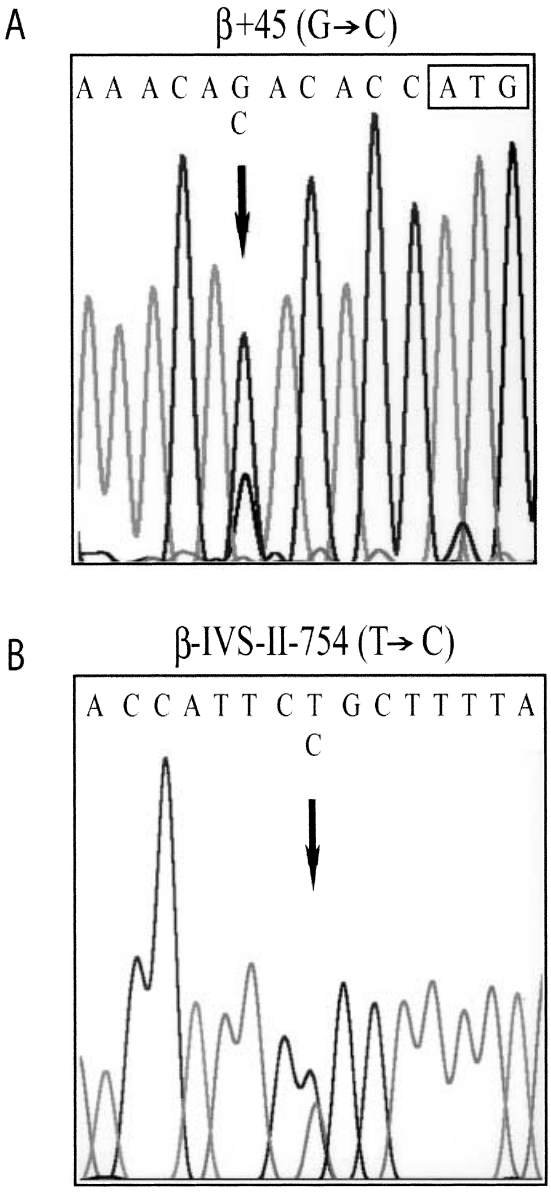


Fig 2. Sequence analysis of the β-globin gene coding strand of the proband showing that he was heterozygous for β+45 G → C (A) and for β-IVS-II-745 T → C (B). The nucleotide substitutions are given at the top of each panel.

had a β/α biosynthetic ratio of 0·27. The results reported here indicate that the β-IVS-II-754 substitution is not responsible for the observed clinical alterations, and must be considered only a neutral substitution because it does not cause splicing defects. In contrast, the results support the hypothesis that the β+45 substitution plays a role in the pathogenesis of thalassaemia intermedia syndrome in the proband. Experiments of transcription/translation *in vitro* have shown that the β+45 impaired translation because the allele produced about 67% of the amount of β-globin chains of the wild type. This translation defect is probably balanced in the carriers (the father and one brother) who had blood counts, Hb A2 levels

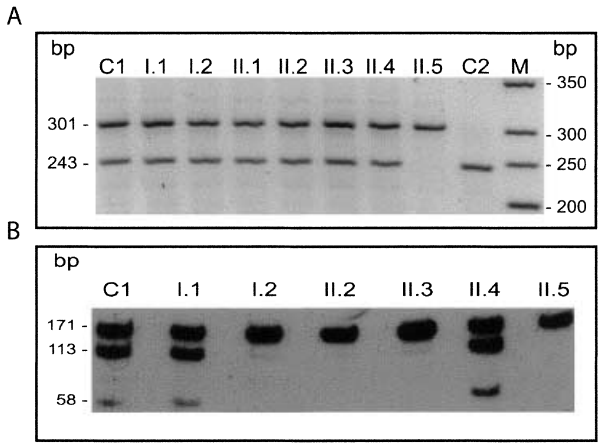


Fig 3. Analysis of the mRNA transcribed by the allele with the β+45 G → C and β-IVS-II-745 T → C substitutions. The analysis was performed taking advantage of the fact that the new allele was associated with codon 2 'T', βA was associated with codon 2 'C' and that the codon 2 C/T polymorphism can be recognized with the *Alw*44I restriction enzyme. Each family member is indicated as in Table I. (A) The 301 bp fragment was amplified from DNA, digested with *Alw*44I and analysed with agarose gel electrophoresis. The codon 2 polymorphism T/C was indicated by fragments of 301 bp or 243 bp and 58 bp respectively. The 58 bp bands are not visible in the gel. All the family members, except II-5, were heterozygous for the polymorphism. (B) The cDNA fragment of 171 bp was digested with *Alw*44I and analysed with polyacrylamide gel electrophoresis. The codon 2 polymorphism T/C was indicated by fragments of 171 bp or 113 bp and 58 bp respectively. In I-2, II-2 and II-3 the 113 and 58 bp bands, obtained from the allele carrying the β-IVS-II-654 C → T mutation, were visible only after long exposure. C1, subject heterozygous for C/T at codon 2; C2, subject homozygous for C at same codon; M, DNA size marker.

and β/α biosynthetic ratio in the normal ranges. Contrarily, it becomes effective in the presence of an alteration of the β/α biosynthetic ratio. The two sisters, who were compound heterozygotes (β IVS-II-654/β+45), showed a β/α ratio (0·38–0·39) at the lower limit of β-thalassaemia heterozygotes and haematological alterations that were more severe than β-thalassaemia heterozygotes. Furthermore, the mother and proband were both double heterozygotes for the β-IVS-II-654 and α-globin gene triplication but only the proband, who also carried the β+45 allele, was affected with 'thalassaemia intermedia'. Comparative analysis of the phenotypes was informative about the extent of the defect. The mother and sisters had the same β-IVS-II-654, associated in the mother with the α-globin gene triplication and in the sisters with the β+45 mutation. In spite of this, the sisters had a β/α biosynthetic ratio (0·38–0·39) lower than the mother (β/α = 0·48) and more severe haematological alterations. This observation suggests that the β+45 mutant has a similar or stronger influence than the α-globin gene triplication on the balance of globin chain synthesis and on the severity of the β-thalassaemic phenotype.

Another hypothesis can be proposed regarding the pathogenesis of the disorder in the proband. A few cases of

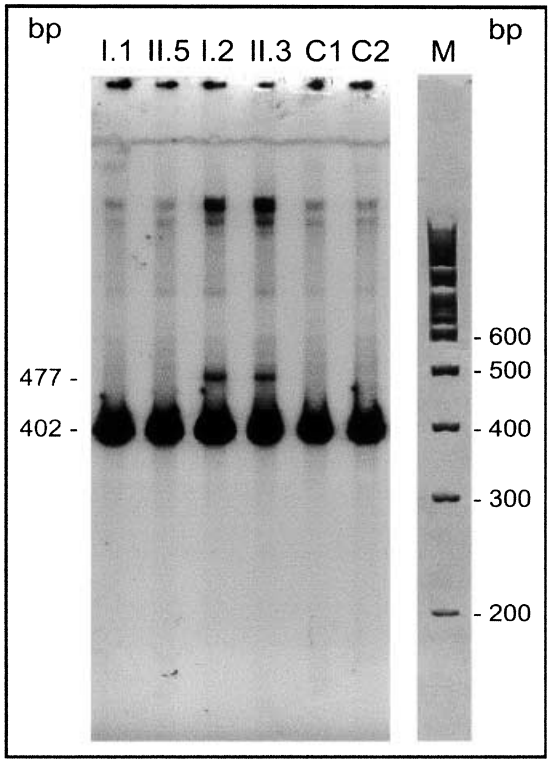


Fig 4. Detection of the abnormally spliced mRNA. Polyacrylamide gel electrophoresis of the cDNA. The fragment of 402 bp indicates the normal allele; the fragment of 477 bp indicates the β-IVS-II-654 allele. No additional bands were present in the three carriers of the new allele. C1 and C2, control subjects; M, DNA size marker.

patients heterozygous for the β-IVS-II-654 with thalassaemia intermedia phenotype have been reported (Gasperini *et al*, 1998; Ho *et al*, 1998). The absence of other globin gene alterations led the authors to hypothesize the inheritance of an unknown factor, unlinked to the β-globin gene cluster, affecting globin gene production but silent in the carriers. We cannot exclude that the proband could have inherited such a factor from his father, which would increase the complexity of the genotype in our patient. However, in our opinion, the first hypothesis is more likely, considering the co-segregation of clinical phenotypes and the β+45 allele.

The G at position +45 of the β-globin gene is one of the nucleotides of the Kozak sequence ($GCC^{A}/_{G}CCAUGG$), an element highly conserved in the eukaryotic genomes, which represents the most efficient context for the correct translation initiation (Kozak, 1994). In particular, the G localized in position -6 with respect to the AUG, is present in 44% of the 699 vertebrate mRNA sequences analysed (Kozak, 1987). The high conservation of the G in position -6 suggests that this G has a role in the initiation process of the translation. In rabbit β-globin 5′UTR (with a Kozak sequence that is slightly different from the human one) the substitution of this G with a T reduced the efficiency of the translation initiation process in experiment *in vitro* (Kozak, 1994).

Only a few substitutions in the Kozak sequence have been reported to date. Three of them were polymorphisms involved in the reduction of the coagulation factor XII (-4 C/T) (Kanaji *et al*, 1998) and the other two affected susceptibility to cardiovascular diseases: the -5 T/C in the glycoprotein Ibα gene (Afshar-Kharghan *et al*, 1999) and the -1 C/T in the annexin V gene (Gonzalez-Conejero *et al*, 2002). Other mutations have been described in few patients with different pathologies, such as: α-thalassaemia (-2/-3 AC deletion) (Morle *et al*, 1985), cystic fibrosis (-4 G → C) (Zielenski *et al*, 1991) and ataxia (-1 C → T) (Usuki & Maruyama, 2000).

To trace the origin of the new allele is extremely difficult because it is characterized by two new substitutions and, in addition, it is associated with the haplotype IV and the Asian framework, which are rare in our area. Because of this complexity neither the autochthonous origin, the spread from other geographic areas or a more intricate hypothesis can be ruled out.

In conclusion, data reported herein supports the hypothesis that the β+45 G → C is a novel β-globin gene mutation that impairs β-globin chain translation. The slight impairment

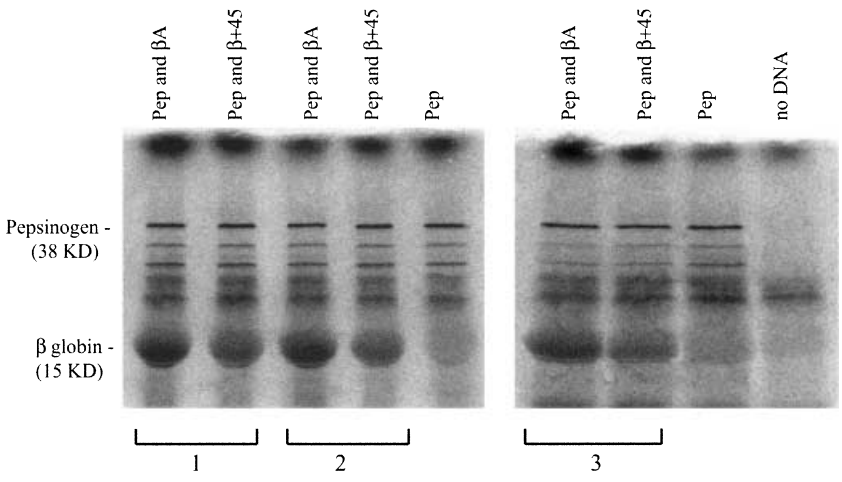


Fig 5. Sodium dodecyl sulphate polyacrylamide gel electrphoresis of *in vitro* translated proteins from the wild type (βA) and the mutated (β+45) allele. Each reaction included the control plasmid containing the pepsinogen (Pep) cDNA from *Trematomus bernacchii*, an Antarctic fish. Three experiments are shown; the three pairs (1–3) of β+45 and βA plasmids used were obtained from independent preparations. Each pair of plasmids was used in three different experiments with similar results. Control reactions without DNA (no DNA) or only with control plasmid (Pep) are shown.

(about 30%) shown *in vitro* is consistent with the clinical, haematological and biosynthetic phenotype observed in the proband and in the other family members. It is the first substitution in the Kozak sequence of the β-globin gene and the first one in humans involving the position -6. The demonstration that the β+45 acts as silent thalassaemia mutation confirms the hypothesized role of the G in position -6 of the Kozak sequence (Kozak, 1994) in the optimization of the translation process.

Acknowledgments

The authors acknowledge the family for their co-operation, Mr Romeo Prezioso from IGB for his collaboration and Dr Gaetano Savia from Hospital 'Giovanni XXIII' for the haematological and serological determinations. Also thanks Dr Clemente Capasso for the kind gift of the Pep plasmid. The project was supported by grants given by Ministero Università, Ricerca Scientifica e Tecnologica, Legge 488/92 Cluster C02, PE2.

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