β-thalassemia major evolution from β-thalassemia minor is associated with paternal uniparental isodisomy of chromosome 11p15

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ABSTRACT

β-thalassemia major can be caused by homozygosity or compound heterozygosity for β-globin gene mutations (*HBB* gene). Most cases are inherited from parents who both have diseased alleles of the *HBB* gene. We report a patient with late-onset β-thalassemia major that evolved from β-thalassemia minor in which only one of her parents had the diseased *HBB* gene. To study the cause of β-thalassemia major in this patient, we performed the 100K single nucleotide polymorphism genotyping assay, fluorescence *in situ* hybridization, and DNA methylation analysis of the imprinting genes near the *HBB* gene. The results showed a loss of heterozygosity in the region of chromosome 11p14.3 to 11p15.5, which perfectly matched one allele of her father. Our study demonstrates that paternal uniparental isodisomy of chromosomal 11p15.5 is associated with the β-thalassemia major in this patient.

Key words: β-thalassemia major, uniparental isodisomy, mosaicism.

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Introduction

Thalassemia is the most common recessive single gene disease in humans which is caused by inheritance of an affected allele from both parents.[1] The people of Taiwan have a high prevalence of β-thalassemias.[2] Various types of molecular defects, most of which are point mutations, affect the expression of the β-globin gene (HBB gene) (GenBank accession n. NC_000011.8) causing β-thalassemia (MIM# 141900). β-thalassemia major is caused by inheritance of two diseased alleles of the HBB gene, one from each parent, and is characterized by severe anemia at an early age, transfusion dependency, and life-limiting complications of iron overload. More than 20 different β-thalassemia mutations are reported in the Taiwanese population, and four of the mutations account for more than 90% of mutant alleles.[2,3] We report a β-thalassemia major patient diagnosed at 28 years of age, in which β-thalassemia major developed from β-thalassemia minor.

Design and Methods

Patient

The patient, a 28-year-old woman did not report a history of anemia but first received medical attention for progressive dyspnea and easy fatigue in October, 2005. Her hemogram showed the following results: hemoglobin (Hb) of 2.1 g/dL, RBCs of 1.03×10^{12}/L, hematocrit of 8.4%, and mean corpuscular volume of 68.3 fl. The percentages of the Hb fractions on Hb electrophoresis were Hb A1, 86.8%, Hb A_2, 5.0%, and Hb F 8.2% (after packed RBC transfusion). The patient's childhood blood counts were not available, but at 20 years of age (October 1998) her hemoglobin level (8.6 g/dL) and mean corpuscular volume (62.4 fL) were low. Family history revealed that her father also had microcytic hypochromic anemia, with an elevated Hb A_2 level. Upon the diagnosis of β-thalassemia major, further studies were approved by the Institutional Review Board of Kaohsiung Medical University, and written informed consent was obtained from the patient and her family.

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DNA preparation and genotyping

DNA was isolated from peripheral blood mononuclear cells, bone marrow, hair follicles, and oral mucosal epithelium by standard methods. The polymerase chain reaction (PCR)-restriction fragment length polymorphism and direct sequencing for the *HBB* gene mutation analyses were performed using the protocols described by Chang *et al*.[2]

RNA preparation and globin messenger RNA quantification

RNA was extracted from peripheral reticulocytes and amplification of α/β globin mRNA by reverse transcription PCR was followed by silver staining of the PCR products according to the protocol described by Lin *et al*.[4]

High-density single nucleotide polymorphism genotyping arrays

The single nucleotide polymorphism (SNP) genotyping assay was performed according to the Affymetrix Gene-Chip Mapping 100K Assay Manual. The Affymetrix Mapping 100 K Set contained 116,204 SNPs (consisting of the 50 K Xba and the 50 K Hind arrays that each contained approximately 58,000 SNPs).[5] (Data submitted to the GEO repository with Accession Number GSE7847; user name d730093, password m12045).

Fluorescence in situ hybridization

Metaphase spreads of peripheral blood mononuclear cells were prepared for fluorescence *in situ* hybridization (FISH) using standard methods.[6] Slides were then hybridized with three biotinylated fluorescence isothiocyanate-labeled, plasmid DNA probes (pHS1234 miniLAR, puLCR/γ and pβ) from the human β-globin gene region on chromosome 11 (11p15.5). The insert of miniLAR, with a size of 8.3 kb, detected the β-locus control region (β-LCR). The puLCR/γ, with an insert size 10.1 kb, detected the β-LCR and the γ region of the β-gene cluster, whereas the 4.9 kb pβ insert detected the β-globin region of the β-gene cluster.

Loss of imprinting of the H19 differentially methylated region

Bisulphite treatment of genomic DNA was performed as previously described.[7] Bisulphite-modified DNA was amplified by PCR. CpG methylase (Sss I)-treated genomic DNA was used as the positive control. To ensure the specificity of the H19 differentially methylated region (DMR) primer sets for bisulphite DNA treatment, amplification using unmodified genomic DNA samples from the proband was also carried out. The primers for the *H19 DMR* were: forward 5'-TGTTGAAGGTTGGGGAG ATGGGA-3' and reverse 5'-CCCAAACCATAACACTAAAACCC-3' (GenBank accession n. NC_000011.8). The PCR products were sequenced using the ABI 310 DNA Sequencer.

Results and Discussion

We found that the patient had homozygous *HBB* [c.52A>T] + [c.52A>T] (homozygous codon 17 A→T) mutations. Her father had a heterozygous *HBB* [c.52A>T] + [wt] mutation. However, her mother had a normal HBB genotype. Surprisingly, DNA from her hair follicles and oral mucosal epithelium was heterozygous for the *HBB* [c.52A>T] mutation. The results of PCR-restriction fragment length polymorphism genotyping are shown in Figure 1A. The peripheral reticulocyte α/β globin mRNA ratio was 34.8 and compatible with the diagnosis of β-thalassemia major.

The homozygosity of the *HBB* gene mutation [c.52A>T] + [c.52A>T] in the patient and a heterozygous *HBB* mutation [c.52A>T] + [wt] in her father were discrepant with Mendelian law. Because the acquired chromosome 11p15.5 deletion is frequently observed in a variety of malignancies,[8] and acquired deletion of the *HBB* gene is also described by Badens and by Galanello *et al*.,[9,10] we suspected that the patient may have a hemizygosity caused by acquired deletion of chromosome 11p15.5 harboring the *HBB* gene. However, FISH analysis showed that both chromosome 11s had positive hybridization signals with all three probes studied (β-LCR, *HBG* and *HBB* gene probes), indicating that the *HBB* gene cluster was probably intact (Figure 1B). To further clarify the cause of the patient's homozygous *HBB* mutation, we genotyped the family using a SNP array. The results showed a loss of heterozygosity in the region of chromosome 11p14.3 to 11p15.5 (166 informative markers mapped to the physical distance of 2.73 Mb to 22.39 Mb) which perfectly matched one allele of the father (Figure 2A). Nevertheless, the patient's hair follicle and oral mucosal cell DNA showed heterozygosity in this region. The results suggest that paternal uniparental isodisomy and mosaicism[11,12] of chromosomal region 11p14.3-11p15.5 could be associated with the β-thalassemia major in this patient.

Since the SNP genotyping assay covered only a small part of chromosomal region 11p15.5, we extended the study to the more distal segment of 11p15.5. We investigated the methylation status of the differentially methylated region upstream of the *H19* gene (*H19 DMR*) using the bisulphite direct sequencing method. *H19 DMR* controls the imprinting of the maternally expressed *H19* and the paternally expressed insulin-like growth factor 2 (*IGF2*) genes (physical distance of 1.97 Mb to 2.11 Mb).[13,14] Loss of imprinting (LOI) of *H19 DMR* (hypermethylated) is associated with the silencing of paternally derived *H19* and the activation of paternally derived *IGF2*. The methylation status analysis of *H19 DMR* showed LOI in our patient's peripheral blood and bone marrow DNA whereas no LOI was found in the patient's oral mucosal epithelium DNA or her parent's DNA samples (Figure 2B). The evidence for this study shows that β-thalassemia major in our patient was associated with mosaic paternal iUPD of chromosome 11 p15. Firstly, the *HBB* [c.52A>T] + [c.52A>T] mutation was only found in the patient's hemopoietic tissue, not in her other tissues, and only one of her parents had the

HBB [c.52A>T] mutation. Secondly, the SNP assay showed shared SNP homozygosity in the chromosome region from 11p14.3 to11p15.5, which perfectly matched one allele of the patient's father, and this phenomenon was not observed in other tissues. Thirdly, two copies of the *HBB* gene were found during FISH analyses, and the results confirmed that the homozygous informative SNPs were derived from paternal uniparental isodisomy. These results suggest mosaicism of the two different clones of cells from the heterozygous and homozygous *HBB* [c.52A>T] mutations.[15] The

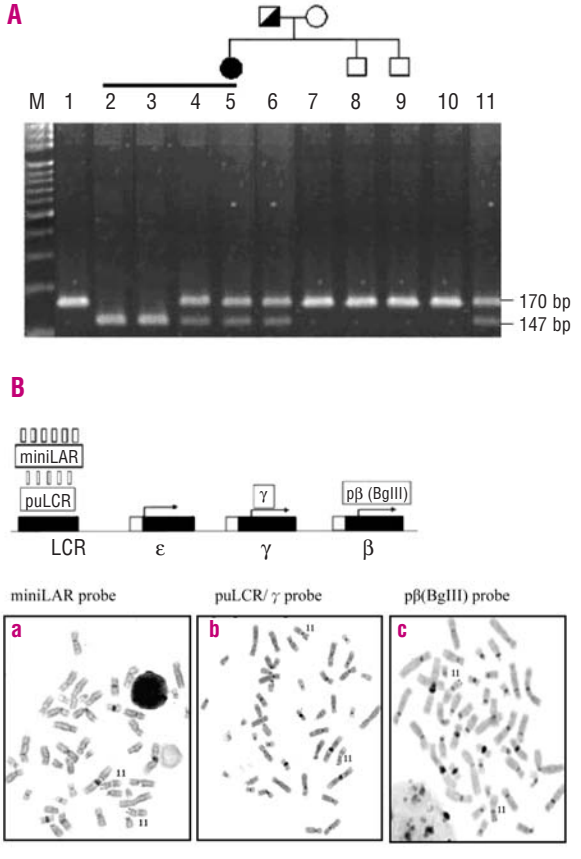

Figure 1. (A) PCR-RFLP results for diagnosis of *HBB* gene [c.52A>T] mutations. Lane 1 is the uncut control fragment of 170 bp. Lanes 2–5 show the results from the patient's peripheral blood (PB), bone marrow (BM) (homozygote of 147 bp), hair follicles, and oral mucosal epithelium (heterozygote of 170 and 147 bp). Lanes 6–9 are the results of the patient's father (heterozygote of 170 and 147 bp), mother (normal), elder brother, and younger brother. Lanes 10, 11 are the normal control and positive control for heterozygosity of the *HBB* gene [c.52A>T] mutation. Lane M represents a 100-bp ladder marker. (B) Fluorescence *in situ* hybridization analysis of patient's PB metaphase spreads. The location of the three biotinylated FITC-labeled, plasmid DNA probes (pHS1234 miniLAR, puLCR/γ and pβ) related to *HBB* gene cluster on chromosome 11 is shown at upper panel. Positive hybridization signal (red fluorescent signal indicated) with miniLAR probe on the terminal short arm of both chromosome 11s (11p15.5) is see at panel a, positive hybridization signals also observed with puLCR/γ probe and p, probe on 11p15.5 of both chromosome 11s respectively (b and c). The results showed that each PB cell carried two copies of *HBB* genes with no deletion found using the above three gene probes.

A

Chr11	snpid	Position Ref: NT 009237	rsid	Blood	Hair	Oral swab	FA	MA	Location
11p15.5	SNP_A-1642499	g.2853359 G>T	rs10488671	BB	AB	AB	A B	A A	11p15.5
11p15.4	SNP_A-1642499	g.4628914 G>A	rs10500608	BB	AB	AB	B B	A B	11p15.4
	SNP_A-1755530	g.4630223 G>A	rs4910524	AA	AB	AB	B A	B B	11p15.4
11p15.3	SNP_A-1716614	g.4885637 G>C	rs10500627	AA	AB	NoCa	B A	B B	11p15.4
	SNP_A-1746262	g.4911183 G>A	rs10500622	AA	AB	AB	A A	B B	11p15.4
11p15.2	SNP_A-1717971	g.4937257 G>A	rs3850508	BB	AB	AB	B B	A A	11p15.4
11p15.1	SNP_A-1751101	g.5034508 T>A	rs2034834	BB	AB	AB	B B	A B	11p15.4
	SNP_A-1703758	g.5373189 C>A	rs1532514	BB	AB	AB	B B	A B	11p15.4
11p14.3	SNP_A-1722501	g.6678136 C>T	rs1960352	AA	AB	AB	B A	B B	11p15.4
11p14.2	SNP_A-1662445	g.6735914 C>G	rs1506969	AA	AB	AB	B A	A B	11p15.4
11p14.1	SNP_A-1656250	g.6840953 A>C	rs2595457	AA	AB	AB	A A	B A	11p15.4
11p13	SNP_A-1755833	g.6915170 T>C	rs1602569	AA	AB	AB	A A	B B	11p15.4
	SNP_A-1696877	g.7707730 G>C	rs7113128	AA	AB	AB	A A	B A	11p15.4
11p12	SNP_A-1741152	g.7832560 A>G	rs10500701	AA	AB	AB	B A	A B	11p15.4
11p11.2	SNP_A-1680035	g.8156792 T>C	rs1528125	BB	AB	AB	A B	A B	11p15.4
	SNP_A-1730690	g.9702777 A>G	rs1695613	AA	AB	AB	A A	B B	11p15.4
11p11.12	SNP_A-1699006	g.10160186 G>A	rs10500721	AA	AB	AB	B A	B B	11p15.4
11p11.1	SNP_A-1723239	g.11167698 A>G	rs10500740	AA	AB	AB	B A	B B	11p15.3
11q11	SNP_A-1664179	g.12876461 C>G	rs10500765	BB	AB	AB	B B	A B	11p15.3
	SNP_A-1659123	g.13442481 A>G	rs3814236	BB	AB	AB	B B	A B	11p15.2
	SNP_A-1720910	g.15692229 G>A	rs3886607	BB	AB	AB	A B	A A	11p15.2
	SNP_A-1748030	g.16214298 C>T	rs10500829	AA	AB	AB	A A	A B	11p15.2
	SNP_A-1687525	g.17397566 C>G	rs2299641	BB	AB	BB	A B	A A	11p15.1
	SNP_A-1707690	g.18595288 T>A	rs10500834	AA	AB	AB	A A	B A	11p15.1
	SNP_A-1729933	g.19860333 C>T	rs2625312	BB	AB	AB	B B	A A	11p15.1
	SNP_A-1660558	g.20058500 T>G	rs1867116	BB	AB	AB	A B	A A	11p15.1
	SNP_A-1723227	g.21122448 A>G	rs1945319	BB	AB	AB	A B	B A	11p15.1
	SNP_A-1713403	g.22398998 T>C	rs10500927	BB	AB	AB	B B	A A	11p14.3
	SNP_A-1695913	g.22349700 T>C	Rs764021	AA	AB	AB	BA	B B	11p14.3

B

C G T T T C G G G T T A T T T A A G T T A C G C G T C G

C

N G T T T N G G G T T A T T T A A G T T A N G N G T N G

Figure 2. (A) Haplotype screening of the informative biallelic markers with chromosomal locations in the patient's PB, hair follicles, oral mucosa cells, and PB from the patient's father and mother. Twenty-nine representative SNPs of the 166 homozygosity SNPs on chromosome 11p are shown. DNA mutation name, reference SNP identity number, and the respective genotypes of the allelic markers are presented (AA: homozygous for one allele, BB: homozygous for the other allele, AB: heterozygous).The chromosomal locations of the SNPs are shown in the left column. Markers on 11p14.3-15.5 show homozygosity and are not interrupted by heterozygous SNPs in the patient's blood. (B) Direct sequencing results of the 6259~6287 CpG site of the H19 DMR. Modified DNA of patient's peripheral blood with all Cs in CpG dinucleotides remaining as C (underlined, upper panel), and patient's father with heterozygous methylation, one methylated (C) and one unmethylated (T) allele (lower panel).

paternal uniparental isodisomy of the 11p15 clone contained the *HBB* [c.52A>T] + [c.52A>T] mutation and the LOI of *H19 DMR* would result in *IGF2* activation and overexpression, and could play a role in cellular proliferation and growth over the clone with the heterozygous *HBB* [c.52A>T] mutation. Through proliferation, this clone became the only hemopoietic cell in the bone marrow and peripheral blood and resulted in the β-thalassemia major phenotype, a phenomenon found in a variety of malignancies.[16,17]

We conclude that the patient's β-thalassemia major involved inheritance of paternal uniparental isodisomy of chromosome 11p15 harboring the *HBB* [c.52A>T] allele, which was mixed with β-thalassemia minor mosaicism caused by normal biparental inheritance. Though homozygosity of autosomal recessive gene mutations was described in uniparental disomy of other chromosomes, homozygosity of *HBB* gene mutations associated with paternal uniparental isodisomy of 11p15 is reported here for the first time. It is likely that more cases will be recognized in areas with high frequencies of β-thalassemia. Paternal uniparental isodisomy of chromosome 11p15 should be considered in cases of unexplained late-onset of β-thalassemia major, and analysis of *HBB* gene mutations should not be restricted to the blood only, but should also investigate other tissues.

Authorship and Disclosures

T-CL designed the study, participated in data collection, analysis, and interpretation, wrote the draft of the manuscript, and approved the final version; J-GC participated in designing the study, and in data collection, analysis, and interpretation, and approved the final version; W-CT, I-WC, C-SC and C-CL participated in data collection, analysis and interpretation, and approved the final version of the report. The authors reported no potential conflicts of interest.

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