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Original Article

Transfusion-independent β^0-thalassemia after bone marrow transplantation failure: proposed involvement of high parental HbF and an epigenetic mechanism

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Abstract: Currently, bone marrow transplantation is the only curative treatment for β-thalassemia and sickle cell disease. In rare cases, sustained and full fetal hemoglobin production was observed in patients after failure of bone marrow transplantation. This rendered the patients transfusion-free, despite genetic disease and transplant rejection. The mechanisms underlying this phenomenon remain unexplored. We have studied a trio (father-mother-child) in which the affected child became transfusion-independent after rejection of an allogeneic bone marrow graft. Remarkably, we found that his non-thalassemic mother also expressed unusually high levels of γ-globin. High HbF in one of the parents may therefore be of prognostic value in these rare cases. Genotyping of the *HBB* locus and the HbF quantitative trait loci *HBS1L-MYB*, *KLF1* and *BCL11A*, and protein expression analysis of KLF1 and BCL11A, failed to explain the increased HbF levels, indicating that an as yet unidentified HbF modifier locus may be involved. We hypothesize that epigenetic events brought about by the transplantation procedure allow therapeutic levels of HbF expression in the child. Potential implications of our observations for reactivation of γ-globin expression and interpretation of the French globin gene therapy case are discussed.

Keywords: β-thalassemia, bone marrow transplantation, HbF, transfusion-independence, epigenetics, *MYB, KLF1, BCL11A, HBB*

Introduction

Hereditary anemias, in particular sickle cell disease (SCD) and β-thalassemia, are the most common monogenic disorders in the human population. It is estimated that ~300,000 severely affected new patients are born annually. Bone marrow transplantation (BMT) is the only curative treatment currently available to the patients, and has been used with considerable success. Given the requirement for a suitable donor and sophisticated health care infrastructure, BMT is available to a minority of the patients. A few rare cases have been reported in which, although the transplant was rejected, the patients became transfusion-independent due to sustained expression of high levels of fetal hemoglobin (HbF) [1, 2]. Here we have started to explore the molecular mechanisms underlying this phenomenon by studying a trio (father, mother, child) in which the affected child became transfusion-independent after rejection of an allogeneic bone marrow graft. Our data indicate that an as yet unidentified modifier locus is responsible for increased HbF expression occurring in some of the β-thalassemic patients undergoing autologous reconstitution following allogeneic bone marrow transplantation. We propose that the transplantation procedure triggers epigenetic events which

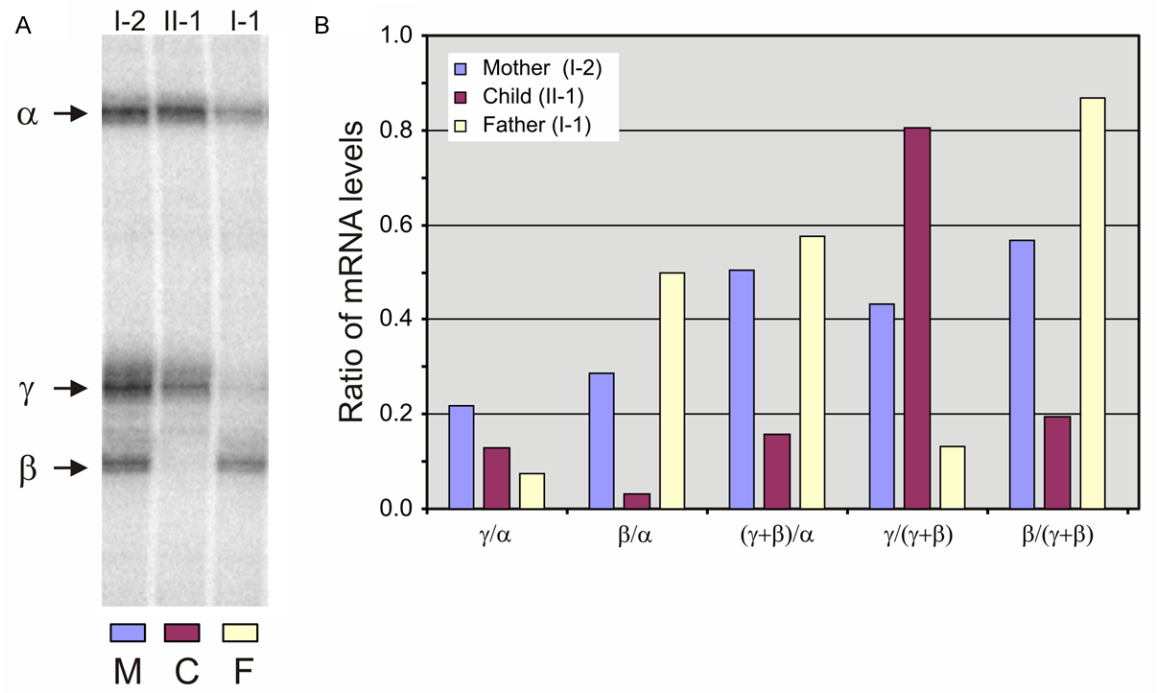

Figure 1. *Globin mRNA expression in primary erythroid progenitors derived from the trio.* Proerythroblasts were cultured from peripheral blood buffy coats and total RNA was extracted as described [4, 5]. A: To measure globin mRNA levels directly, we used quantitative S1 nuclease protection assays [5]. Protected fragments are indicated: α-globin (α); γ-globin (γ); β-globin (β). B: Quantification was performed using a Typhoon Trio PhosphorImager (GE Healthcare) and corrected for specific activity of the probes. M=mother, C=child, F=father.

allow sustained and high level expression of HbF in these rare cases. Furthermore, high HbF levels in at least one of the (non-thalassemic) parents could be a predictor of this phenotype.

Materials and methods

Subjects

The patient has been reported previously [2, 3]. Informed consent was obtained from the patient and his parents. The present study was carried out in full compliance with the guidelines of the Mediterranean Institute of Hematology, Policlinic of Tor Vergata, Rome, Italy.

Cells and cell culture

Buffy coats were collected from 8-25 ml of peripheral blood, and cryopreserved at a cell density of 7-30 × 10^6 per ml. Upon thawing, cells were expanded under erythroid growth conditions as described [4]. After 7-14 days of culture, the cells were harvested and used for isolation of DNA, RNA and protein [5].

Genotyping and sequencing

DNA isolated form the proerythroblast cultures was used for genotyping of the *HBB, BCL11A and HMIP2* loci [5-8]. For *KLF1*, the promoter and exons including exon-intron junctions were amplified by PCR and the amplification products were sequenced directly [5].

Expression of globin mRNAs

Quantitative S1 nuclease protection assays were used to measure the expression levels of α-globin, γ-globin and β-globin mRNA in total RNA samples isolated from the cultured proerythroblasts [5, 9]. For each assay, 2-2.5 μg of total RNA was used. Sizes of protected fragments were: α-globin: 218 nt; γ-globin: 165 nt; β-globin: 155 nt. Quantification was performed using a Typhoon Trio Phosphorimager (GE Healthcare) and corrected for specific activity of the individual probes.

Expression of BCL11A and KLF1 proteins

Whole cell protein lysates were prepared from the cultured proerythroblasts, and size-frac-

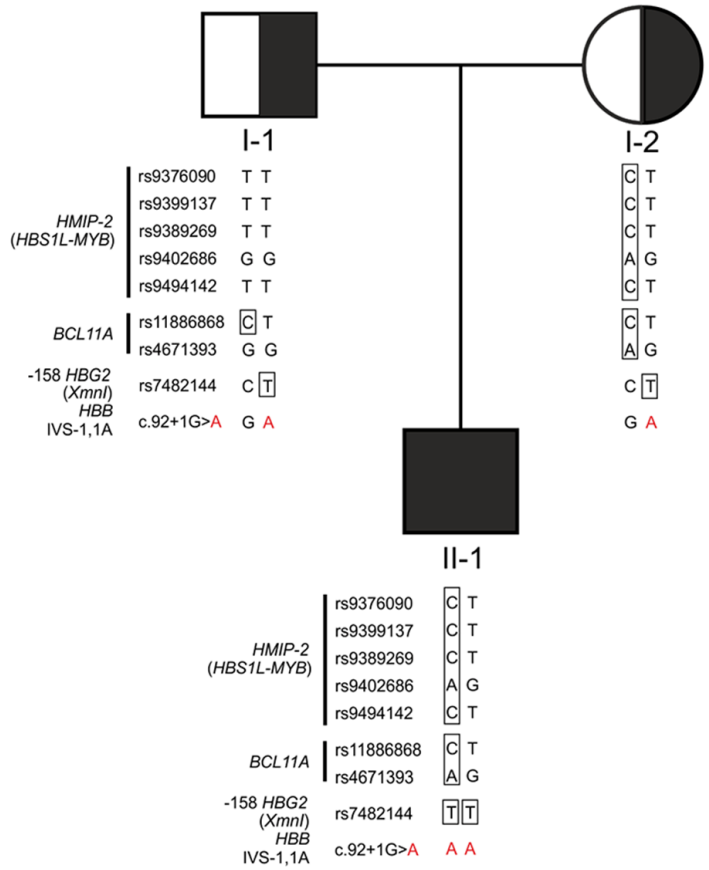

Figure 2. *Genotypes of the HMIP-2, BCL11A, and HBB loci.* SNPs associated with increased HbF are boxed. The pathogenic mutation in the *HBB* gene is indicated in red. Promoter and exon sequencing of *KLF1* revealed the presence of exclusively wildtype alleles for all three individuals.

tionated by SDS-PAGE. The gels were transferred to nitrocellulose membranes and probed with antibodies recognizing BCL11A (sc-56013, Santa Cruz Biotechnology) and KLF1 (homemade rabbit polyclonal) [5]. An antibody specific for NPM1 (ab10530, Abcam) was used as a loading control. For detection, the appropriate secondary antibodies were used. The Odyssey Infrared Imaging System (Li-Cor Biosciences) was used to develop the membranes.

Results

An initial clinical study conducted by KP and GL reported on an adult patient affected by β^0-thalassemia who received an allogeneic BMT from a HLA-matched related donor [3]. Fourty days after BMT, allogeneic engraftment failure and autologous β^0-thalassemic bone marrow recovery were documented. Remarkably, hemoglobin (Hb) levels stabilized at ~12 g/dl throughout follow-up (currently >8 years), rendering the patient transfusion-independent. Hemoglobin (Hb) analysis showed 100% HbF, indicating that the *HBG* genes (encoding fetal γ-globin) had been fully activated [2].

Taking advantage of a recently developed culture method for primary human proerythroblasts [4], we expanded peripheral blood buffy coat cells from the patient (II-1; cells taken after failed BMT) and his parents (father I-1 and mother I-2). We first determined globin mRNA expression quantitatively (**Figure 1**). As expected, this demonstrated virtual absence of β-globin mRNA in the patient (II-1) sample due to homozygosity for the IVS-I, 1A (c.92+1 G>A) mutation, and high levels of γ-globin mRNA. The father (I-1) expressed β-globin mRNA but only a small amount of γ-globin mRNA. The mother (I-2) also expressed β-globin mRNA. Remarkably, in contrast to the father she expressed unexpectedly high levels of γ-globin mRNA. The mother is a carrier of the IVS-I, 1A *HBB* mutation, and she is heterozygous for the T allele of the *XmnI* polymorphism in the *HBG2* gene (**Figure 2**), which is associated with increased HbF [10]. Given that the father also carries these mutations (**Figure 2**), this cannot fully explain the high level of γ-globin expression observed in the mother. We therefore explored the three loci that are currently known to be firmly associated with HbF levels, *HMIP-2* (the intergenic region of the *HBS1L* and *MYB* genes [7]), *BCL11A* [6, 8] and *KLF1* [5, 11, 12]. Genotyping of single nucleotide polymorphisms (SNPs) associated with HbF in the *HMIP-2* and *BCL11A* loci revealed that none of the individuals was homozygous for a particularly advantageous combination of these SNPs (**Figure 2**). Moreover, sequencing of the exons of the *KLF1* gene revealed that the family members exclusively carried wildtype alleles for this gene. Since reduced expression of BCL11A [13] and KLF1 [5] has been linked directly to increased HbF we assessed expression of these proteins

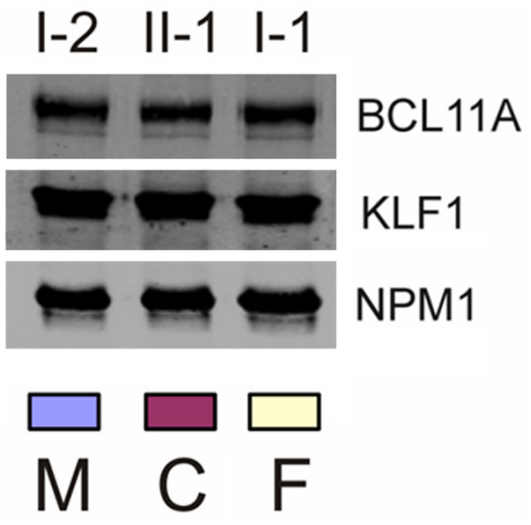

Figure 3. *Protein expression levels of KLF1 and BCL11A in proerythroblasts of the trio.* Proteins extracts were prepared from cultured proerythroblasts and subjected to Western blotting as described [5]. NPM1 served as a loading control. M=mother, C=child, F=father.

by Western blotting [5]. Using nucleophosmin (NPM1) as a loading control, we observed virtually identical expression levels of BCL11A and KLF1 between the family members (**Figure 3**). Thus, reduced expression of BCL11A or KLF1 at the protein level does not appear to be involved in the high HbF phenotype of the mother and child.

Discussion

As stated previously, cases such as II-1 demonstrate that "full HbF production in a β^0-thalassemia patient may be developed in adulthood and that HbF possesses the disease-specific function to reduce chain imbalance, thereby abolishing the transfusion therapy requirement" [2, 3]. Since patient II-1 is now transfusion-independent for over 8 years, it is worth reinforcing this statement. The increase in HbF production, which corrected the α/non-α ratio from 2.47 to 0.97 and increased total Hb from 8.3-9.0 to 11.8-13 g/dl, is apparently very stable and not dependent on ongoing medical intervention [2, 3]. We note that II-1 is genetically pre-disposed for high HbF expression, given that his pre-transplant HbF levels were high (73%) and that his non-thalassemic mother I-2 displays remarkably high γ-globin mRNA levels. The observation that these high γ-globin expression levels are maintained in proerythroblast cultures of 1-2 and II-1 while remaining low in those of 1-I, argues strongly in favor of a cell-intrinsic mechanism rather than involvement of a stromal component in the high HbF phenotype. We therefore focused our attention on the known regulators of HbF in adults. Remarkably, we find no significant differences in protein expression levels of the KLF1 and BCL11A transcription factors between father I-1, mother I-2 and child II-1. Since *HMIP-2* is thought to exert its effect on HbF through regulation of KLF1 levels via MYB [14], our genetic and molecular data collectively rule out alterations in the MYB-KLF1-BCL11A axis as the mechanism underlying high HbF in mother I-2, and patient II-1 after transplantation. Variants in the *HBB*, *BCL11A* and *HBS1L-MYB* loci together account for ~50% of the variation in γ-globin expression [15]. The remaining variation could be accounted for by loci with relatively small impact, and by rare variants with significant quantitative effects on γ-globin expression that are typically missed by population studies. Mutations in *KLF1* are an example of the latter [5, 12] but the family reported here does not carry *KLF1* mutations. We therefore hypothesize that mother I-2 carries an allele of a currently unknown locus that modulates γ-globin expression levels. Her son II-1 inherited this allele but it was subjected to epigenetic regulation during development. After transplantation, the epigenetic status of the maternal allele was reset, enabling full γ-globin expression and rendering the child transfusion-independent. Thus, we suggest that high HbF in one of the parents could be of prognostic value in these rare cases of transfusion-independence acquired after failed allogeneic BMT.

This hypothesis proposes the existence of an additional HbF modifier locus that is sensitive to epigenetic regulation. This notion offers support to efforts aimed at epigenetic reactivation of γ-globin expression. Notably, experimental drugs such as 5-azacytidine [16], short-chain fatty acids/butyrates [17], monoamine oxidase inhibitors [18] and histone deacetylase inhibitors [19], which all have been proposed as HbF inducing agents, are believed to act at least partially through epigenetic mechanisms.

The observations reported here could be relevant to the interpretation of the outcome of the French β-thalassemia gene therapy trial [20]. After the gene therapy procedure, which

involved an autologous BMT, the patient displayed an Hb of 10 g/dl of which 1/3 was HbF derived from the endogenous *HBG* genes. Upon gene therapy, HbF increased from around 1 g/dl to 3-3.5 g/dl. This high level of HbF was sustained over a long period (>60 months [20, 21]) and contributed significantly to the transfusion-independent status achieved after gene therapy. This raises the prospect that also in this case epigenetic events connected to the BMT procedure enabled increased γ-globin expression, and, importantly, that this phenomenon is not connected to allogeneic BMT *per se* but could be primarily dependent on the conditioning regime.

Conclusions

In rare cases, β-thalassemia patients may become transfusion-independent after a failed BMT due to increased HbF levels derived from autologous bone marrow cells. We propose that, secondary to the BMT procedure, epigenetic changes in the hematopoietic compartment affect the expression status of a novel HbF modifier locus thereby facilitating the observed increase in HbF levels. This supports research aimed at epigenetic reactivation of the *HBG* genes in β-thalassemia and SCD patients. Identifying the proposed novel HbF modifier locus will be a challenge for the future. Furthermore, our proposal that high HbF in one of the parents is of prognostic value for transfusion-independence acquired after failed allogeneic BMT should be tested in more cases. If these analyses confirm this notion, it could lead to a new treatment for similar cases involving conditioning of the patients without the need for allogeneic BMT.

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Disclosure of conflict of interest

None to declare.

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