

# Myelodysplastic syndrome unrelated to lentiviral vector in a patient treated with gene therapy for sickle cell disease

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## Key Points

- Ability to accurately attribute adverse events post-gene therapy is required to describe the benefit-risk of these novel treatments.
- A SCD patient developed myelodysplastic syndrome post-LentiGlobin treatment; we show how insertional oncogenesis was excluded as the cause.

## Introduction

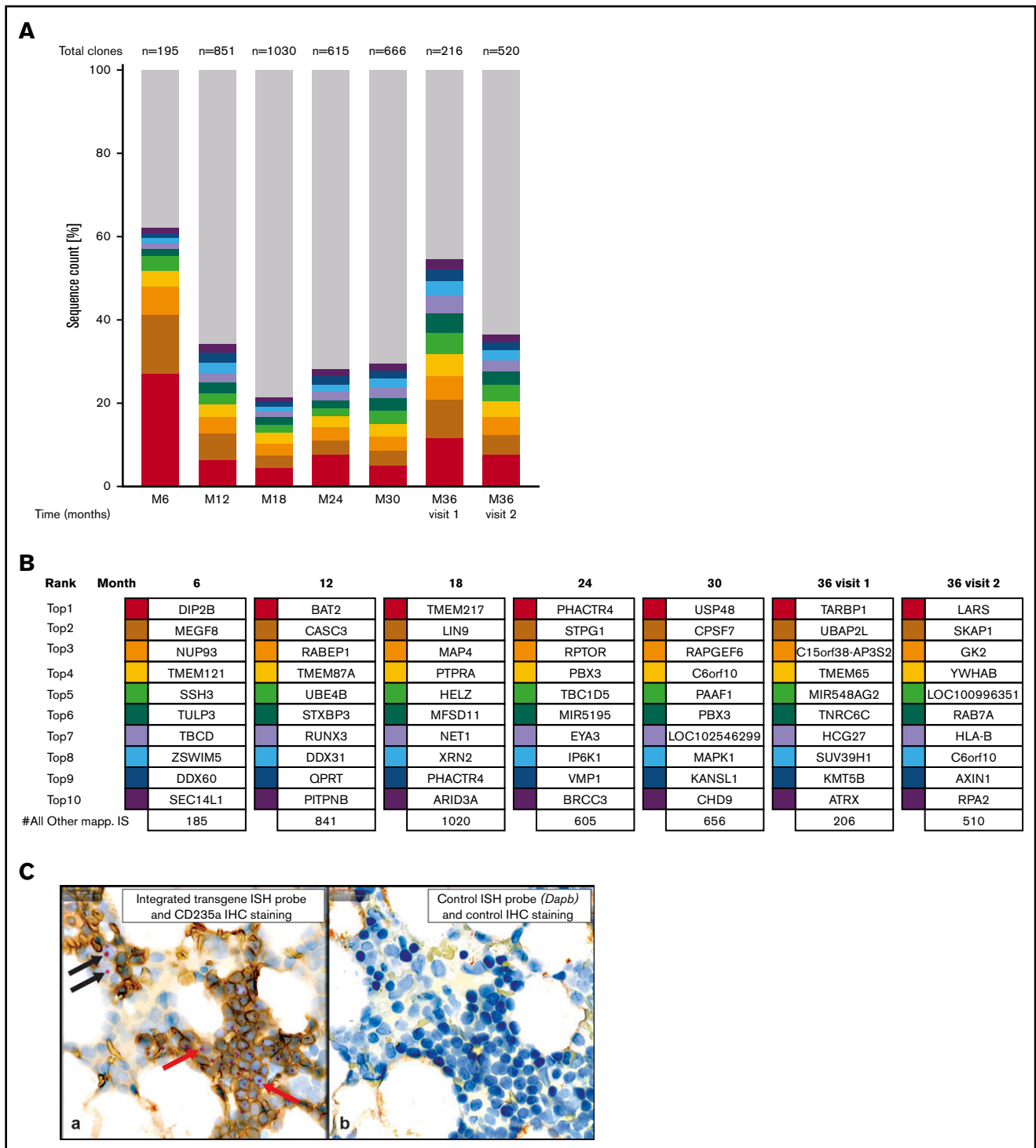
HGB-206 (registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) as #NCT02140554) is a multicenter, phase 1/2 study evaluating the safety and efficacy of LentiGlobin for sickle cell disease (SCD). The LentiGlobin drug product (DP) contains autologous CD34<sup>+</sup> hematopoietic stem cells (HSCs) transduced ex vivo with the BB305 lentiviral vector (LVV) encoding antisickling  $\beta$ -globin,  $\beta^{A-T87Q}$ .<sup>1,2</sup> Following myeloablative busulfan conditioning, patients who receive LentiGlobin DP are monitored for 2 years in HGB-206 and for an additional 13 years in a long-term follow-up study.

Although gene therapy has the potential for curative outcome, it may have associated risks. Most existing gene therapy and allogeneic HSC transplantation (HSCT) protocols require myeloablation with an alkylating agent, which carries a risk of secondary malignancy, including acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS).<sup>3,4</sup> Hematologic malignancies due to insertional oncogenesis were previously reported following gene therapy with  $\gamma$ -retroviral vectors in several hematologic diseases.<sup>5</sup> We have thus used a modified LVV developed to mitigate the potential risk of insertional oncogenesis, with no such cases reported for >110 patients treated with LVV-based HSC gene therapy in bluebird bio-sponsored clinical trials ( $\leq 5$  years follow-up) or in >200 patients described in the literature who received any LVV-based HSC gene therapy ( $\leq 12$  years follow-up)<sup>6</sup>; however, a theoretical risk remains.

This case describes the approaches used to assess whether MDS, diagnosed 36 months post-LentiGlobin infusion in a patient enrolled in HGB-206, was due to vector-mediated insertional oncogenesis.

## Methods

The patient, aged 42 years at consent, presented with SCD ( $\beta^S/\beta^S$  genotype) and a history of vaso-occlusive pain, asthma, hypertension, iron overload, leg ulcers, depression, transaminitis, and gallbladder disease. For 8 years before study entry, the patient received hydroxyurea (HU), which was discontinued 6 months pre-LentiGlobin treatment. This patient was a part of the initial cohort of 7 HGB-206 patients (group A) for whom the HSCs were collected by bone marrow (BM) harvest and DPs were made using the original manufacturing process, which was later modified for subsequent patients enrolled.<sup>7</sup> The LentiGlobin DP for this patient had a vector copy number (VCN) of 1.3 copies per diploid genome (c/dg), a cell dose of  $2.8 \times 10^6$  CD34<sup>+</sup> cells per kilogram, and 29% LVV-containing HSCs. Before LentiGlobin infusion, the patient received myeloablative conditioning with IV busulfan 3.3 mg/kg (200 mg) daily over 4 days. Per protocol, the goal for busulfan exposure is an area under curve (AUC) of 4000 (minimum, 3600; maximum, 5000)  $\mu\text{m} \times \text{minutes}$ . The busulfan pharmacokinetic monitoring was performed locally and a manually calculated AUC was 3460  $\mu\text{m} \times \text{minutes}$ , based on the partial actual busulfan level (5 of 6 planned busulfan pharmacokinetic measurements are available; the first value is missing). Neutrophil engraftment (absolute neutrophil counts  $\geq 0.5 \times 10^9/\text{L}$  for 3 consecutive days) and platelet engraftment (the first day of 3 consecutive platelet measurements  $\geq 50 \times 10^9/\text{L}$  without platelet



**Figure 1. Evaluation of ISs in whole blood and integrated transgene abundance in bone marrow precursors.** (A-B) IS analysis was done, using whole blood samples, by nonrestrictive linear amplification-mediated polymerase chain reaction [(nr)LAM PCR] and sequencing as described previously.<sup>2</sup> (A) IS analysis for months 6 through 36 showing distribution of gene-marked cell clones. Each of the top 10 most represented unique ISs is indicated by a different color, with gray showing the cumulative proportion of all other unique ISs. The total number of ISs is indicated at the top of each column. None of the clones showed a clonal contribution of >30% of the total retrieved ISs. Month 36 (M36) visit 1 was the regular study follow-up. M36 visit 2 was at the time of MDS diagnosis. (B) IS clonal abundance for months 6 to 36. For individual samples, sequence data from all (nr)LAM-PCR amplicons were combined. RefSeq gene names for the top 10 genes located next to or at the IS are shown. None of the top 10 most prominent clones appeared in >2 samples collected at different time points. (C) A core BM biopsy was collected from the patient 20 days post-MDS diagnosis, fixed in formalin, decalcified, and routinely processed. (a) In situ hybridization (ISH) was performed on a tissue section using a specific probe to detect integrated transgene DNA, and immunohistochemistry (IHC) for CD235a (glycophorin A) protein was used to identify erythroid precursors. Red and black arrows indicate examples of the integrated

transfusions for 7 days) occurred on days 17 and 29, respectively. One grade 3 serious adverse event of iron overload, assessed unrelated to LentiGlobin, occurred ~9 months post-LentiGlobin infusion.

Due to persistent anemia, HU was restarted and darbepoetin introduced at 1 and 2 years post-LentiGlobin infusion, respectively. Peripheral blood (PB) VCN over 3 years post-LentiGlobin infusion ranged from 0.08 to 0.15 c/dg and the resulting gene therapy-derived hemoglobin was 0.1 to 1.23 g/dL.

During a routine clinical visit 36 months post-LentiGlobin infusion, the patient had a  $3.43 \times 10^9/L$  white blood cell count,  $0.79 \times 10^9/L$  absolute neutrophil counts, a  $131 \times 10^9/L$  platelet count, and 7.3 g/dL hemoglobin. Routine blood tests revealed 3% blast-like cells in the PB, which increased to 6% in 9 days. Per BM biopsy, ~10% of BM cells were malignant myeloblasts. The patient was diagnosed with MDS with excess blasts (MDS-EB-2), designated a grade 4 serious adverse event. Post-MDS diagnosis, the patient received standard treatment including 5-azacytidine and decitabine, and, while on treatment, was diagnosed with AML. AML was treated by 7+3 induction chemotherapy of idarubicin/cytarabine, followed by reinduction with cladribine, high-dose cytarabine, and granulocyte-colony-stimulating factor. The patient subsequently received myeloablative doses of melphalan, fludarabine, and 200 rad of total-body irradiation, followed by an HLA-haploidentical HSCT and 2 doses of cyclophosphamide posttransplant. At 3 months post-transplant, the patient was in remission with BM findings consistent with posttransplant recovery, normal cytogenetics, no blasts in the PB, and no graft-versus-host disease. At 6 months posttransplant, blasts (confirmed by flow cytometry) were detected in the PB and BM, and treatment with a hypomethylating agent and venetoclax was initiated.

The study protocol was reviewed and approved by the Institutional Review Boards/Ethics Committees at all HGB-206 clinical sites, including the General Medicine 1 Institutional Review Board (Bethesda, MD).

## Results and discussion

Multiple cytogenetic and molecular assays were performed to investigate the MDS etiology. Malignant blasts had monosomy 7 and structurally abnormal chromosome 19p in 8 of 20 metaphases. Archived BM samples, collected preconditioning, were negative for monosomy 7 per fluorescence in situ hybridization (NeoGenomics, Fort Myers, FL) and negative for the 54 mutations associated with myeloid disorders per next-generation sequencing-based analysis (NeoTYPE; NeoGenomics, Fort Myers, FL).

Routine integration site (IS) analyses performed at 6-month intervals post-LentiGlobin infusion showed no protocol-defined clonal dominance through 36 months (Figure 1A).<sup>2</sup> No single IS represented >30% of the total sites, and the top 10 prevalent clones were transitory (Figure 1B).

The frequency of CD34<sup>+</sup> cells was stable: 1.83% at baseline in BM and 1.5% in PB at month 21 post-LentiGlobin infusion. RUNX1,

**Table 1. VCN analysis of CD34<sup>+</sup> and CD34<sup>-</sup> cells post-MDS diagnosis and post-AML recurrence**

Sample	Post-MDS diagnosis		Post-AML recurrence*
	Purity, %	VCN, c/dg	VCN, c/dg
<b>BM</b>			
Unsorted BM sample	n/a	0.14 ± 0.0	nd
CD34 <sup>-</sup> selected	98	0.21 ± 0.03	<LOQ
CD34 <sup>+</sup> selected for myeloblasts	93	0.02 ± 0.01 (<LOQ)	<LOQ
<b>PB</b>			
Unsorted PB sample	n/a	0.10 ± 0.0	nd
CD34 <sup>-</sup> selected	99	0.07 ± 0.01	<LOQ
CD34 <sup>+</sup> selected for myeloblasts	53	0.08 ± 0.01	<LOQ

Cells were collected from the patient at diagnosis (PB), 20 days postdiagnosis of MDS (BM), and 8 days post-AML recurrence (PB and BM). Ficoll-Paque density gradient or hypotonic ammonium chloride solution (ACK Lysing Buffer; Gibco) were used to deplete BM and PB of red blood cells (RBCs). Post-RBC depletion,  $\sim 2 \times 10^5$  mononuclear cells (MNCs) were set aside for VCN analysis (unsorted BM sample). For PB, the remaining cells were stained with anti-CD34 BV421 antibody (BD Biosciences) and separated into CD34<sup>+</sup> and CD34<sup>-</sup> fractions using fluorescence-activated cell sorting (SH800 cell sorter; Sony Biotechnology). For BM, remaining MNCs were stained with an anti-CD34-allophycocyanin, washed, and sorted via a Beckman Coulter MoFlo Astrios EQ cell sorter to isolate CD34<sup>-</sup> (CD34<sup>-</sup> selected) and CD34<sup>+</sup> (with malignant blasts as main contributors) cells. Purity of sorted cell populations was subsequently determined. Additionally, sorted cells were counted by trypan blue exclusion on a hemacytometer and cells from each population were used for VCN analysis, conducted as previously described.<sup>32</sup> For post-MDS diagnosis VCN, mean ± standard deviation is shown.

LOQ, limit of quantitation; n/a, not applicable; nd, not determined.

\*After haploidentical transplant.

KRAS, and PTPN11 mutations were detected by the NeoType analysis in the BM sample at MDS diagnosis (NeoGenomics). Malignant blasts were determined to be CD34<sup>+</sup>. Therefore, the enrichment of CD34<sup>+</sup> cells from BM and PB would be expected to predominantly yield malignant blasts, which would have a VCN  $\geq 1$  c/dg if LVV-mediated insertional mutagenesis was causing cell transformation. BM cells were sorted into CD34<sup>-</sup> (purity 98%) and CD34<sup>+</sup> (purity 93%) fractions and compared with unsorted cells in BM aspirate collected post-MDS diagnosis (Table 1). In unsorted and CD34<sup>-</sup> cells, the VCNs were 0.14 c/dg and 0.21 c/dg, respectively. In CD34<sup>+</sup> cells, mostly containing malignant blasts, the VCN was 0.02 c/dg, which was less than the lower limit of quantitation of 0.07 c/dg. This is consistent with the absence of vector integration in the CD34<sup>+</sup> blast cells. Similarly, enriched CD34<sup>+</sup> cells from PB did not have increased VCN compared with the unsorted sample (Table 1). In the CD34<sup>+</sup> and CD34<sup>-</sup> samples from BM post-AML recurrence after HLA-haploidentical HSCT, no LVV was detected.

DNA in situ hybridization of a BM sample collected post-MDS diagnosis with an integrated transgene-specific probe showed nuclear signals in isolated clusters of CD235<sup>+</sup> erythroid precursors and rarely within CD235<sup>-</sup> nonerythroid cells (Figure 1C).

LVV gene therapy has been safe in animal models,<sup>8-11</sup> and there have been no published cases of insertional oncogenesis in patients thus far.<sup>6,12-15</sup> The patient received HU for several years,

**Figure 1. (continued)** transgene signal within erythroid (CD235a<sup>+</sup>) and nonerythroid (CD235a<sup>-</sup>) precursors, respectively. (b) A serial section stained with a probe specific to an absent gene (the bacterial gene *Dapb*) to control for nonspecific ISH signals and with the isotype control antibody to assess the background IHC signal. Counterstaining with hematoxylin was also performed. Scale bars, 20  $\mu$ m.

but evidence supporting a relationship between HU use and myeloproliferative disease in SCD is lacking. Although MDS has been reported in 2 individuals with SCD after 14 and 15 years of HU treatment, respectively,<sup>16,17</sup> a systematic literature review (with up to 9 years of patient follow-up) concluded that HU use is not leukemogenic in patients with SCD.<sup>18</sup> Whether underlying SCD increases the risk of myeloid neoplasms is still under investigation, with recent epidemiology reports in California and the United Kingdom suggesting a higher risk of hematologic malignancies for patients with SCD. In the Seminog et al study, patients in the United Kingdom SCD cohort had significantly higher rate ratios for all cancers combined (2.1;  $P < .001$ ) and for hematologic malignancies (2.6-10.0;  $P$  range from  $<.001$  to  $.006$ , depending on type) compared with the non-SCD patient cohort.<sup>19</sup> Similarly, the Brunson et al study reported a 72% higher risk of hematologic malignancies for patients with SCD in California compared with the general population of California; the risk of solid tumors was 38% lower.<sup>20</sup> High cellular turnover in the BM and chronic inflammation associated with SCD have been discussed as factors that may contribute to leukemic transformation, but confirmatory mechanistic evidence is lacking.<sup>20</sup> In the patient described in this case study, the persistence of unmodified progenitor cells postablation and post-infusion may have occurred due to a low proportion of LVV-containing HSCs in the drug product ( $<30\%$ ), preserving a highly proliferative BM characteristic of hemoglobinopathies, which may have contributed to the development of MDS.<sup>21,22</sup> Additionally, given that the patient's actual busulfan AUC was lower than the goal per protocol, it is possible that residual unablated HSCs may have persisted postconditioning. The genomic stability of the patient's HSCs might have been compromised by hematopoietic stress associated with SCD and further disrupted by chemotherapy, potentially resulting in MDS, given that MDS and secondary AML are identified risks following HSCT using alkylating agents.<sup>4</sup> The finding of monosomy 7 in this patient is expected in secondary myeloid neoplasia, including following exposure to alkylating agents such as the busulfan.<sup>23</sup> Reports describe MDS in patients with SCD who experienced graft failure and autologous reconstitution post-HLA-haploidentical HSCT using nonmyeloablative conditioning regimens, typically with alkylating agents. One report showed that 2 of 23 patients with hemoglobinopathies (21 with SCD and 2 with thalassemia) who received 400 cGy total-body irradiation and/or posttransplant addition of alkylating agent cyclophosphamide developed high-grade MDS.<sup>24</sup> Another report highlighted a patient with SCD who underwent prolonged HU treatment ( $>5$  years) and developed a rapidly progressing MDS that transformed into AML after graft failure following an HLA-haploidentical transplant that included both radiotherapy and alkylating agents.<sup>25</sup> Currently, ex vivo HSC gene therapy requires myeloablative chemotherapy to ensure engraftment of gene-modified cells, but alternative nonchemotherapy and nonirradiation approaches aimed at reducing short- and long-term toxicities associated with conditioning warrant further investigation. Early protocols and clinical studies that explored naked antibody-based conditioning for HSCT still required the use of chemotherapy and/or radiation,<sup>26</sup> or application to particular disease states.<sup>27</sup> More recently, preclinical models were used to investigate approaches coupling cytotoxic conjugates, such as saporin, to antibodies targeting either CD45, expressed exclusively on most hematopoietic cells including HSCs, or CD117 (c-Kit), a marker of HSCs and hematopoietic progenitors.<sup>28,29</sup> Additionally, anti-c-Kit antibodies have been tested in mice in combination with antagonists

targeting a "don't eat me" signal CD47, with the goal of activating macrophage-assisted depletion of anti-c-Kit-antibody-marked HSCs.<sup>30</sup> Although these methods have enabled varying levels of HSC clearance in animal models, their efficacy and safety in human studies, in particular with respect to off-target effects, the impact on the body's ability to fight opportunistic infections, and potential to result in long-term multilineage engraftment of transplanted cells, remain to be determined.<sup>31</sup>

In summary, a patient in HGB-206, who did not have MDS prior to myeloablation, developed MDS post-LentiGlobin treatment. Multiple independent assays demonstrated the absence of vector integration in the CD34<sup>+</sup> blasts and excluded LVV-mediated oncogenesis as the MDS cause. The data presented here show that the MDS in this patient was unrelated to LentiGlobin.

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## Authorship

Contribution: A.A.T., M.A., M.B., M.M.H., J.K., F.J.P., J.F.T., and M.C.W. contributed to the conception, design, and planning of the analysis; M.A., M.B., F.J.P., J.R., J.F.T., L.D., M.S., and N.U. contributed to data analysis; M.B., M.M.H., F.J.P., J.R., M.S., and N.U. contributed to data acquisition; A.A.T., M.A., M.B., M.M.H., J.K., F.J.P., J.F.T., and M.C.W. contributed to interpretation of the results; and all authors contributed to critically reviewing or revising the manuscript.

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