ORIGINAL ARTICLE

Hematologic Cancer after Gene Therapy for Cerebral Adrenoleukodystrophy

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ABSTRACT

BACKGROUND

Gene therapy with elivaldogene autotemcel (eli-cel) consisting of autologous CD34+ cells transduced with lentiviral vector containing *ABCD1* complementary DNA (Lenti-D) has shown efficacy in clinical studies for the treatment of cerebral adrenoleukodystrophy. However, the risk of oncogenesis with eli-cel is unclear.

METHODS

We performed integration-site analysis, genetic studies, flow cytometry, and morphologic studies in peripheral-blood and bone marrow samples from patients who received eli-cel therapy in two completed phase 2–3 studies (ALD-102 and ALD-104) and an ongoing follow-up study (LTF-304) involving the patients in both ALD-102 and ALD-104.

RESULTS

Hematologic cancer developed in 7 of 67 patients after the receipt of eli-cel (1 of 32 patients in the ALD-102 study and 6 of 35 patients in the ALD-104 study): myelodysplastic syndrome (MDS) with unilineage dysplasia in 2 patients at 14 and 26 months; MDS with excess blasts in 3 patients at 28, 42, and 92 months; MDS in 1 patient at 36 months; and acute myeloid leukemia (AML) in 1 patient at 57 months. In the 6 patients with available data, predominant clones contained lentiviral vector insertions at multiple loci, including at either *MECOM–EVI1* (MDS and EVI1 complex protein EVI1 [ecotropic virus integration site 1], in 5 patients) or *PRDM16* (positive regulatory domain zinc finger protein 16, in 1 patient). Several patients had cytopenias, and most had vector insertions in multiple genes within the same clone; 6 of the 7 patients also had somatic mutations (*KRAS*, *NRAS*, *WT1*, *CDKN2A* or *CDKN2B*, or *RUNX1*), and 1 of the 7 patients had monosomy 7. Of the 5 patients with MDS with excess blasts or MDS with unilineage dysplasia who underwent allogeneic hematopoietic stem-cell transplantation (HSCT), 4 patients remain free of MDS without recurrence of symptoms of cerebral adrenoleukodystrophy, and 1 patient died from presumed graft-versus-host disease 20 months after HSCT (49 months after receiving eli-cel). The patient with AML is alive and had full donor chimerism after HSCT; the patient with the most recent case of MDS is alive and awaiting HSCT.

CONCLUSIONS

Hematologic cancer developed in a subgroup of patients who were treated with eli-cel; the cases are associated with clonal vector insertions within oncogenes and clonal evolution with acquisition of somatic genetic defects. (Funded by Bluebird Bio; ALD-102, ALD-104, and LTF-304 ClinicalTrials.gov numbers, NCT01896102, NCT03852498, and NCT02698579, respectively.)

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Adrenoleukodystrophy is an X-linked metabolic disease caused by mutations in the *ABCD1* gene that lead to a deficiency in encoding peroxisomal transporter ATP-binding cassette domain 1 (adrenoleukodystrophy protein) and the buildup of very-long-chain fatty acids in tissue and plasma.[1,2] Cerebral adrenoleukodystrophy develops in approximately 35% of affected boys before adulthood[1,3] and results in progressive destruction of white matter, loss of cognitive and neurologic function, and early death if untreated.[3,4]

To express *ABCD1* complementary DNA (cDNA), the elivaldogene autotemcel (eli-cel) gene therapy, which contains autologous CD34+ cells transduced with Lenti-D lentiviral vector, uses a virally derived synthetic regulatory element that includes the U3 segment of the myeloproliferative sarcoma virus long terminal repeat with the negative control region deleted and the DL587 endogenous retrovirus primer binding site substituted (MNDU3).[5-7] When the eli-cel studies were initiated (approximately 15 years ago), the cell types that were needed to express functional adrenoleukodystrophy protein and the level of therapy necessary to stabilize disease progression were unknown. Therefore, MNDU3 was chosen because it is a strong, ubiquitous promoter–enhancer that drives consistent gene expression across different cell lineages.[8,9] In 2009, in a study involving a small number of patients with cerebral adrenoleukodystrophy, Cartier et al. showed that gene therapy that used an MNDU3-based vector provided relative neurologic stability.[6]

We evaluated eli-cel in two completed clinical studies (ALD-102 and ALD-104), with ongoing long-term follow-up in one integrated study (LTF-304) involving the patients in both the ALD-102 and the ALD-104 study. The study design of ALD-102 has been described previously.[7] As reported in this issue of the *Journal*,[10] boys 17 years of age or younger with early, active cerebral adrenoleukodystrophy who were treated with eli-cel in the ALD-102 study have been followed for up to 8.9 years in the LTF-304 study. As of February 1, 2023, 26 of 32 patients (81%) remained in the study and were free of major functional disabilities (Kaplan–Meier estimate of survival free of major functional disabilities, 75%; overall survival, 94%). In the phase 3 ALD-104 study, all 35 patients who were treated with eli-cel completed 24 months of follow-up and enrolled in the long-term follow-up study.

Although the integration of lentiviral vectors has proved to be safe in previous clinical studies of gene therapy,[11] two reports have described clonal expansions related to lentiviral vector insertions associated with abnormal splicing events.[12,13] We comprehensively characterize the clinicopathological and genetic features of seven cases of hematologic cancer (as of April 25, 2024) in patients who received eli-cel (one patient in the ALD-102 study and six patients in the ALD-104 study).

Methods

eli-cel Drug Product

The eli-cel drug product was manufactured by transduction of autologous enriched CD34+ hematopoietic progenitor cells with Lenti-D lentiviral vector encoding the *ABCD1* cDNA under the control of an internal MNDU3 promoter–enhancer; CD34+ cells were collected after the patients received granulocyte colony-stimulating factor (G-CSF) with or without plerixafor mobilization (in the ALD-102 study) or G-CSF with plerixafor mobilization (in the ALD-104 study) (see Fig. S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org). The conditioning agents were busulfan–cyclophosphamide in the ALD-102 study and busulfan–fludarabine in the ALD-104 study. The multiplicity of infection (the number of transducing viral particles present relative to host cells) of the lentiviral vector ranged from 29 to 46 for the ALD-102 study and was fixed at 40 for the ALD-104 study.

Analyses and Examinations

Integration-site analysis was performed, according to published methods,[14,15] on the peripheral blood of the patients who were treated with eli-cel to determine patterns of integration of the proviral Lenti-D lentiviral vector DNA in genomic DNA (gDNA) (see the Supplementary Appendix). Additional analyses including physical examinations, blood tests, bone marrow examination, and imaging tests were performed as specified in the study protocol, available at NEJM.org. Bone marrow aspirates, biopsy specimens, and accompanying whole-blood specimens were obtained for

storage at the time of the collection of bone marrow samples to facilitate subsequent exploratory analyses carried out either at a central laboratory or at the treating physician's preferred laboratory. These analyses included integration-site analysis and vector copy number; targeted sequencing of a panel of genes associated with leukemias, myelodysplastic syndromes, and myeloproliferative neoplasms by means of the Rapid Heme Panel; gene expression studies; chromosome analysis; fluorescence in situ hybridization; and flow cytometry. Additional clinical laboratory tests were performed at the investigator's discretion. Definitions of successful engraftment, normal complete blood count, oligoclonality, persistent oligoclonality, and current persistent oligoclonality can be found in the Supplementary Appendix, along with flow cytometry methods.

RESULTS

PATIENTS

A total of 67 patients received eli-cel infusion during the completed studies (32 patients in the ALD-102 study and 35 patients in the ALD-104 study). Mobilization, apheresis, conditioning, and engraftment details for the patients who received eli-cel are shown in Table S1 in the Supplementary Appendix. As of April 25, 2024, the probability of event-free survival (survival without major functional disabilities, hematologic cancer, or rescue-cell administration or allogeneic hematopoietic stem-cell transplantation) at 4 years was 81.0% (95% confidence interval [CI], 68.8 to 88.8) (Fig. 1).

Hematologic cancer developed in 7 patients (incidence rate, 2.1 per 100 person-years [95% CI, 0.8 to 4.2]; 338 person-years of total follow-up): 1 who had been treated in the ALD-102 study (incidence rate, 0.5 per 100 person-years [95% CI, 0 to 2.5]; 222 person-years of follow-up) and 6 in the ALD-104 study (incidence rate, 5.2 per 100 person-years [95% CI, 1.9 to 10.9]; 116 person-years of follow-up). Table 1 lists the baseline and treatment characteristics of the patients, and Table 2 lists the pathological and genetic findings (up to April 25, 2024) for the 6 patients in whom myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) developed, along with limited available data for a recently identified 7th patient in whom MDS developed.

BASELINE, ENGRAFTMENT, AND COMPLETE BLOOD COUNT DATA

The patients with hematologic cancer had received eli-cel when they were between 5 and 13 years of age and had no history of blood disorders. The baseline blood counts and characteristics of the conditioning agents and the drug product did not differ materially between the patients with hematologic cancer and those without hematologic cancer (Table 1). After receiving eli-cel, Patient 36 and Patient 46 had delayed platelet engraftment (>100 days postinfusion) (Fig. 2). Patient 3 had prolonged mild thrombocytopenia after platelet engraftment; however, platelet counts, white-cell counts, and hemoglobin levels ultimately returned to normal levels (Fig. 2). Persistent thrombocytopenia developed in Patient 54 at month 23.

DEVELOPMENT OF HEMATOLOGIC CANCER

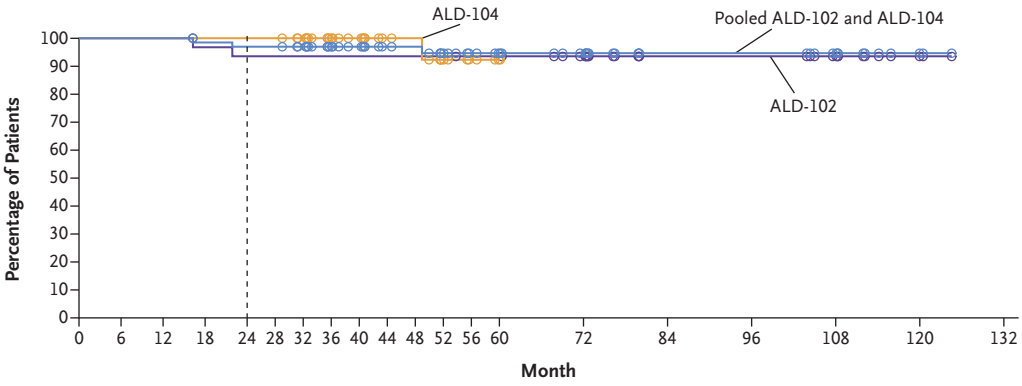
Hematologic cancer developed 14 to 92 months after the receipt of gene therapy (Table 2). Three patients received a diagnosis of MDS with excess blasts, two patients received a diagnosis of MDS with unilineage dysplasia, one patient received a diagnosis of AML, and one patient received a diagnosis of MDS. In 6 of the 7 patients, hematologic cancer was associated with the presence of somatic mutations (*KRAS*, *NRAS*, *WT1*, *CDKN2A* or *CDKN2B*, or *RUNX1*); in 1 patient, the cancer was associated with monosomy 7. Histopathological features of a patient with MDS are shown in Figure S2. Further information on the diagnosis of hematologic cancer can be found in the Supplementary Appendix.

INTEGRATION-SITE ANALYSIS AND CLONAL ANALYSES

All Patients

As of April 25, 2024, among all 67 patients involved in both studies, the median highest total number of unique mappable insertion sites was 6973 (range, 582 to 15,683) (Table 3). Of the 10 genes with the highest frequency of insertions reported in patients, *SMG6*, *MECOM*, *CCND2-AS1*, *MPL*, and *C6ORF10* were the most abundant in the study population (Table S2). The total number of unique mappable insertion sites over time and genes with the greatest number of unique insertions across the two ALD trials and all the related Bluebird Bio–sponsored studies involving lentiviral vector therapy can be found in Table S3 and Figure S3, respectively. Across the ALD-102

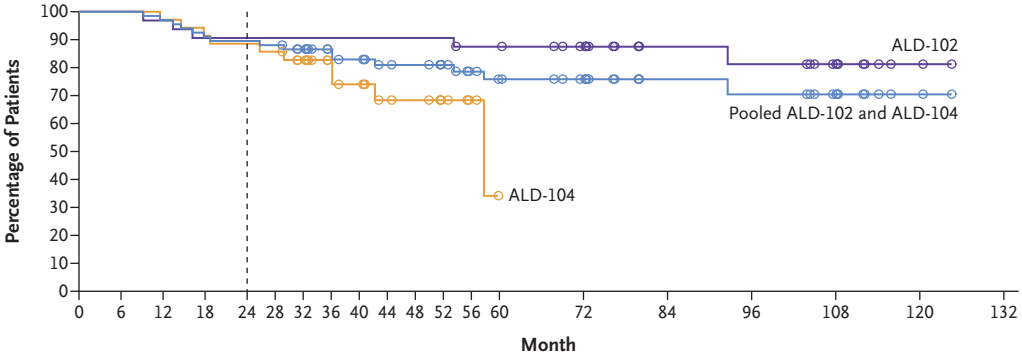
A Overall Survival



No. at Risk

ALD-102	32	32	32	30	29	29	29	29	29	29	29	28	28	24	14	14	10	3	0
ALD-104	35	35	35	35	35	35	32	24	20	14	13	8	4	1	0				
Pooled ALD-102 and ALD-104	67	67	67	65	64	64	61	53	49	43	42	37	32	29	24	14	14	10	3	0

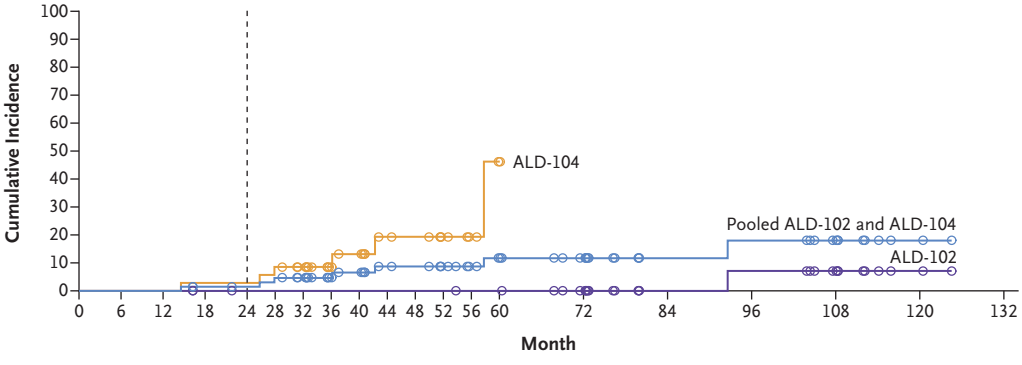
B Event-free Survival



No. at Risk

ALD-102	32	32	31	29	29	29	29	29	29	29	29	27	27	23	14	13	9	2	0
ALD-104	35	35	34	32	31	30	26	19	16	11	10	6	3	0					
Pooled ALD-102 and ALD-104	67	67	65	61	60	59	55	48	45	40	39	35	30	27	23	14	13	9	2	0

C Hematologic Cancer among Patients Treated with Eli-Cel in ALD-102 and ALD-104



No. at Risk

ALD-102	32	32	32	30	29	29	29	29	29	29	29	28	28	24	14	13	9	2	0
ALD-104	35	35	35	34	34	32	29	21	18	12	11	7	4	1	0				
Pooled ALD-102 and ALD-104	67	67	67	64	63	61	58	50	47	41	40	36	32	29	24	14	13	9	2	0

Figure 1 (facing page). Overall Survival, Event-free Survival, and Hematologic Cancer.

Shown are Kaplan–Meier curves for overall survival (Panel A), event-free survival (Panel B), and hematologic cancer (Panel C) in patients treated with eli-cel. Purple lines represent the ALD-102 study, orange lines represent the ALD-104 study, and blue lines represent pooled date from the ALD-102 and ALD-104 studies. The ongoing long-term follow-up study (LTF-304) involving patients from both the ALD-102 and ALD-104 studies began after month 24 (dashed line). Results for cumulative incidence curves of hematologic cancer were consistent when death and allogeneic hematopoietic stem-cell transplantation (HSCT) for progression or patient withdrawal were included as competing events. Exposure-adjusted incidence rate per 100 person-years was calculated as 100 times the number of events divided by the total person-years of follow-up. For patients who received a diagnosis of hematologic cancer or who underwent allogeneic HSCT, the follow-up time is up to the earliest time the diagnosis was received or when the patient underwent allogeneic HSCT. The data shown are as of April 25, 2024.

and ALD-104 studies, 29 patients (43%) met the criteria for persistent oligoclonality at any time during the study, of whom 20 patients (69%) had persistent oligoclonality at the most recent follow-up or before undergoing allogeneic HSCT. A total of 22 patients (33%) had persistent oligoclonality with insertion in a known oncogene at any time (Table 3).

Patients with Hematologic Cancer

Plots of peripheral-blood integration-site analysis over time for individual patients with hematologic cancer are shown in Figure 2, and the vector copy number in peripheral blood and CD14+ cells is shown in Table S4. All seven patients, as compared with 35% of patients without hematologic cancer, had current oligoclonality. Six of seven patients (86%), as compared with 27% of the patients without hematologic cancer, had a persistent integration site in a known oncogene at any time up to the most recent follow-up or before undergoing allogeneic HSCT (Table 3). Patient 54 had a current integration site in a known oncogene (*MECOM*) but did not meet the criterion for a persistent integration site because the patient did not have two consecutive oligoclonality measurements before undergoing allogeneic HSCT. Further details of integration-site analysis for the seven patients with hematologic cancer can be found in the Supplementary Appendix.

Genomic Analyses of MECOM *and* PRDM16 *Loci*

A detailed genomic analysis was conducted with the use of data that were collected until April 3, 2024, with a specific focus on the *MECOM* locus (Fig. S5) and a more limited focus on its close homologue, *PRDM16* (Fig. S6). In total, 99% of the patients (66 of 67) who were treated with eli-cel had integrations in *MECOM*, with an average of 47 integration sites present in cells from the peripheral blood (range, 0 to 180). The pattern of integrations did not differ between patients with maintained polyclonality and those with persistent oligoclonality, MDS, or both (Fig. S5A).

Integration sites in *MECOM* that were associated with MDS and persistent oligoclonality fell primarily within intron 2 (Fig. S5B), a finding consistent with the preferential intronic lentiviral vector integration across multiple lentiviral vector products that were evaluated in Bluebird Bio–sponsored clinical trials (Fig. S7). A slight increase in *MECOM* insertions was noted in the patients whose disease progressed to MDS, but these insertions did not reach statistical significance as compared with those in patients with persistent oligoclonality or polyclonality. The number of MECOM insertions in patients with expanded *MECOM* clones did not differ from the number in patients with persistent oligoclonality or polyclonality, which suggested that the absolute number of integrations was not associated with clonal expansion (Fig. S5C). Similar results were found with respect to *PRDM16*, albeit with lower frequencies of integration than in *MECOM* (Figs. S6 and S8). Analysis of peripheral-blood samples after engraftment revealed that the fraction of insertions into *MECOM* and *PRDM16* was significantly higher in the patients involved in the ALD-102 and ALD-104 studies than in the patients involved in the other Bluebird Bio–sponsored studies, which used different lentiviral vectors (Fig. S9).

OUTCOMES IN PATIENTS WITH HEMATOLOGIC CANCER

Five patients with hematologic cancer underwent myeloablative conditioning and allogeneic HSCT 3 to 5 months after receiving the diagnosis, with Patients 3, 44, and 54 receiving cytoreductive therapy before transplantation (Table 2). None of these five patients had available HLA-identical donors. More recently, Patient 33 received a diagnosis of AML, and Patient 61 received a diagnosis

Table 1. Selected Baseline and Treatment Characteristics of Presented Patients.*

Characteristic	Patient 3	Patient 46	Patient 36	Patient 44	Patient 54	Patient 33	Patient 61	Patients without Hematologic Cancer (N=60)
				Value				*Median (range)*
Age at eli-cel infusion — yr	5	11	13	10	9	6	7	6 (4–14)
History of blood disease	No	No	No	No	No	No	No	
Baseline blood count†								
Hemoglobin — g/dl	11.7	13.7	12.8	14.9	13.1	12.8	10.2	13.5 (10.5–15.7)
White cells — $\times 10^{-9}$/liter	6.9	4.7	3.2	7.29	4.8	8	6	6.7 (3.5–15.7)
Platelets — $\times 10^{-9}$/liter	347	245	405	336	165	243	157	303 (191–492)
Mobilization regimen	G-CSF	G-CSF and plerixafor	G-CSF and plerixafor	G-CSF and plerixafor	G-CSF and plerixafor	G-CSF and plerixafor	G-CSF and plerixafor	
Conditioning regimen	Busulfan–cyclophosphamide	Busulfan–fludarabine	Busulfan–fludarabine	Busulfan–fludarabine	Busulfan–fludarabine	Busulfan–fludarabine	Busulfan–fludarabine	
Estimated average area under the plasma busulfan concentration–time curve per day — min×μmol/liter	4729	4995	5586	5282	5473	5640	5160	4970 (3478–5695)
VCN in drug product — c/dg	1.6	1.3	1.8	1.2	1.4	3.1	1.1	1.2 (0.5–2.7)
Total cells in drug product — $\times 10^{-6}$/kg of body weight	6	5.7	12.1	15.1	9.6	22.8	7.7	12.0 (5.0–38.2)
Lentiviral vector cells in drug product — %	62	ND	70	45	60	84	41	47 (19–74)
Platelet engraftment — days after drug infusion	37	106	104	24	21	34	58	29 (14–108)
Neutrophil engraftment — days after drug infusion	37	14	12	12	15	13	17	13 (11–41)

* Shown are data as of April 25, 2024. Patient 3 was in the ALD-102 study, and Patients 46, 36, 44, 54, 33, and 61 were in the ALD-104 study. The data from patients without hematologic cancer were pooled from both studies. Eli-cel denotes elivaldogene autotemcel, G-CSF granulocyte colony-stimulating factor, ND not determined, and VCN vector copy number.
† For blood counts, baseline was defined as the last nonmissing assessment before hematopoietic stem-cell mobilization.

of MDS. Patient 33 underwent cytoreductive therapy and chemotherapy followed by myeloablative conditioning for transplantation involving a matched unrelated donor, but Patient 61 had not received any treatment for MDS at the most recent follow-up.

After allogeneic HSCT, chimerism data for Patients 3, 46, 36, and 54 showed that the patients had 100% donor cells in bone marrow and whole blood after allogeneic HSCT; chimerism data for Patient 44 showed that the patient had more than 97% donor cells. Four of five patients (Patients 3, 46, 44, and 54) remain alive after successful allogeneic HSCT, with no graft-versus-host disease (GVHD). One patient (Patient 44) had minimal residual disease that was reported as negative at the most recent follow-up after the infusion of donor leukocytes. Patient 36 was treated for presumptive chronic GVHD of the lung and ultimately died at month 49 from pulmonary hemorrhage resulting from pulmonary aspergillosis. The patient with AML (Patient 33) is alive and had full donor chimerism after HSCT. The patient in whom MDS was most recently diagnosed (Patient 61) is alive and awaiting HSCT.

As of April 25, 2024, the Loes scores of the patients with hematologic cancer ranged from 0 to 13, and the neurologic function score ranged from 0 to 5 (Table S5). The Loes score, which measures adrenoleukodystrophy white-matter lesions and atrophy on MRI, ranges from 0 to 34, with higher scores indicating more extensive disease; the neurologic function scale ranges from 0 to 25, with higher scores indicating more severe deficits. Patient 54 reported a major functional disability (total incontinence) at month 18 (before MDS developed). Patients 3, 33, 44, 46, and 61 remain free of major functional disabilities at the most recent follow-up.

DISCUSSION

As of April 25, 2024, among the 67 patients treated with eli-cel, 7 patients (10%) have received a diagnosis of hematologic cancer (338 person-years of total follow-up; incidence rate, 2.1 per 100 person-years; 95% CI, 0.8 to 4.2). Hematologic cancer was diagnosed in 1 of 32 patients (3%) treated in the ALD-102 study with 222 person-years of follow-up (incidence rate, 0.5 per 100 person-years; 95% CI, 0 to 2.5) and in 6 of 35 patients (17%) in the ALD-104 study with 116 person-years of follow-up (incidence rate, 5.2 per 100 person-years; 95% CI, 1.9 to 10.9). Incidence rates do not include the minimal residual disease in Patient 44. All seven cases are probably mediated by Lenti-D lentiviral vector insertion. Of the six cases with available data, all were found to be associated with expansion of a clone that contains at least one confirmed insertion site in a known proto-oncogene: *MECOM* in five cases and *PRDM16* in 1 case. The seventh case of cancer is undergoing more-detailed investigation.

Six of the seven cases of hematologic cancer occurred in patients who were treated in the ALD-104 study, which seems disproportionate given that the length of follow-up for patients originally enrolled in the ALD-102 study is longer than for those originally enrolled in the ALD-104 study. In the ALD-102 study, 11 of 32 patients (34%) underwent mobilization with both G-CSF and plerixafor. In the ALD-104 study, all the patients underwent mobilization with G-CSF and plerixafor. The conditioning agents were busulfan–cyclophosphamide in the ALD-102 study and busulfan–fludarabine in the ALD-104 study. Previous studies in allogeneic HSCT seem to show similar efficacy but suggest lower systemic toxic effects and a higher incidence of cytopenias with busulfan–fludarabine conditioning than with busulfan–cyclophosphamide.[16-19] An increased incidence of secondary neoplasms has been shown after transplantation in patients who received low-intensity conditioning regimens.[20,21] Whether the differences in mobilization and chemotherapy regimens played a role in the development of hematologic cancer remains unclear.

Five of the seven patients had prolonged cytopenias, particularly thrombocytopenia, after engraftment. The presence of megakaryocyte dysplasia in multiple patients and the recent observations of a more proximate lineage relationship of megakaryocytopoiesis with hematopoietic stem cells[22] (which are the target of autologous gene modifications) may suggest some biologic relevance to prolonged thrombocytopenia during engraftment, the appearance of megakaryocyte dysplasia in this phenotype, or both.

Unlike γ-retroviral vectors, self-inactivating lentiviral vectors lack strong promoter–enhancer long terminal repeat sequences and preferentially integrate in introns, away from the transcription start sites of actively transcribed genes[23]; thus, they are considered to be safe.[23,24] Lentiviral vec-

Table 2. Bone Marrow Findings and Hematologic Cancer Diagnosis and Treatment.*

Characteristic	Patient 3	Patient 46	Patient 36	Patient 44	Patient 54	Patient 33	Patient 61
Bone marrow findings							
Bone marrow cell morphologic characteristics	Mo 92: Mild hypocellularity (60%) Increased myeloid–erythroid ratio and left-shifted myeloid maturation Blasts, 15% Trilineage dysplasia present including abundant micromegakaryocytes	Mo 12: Moderate hypocellularity (40–50%); <10% Atypical megakaryocytes† Blasts, <5% Mo 14 and 18: 15% Hypocellularity with progressive megakaryocytic dysplasia including micromegakaryocytes	Mo 26: Normocellularity (80%), with trilineage hematopoietic maturation Dysplastic megakaryocytes Blasts, 1%	Mo 42: Mild hypocellularity (50–60%) with dysplastic megakaryocytes Myeloblasts, 8%, showing abnormal coexpression of CD7	Mo 28: Myelodysplasia with 18% blasts	Mo 57: AML with myelomonocytic features 48–65% blasts Normocellular bone marrow (80–90%)	Mo 36: Diminished cellularity Presence of a group of myeloid blast cells (7%), including a blast cell with an Auer body consistent with myeloid MDS
Chromosome and karyotype analysis	Normal	Presumed germline aberration at chromosome 14	Normal	Normal	Monosomy 7, 80%	Normal	NA
MDS FISH	Normal	Normal	Normal	Normal	NA	Normal	NA
Targeted deep sequencing with RHP	Somatic mutations in *KRAS* c.35G>C (p.G12A) at 14% VAF, and *NRAS* c.35G>A (p.G12D) at 3% VAF and *JAK2* c.2696T>C (p.I899T), VUS at 48% VAF	Germline VUS in *CDKN2A* c.168C>G (p.S56R) at a VAF of 41%	No somatic mutations in the genes screened	Pathogenic *WT1* c.1142C>A (p.S381‡) at 39% VAF and a VUS in *CDKN2B* c.34G>A (p.G12S) at 38% VAF	Mutation in *RUNX1* c.508+1_508+3delGTAinsAG (splice site) at 4% VAF	Somatic mutation in *KRAS* c.35G>A(p.G12) at 14.6% VAF (206x consensus coverage)	Mutation in *RUNX1* c.496 C>G (p.R166G) at 8.7% VAF (922x consensus frequency)
Diagnosis and treatment of hematologic cancer							
Age at diagnosis — yr	12	12	15	13	11	11	10
Diagnosis	MDS-EB	MDS-ULD	MDS-ULD	MDS-EB	MDS-EB	AML	MDS
Time of diagnosis — mo since eli-cel infusion	92	14	26	42	28	57	36
Pretransplantation therapy	Cytoreductive therapy	NA	NA	Cytoreductive therapy	Cytoreductive therapy	Cytoreductive therapy and chemotherapy	NA

Transplantation therapy	Myeloablative conditioning and allogeneic HSCT	Myeloablative conditioning and allogeneic HSCT	Myeloablative conditioning and allogeneic HSCT	Myeloablative conditioning and allogeneic HSCT	Myeloablative conditioning and allogeneic HSCT	NA	NA
Donor type	Unrelated mismatch cord donor	Parent haplotransplant	Parent haplotransplant	Sibling haplotransplant	Parent haplotransplant	NA	NA
Relative time of allo-HSCT — mo	95	19	29	45	31	NA	NA
Relative time of post–allogeneic HSCT bone marrow investigation — mo	96	21	31	52§	33	NA	NA
Post–allogeneic HSCT bone marrow findings	100% donor cells; morphologic, immunophenotypic, and molecular remission	100% donor cells; flow cytometry, FISH, and karyotype, normal; no mutations detected with RHP	100% donor cells; flow cytometry and morphologic analyses, normal	>97% donor cells,§ trace recipient; morphologic analyses, normal; cytogenetics, normal; MRD, negative	100% donor cells	NA	NA
Current status	Mo 120: alive, free of MFD, MDS resolved	Mo 43: alive, free of MFD, MDS resolved	Mo 49: died from GVHD	Mo 52: alive, free of MFD, MDS relapsed (MRD, negative at last follow-up)§	Mo 37: alive, MDS resolved	Mo 61: alive, free of MFD, AML unresolved	Mo 37: alive, MDS unresolved

* AML denotes acute myeloid leukemia, EB excess blasts, FISH fluorescence in situ hybridization, GVHD graft-versus-host disease, HSCT hematopoietic stem-cell transplantation, MDS myelodysplastic syndrome, MFD major functional disabilities, MRD minimal residual disease, NA not available, RHP Rapid Heme Panel, ULD unilineage dysplasia, VAF variant allele frequency, and VUS variant of unknown significance.
† Megakaryocytes are characterized by small forms with hypolobulated nuclei or separated nuclear lobes.
‡ Shown are data as of 12 June 2024.
§ The patient had minimal residual disease at month 51 and received a donor lymphocyte infusion in the same month. Bone marrow analysis conducted at month 52 showed no evidence of MDS or MRD positivity. He has since received two more donor lymphocyte infusions with a higher cell dose.

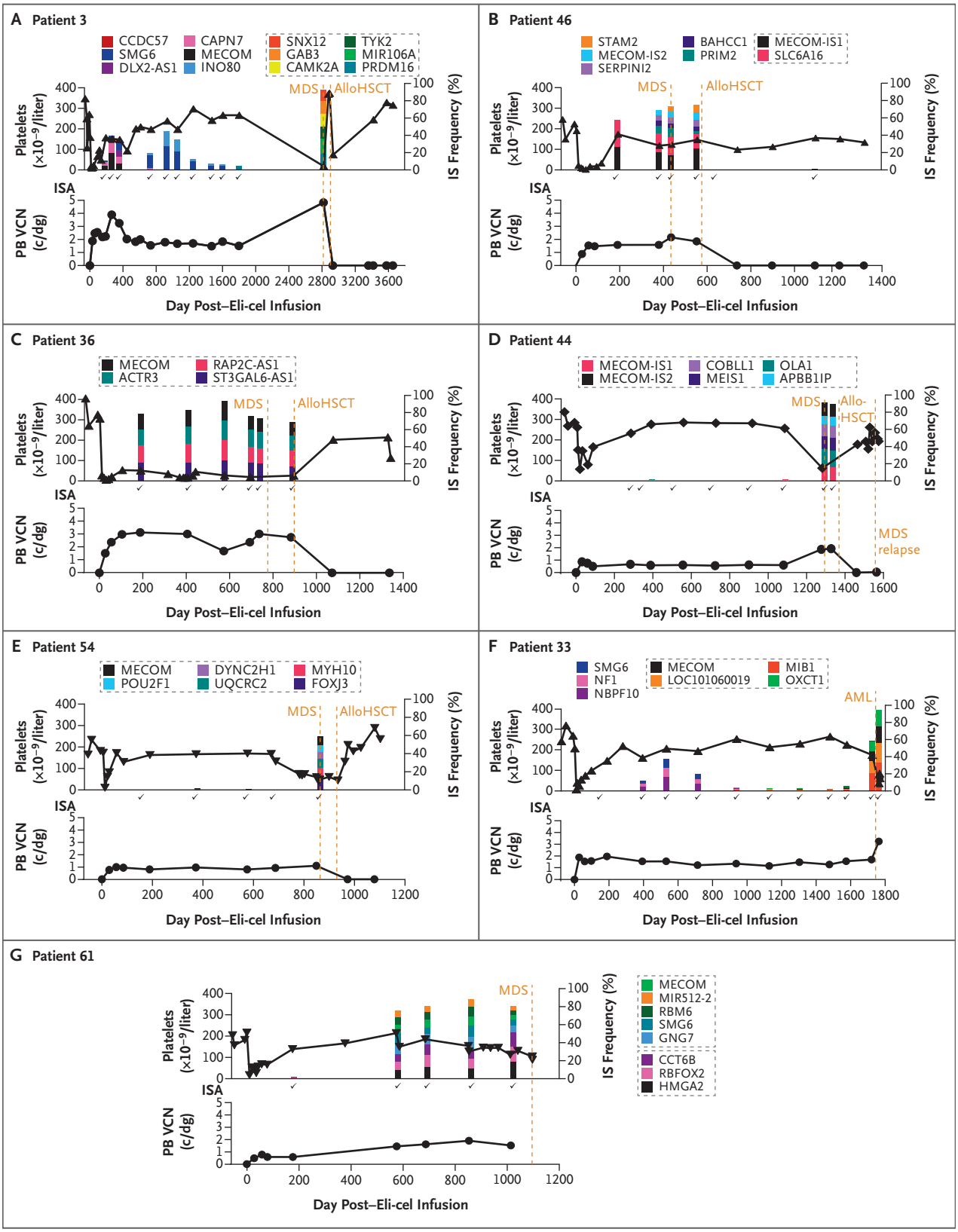

A Patient 3
CCDC57
SMG6
DLX2-AS1
CAPN7
MECOM
INO80
SNX12
GAB3
CAMK2A
TYK2
MIR106A
PRDM16
MDS
AlloHSCT
Platelets (×10⁻⁹/liter)
IS Frequency (%)
ISA
PB VCN (c/dg)
Day Post–Eli-cel Infusion
B Patient 46
STAM2
MECOM-IS2
SERPINI2
BAHCC1
PRIM2
MECOM-IS1
SLC6A16
C Patient 36
MECOM
ACTR3
RAP2C-AS1
ST3GAL6-AS1
D Patient 44
MECOM-IS1
MECOM-IS2
COBLL1
MEIS1
OLA1
APBB1IP
Allo-HSCT
MDS relapse
E Patient 54
MECOM
POU2F1
DYNC2H1
UQCRC2
MYH10
FOXJ3
F Patient 33
SMG6
NF1
NBPF10
MECOM
LOC101060019
MIB1
OXCT1
AML
G Patient 61
MECOM
MIR512-2
RBM6
SMG6
GNG7
CCT6B
RBFOX2
HMGA2

Figure 2 (facing page). Integration-Site (IS) Analysis, Platelet Counts, and Peripheral-Blood Vector Copy Number Over Time for Individual Patients.

Shown are the results (as of April 25, 2024) for Patients 3, 46, 36, 44, 54, 33, and 61. Check marks indicate the timing of the IS analysis (ISA), which was performed on peripheral-blood samples. ISs from different time points that are located within five base pairs are considered the same IS. The plot shows all ISs that occur with a relative frequency of 10% or more at any time point or any two or more ISs in a clone with a relative frequency of 5% or more at any time point. Multiple ISs in the same clone were defined as multiple ISs with a relative frequency within 20% of each other. Genes in gray dashed boxes represent genes within the same clone. The limit of quantification for IS analysis was a relative frequency of 5%. The MECOM gene involved was 3+168905559 for Patient 3, 3+169089800 (MECOM-IS1) and 3-168929610 (MECOM-IS2) for Patient 46, 3+168881163 for Patient 36, 3+169034816 (MECOM-IS1) and 3–168952545 (MECOM-IS2) for Patient 44, 3-168884077 for Patient 54, 3+169089416 for Patient 33, and 3-168890747 for Patient 61. AlloHSCT denotes allogeneic hematopoietic stem-cell transplantation, MDS myelodysplastic syndrome, PB peripheral blood, and VCN vector copy number.

tors and enhancer-deleted γ-retroviral vectors are less likely to transactivate oncogenes and have a lower probability of oncogenic transformation in vitro and in vivo.[25,26] A systematic review and meta-analysis showed that no cases of insertional oncogenesis have been reported for lentiviral vectors through March 2022.[11]

A unique feature of Lenti-D lentiviral vector is the inclusion of an internal MNDU3 promoter–enhancer to drive expression of *ABCD1* in multiple cell types, including cerebral macrophages, microglia, and hematopoietic stem cells. Thus, the integrated provirus contains one intact promoter–enhancer derived from a long terminal repeat. Retroviral vectors have been shown to trigger acceleration of tumor onset in tumor-prone mice that are dependent on long terminal repeat activity, whereas tumorigenesis was unaffected by self-inactivating lentiviral vectors.[24] However, inclusion of an intact long terminal repeat sequence in a lentiviral vector increases the risk of tumorigenesis in this same model.[27] Abnormal dysplastic clonal hematopoiesis was observed in a rhesus macaque that had undergone transplantation with hematopoietic stem and progenitor cells transduced with lentiviral vector containing a strong retroviral murine stem-cell virus constitutive promoter–enhancer in the long terminal repeat sequence.[28] However, there is a clear difference between Lenti-D and the vector used in this previous study.

To date, no cases of insertional oncogenesis have been reported in clinical studies of metachromatic leukodystrophy[29] or in the enhancer-deleted γ-retrovirus trial of X-linked severe combined immunodeficiency,[26] nor in the Bluebird Bio–sponsored studies that use lentiviral vectors with different characteristics than Lenti-D — for example, in the studies for sickle cell disease or β-thalassemia that used the BB305 lentiviral vector (see the Supplementary Appendix). Differences between the lentiviral vectors used in these studies could have led to the varied results; for example, in contrast to Lenti-D, BB305 lentiviral vector leads to lineage-restricted expression of adult hemoglobin with a T87Q amino acid substitution owing to erythroid-specific gene regulatory elements.[30] Although the Lenti-D lentiviral vector should be associated with a low risk of insertional oncogenesis because of its preferential insertion into introns away from the gene transcription start site, the one intact enhancer present in the internal MNDU3 promoter–enhancer does have the potential to drive gene expression of the nearby endogenous gene.

All the patients described here had expanded clones with insertion sites in *MECOM* or *PRDM16*, a gene with 63% sequence homology to *MECOM*.[31] Three of these patients were tested and had evidence of target-gene dysregulation. Both *PRDM16* and *MECOM* encode the family of PR-domain proteins and are involved in chromosomal translocation in MDS and AML; both genes have been implicated in MDS and AML related to vector insertion in human gene therapy studies.[32] *MECOM* encodes several transcripts and protein variants, including MDS1, transcription factor EVI1, and MDS1–EVI1.[33] EVI1 controls stem-cell proliferation and is considered to be a proto-oncogene[34]; MDS1–EVI1 has been proposed to function as a tumor suppressor.[33] Like *MECOM*, full-length *PRDM16* is thought to be a tumor suppressor with expression of an oncogenic shorter isoform.[31] Chromosomal aberrations that dysregulate *EVI1* expression have been observed in primary AML and MDS, with increased expression of *EVI1* linked to poor prognosis.[35] Sporadic myeloid neoplasia with *MECOM* rearrangement manifests as characteristic megakaryocyte dysplasia with frequent micromegakaryocytes and separated nuclear lobes,

Table 3. Results of Integration-Site Analysis.*

Characteristic	Patients with Hematologic Cancer (N=7)	Patients without Hematologic Cancer (N=60)	All Patients (N=67)
Median highest total no. of unique mappable integration sites within each patient across all visits (range)	9748 (2548–14,796)	6732 (582–15,683)	6973 (583–15,683)
Median highest relative frequency of any unique mappable integration site within each patient across all visits (range)	25 (18–34)	11 (0–55)	14 (0–55)
Persistent oligoclonality at any time — no. (%)†			
Yes	6 (86)	23 (38)	29 (43)
No	1 (14)	37 (62)	38 (57)
Current persistent oligoclonality — no./total no. (%)‡			
Yes	5/6 (83)	15/23 (65)	20/29 (69)
No	1/6 (17)	8/23 (35)	9/29 (31)
Current oligoclonality — no. (%)§			
Yes	7 (100)	21 (35)	28 (42)
No	0	39 (65)	39 (58)
Persistent integration site in a known oncogene at any time — no. (%)¶			
Yes	6 (86)	16 (27)	22 (33)
No	1 (14)	44 (73)	45 (67)
Current persistent integration site in a known oncogene — no./total no. (%)‖			
Yes	5/6 (83)	13/16 (81)	18/22 (82)
No	1/6 (17)	3/16 (19)	4/22 (18)
Current integration site in a known oncogene — no. (%)**			
Yes	7 (100)	16 (27)	23 (34)
No	0	44 (73)	44 (66)

* Shown are data as of April 25, 2024. Data for Patient 61 at the time of diagnosis were unavailable. "Persistent oligoclonality" is defined as a relative frequency of at least 10% for the same integration site at two consecutive time points or a relative frequency of at least 5% for the same two or more integration sites at two consecutive time points. "Persistent integration site in a known oncogene" is defined as a relative frequency of at least 5% for the same integration site in a known oncogene at two consecutive time points. "Known oncogene" is defined as a gene classified as Tier 1 in the Cancer Gene Census in the Catalogue of Somatic Mutations in Cancer at the time of the report review of the integration-site analysis. For patients who have undergone allogeneic HSCT, the last assessment is the latest assessment available before allogeneic HSCT.

† Persistent oligoclonality at any time refers to a patient whose results meet the criteria of persistent oligoclonality at any time during the study, including when the criteria are no longer met in later follow-up assessments. The denominator is the number of patients with at least two results from integration-site analysis. These measures were evaluated in genes with a top 10 integration site with the highest relative frequency.

‡ Current persistent oligoclonality is a subgroup of persistent oligoclonality at any time and refers to a patient whose results meet the criteria of persistent oligoclonality at the last two assessments as of the data cutoff date. The denominator is the number of patients whose results met the definition for persistent oligoclonality at any time. These measures were evaluated in genes with a top 10 integration site with the highest relative frequency.

§ Current oligoclonality is defined as a relative frequency of at least 10% for at least one integration site or a relative frequency of at least 5% for two or more integration sites at the last assessment as of the data-cutoff date. The denominator is the number of patients with at least one result from integration-site analysis. These measures were evaluated in genes with a top 10 integration site with the highest relative frequency.

¶ Persistent integration site in a known oncogene at any time refers to a patient whose results meet the criteria of persistent integration site in a known oncogene at any time during the study, including when the criteria are no longer met in later follow-up assessments. The denominator is the number of patients with at least two results from integration-site analysis. These measures were evaluated in genes with a top 10 integration site with the highest relative frequency.

‖ Current persistent integration site in a known oncogene is a subgroup of persistent integration site in a known oncogene at any time, defined as a patient whose results meet the criteria of persistent integration site in a known oncogene at the last two assessments as of the data-cutoff date. These measures were evaluated in genes with a top 10 integration site with the highest relative frequency. The denominator is the number of patients whose results meet the definition for persistent integration site in a known oncogene at any time.

** Current integration site in a known oncogene is defined as at least one integration site with a relative frequency of at least 5% in a known oncogene at the last assessment as of the data-cutoff date. The denominator is the number of patients with at least one result from integration-site analysis. These measures were evaluated in genes with a top 10 integration site with the highest relative frequency at every visit.

findings that are similar to those found in the cases presented in this study. In all six cases of hematologic cancer in the ALD-104 study, lentiviral vector insertion in the expanded clone occurred in intron 2 of the *MECOM* gene; the ability to increase *MECOM* gene expression from an intronic insertion site is possibly related to the unique splicing patterns of the *MDS1–EVI1* locus.

Although *MECOM* is a frequent insertion site in gene therapy, there seems to be rare clonal outgrowth with Lenti-D that results in transformation. In 2021, Reinhardt et al. observed that insertion sites are frequently detected in proto-oncogenes, including *MECOM*, without any oligoclonality or cancer.[36] This observation is supported by our findings, which showed that although *MECOM* insertions were frequently observed among the patients involved in the ALD-102 and ALD-104 studies, most cases, including those with clonal expansion, have not resulted in cancer (Figs. S10 and S11). Thus, additional unknown factors appeared to play a role in the development of cancer in patients who were treated with eli-cel. Expanded clones with insertion sites in *MECOM–EVI1* also carried such lesions in other genes in the same clone. In addition, pathogenic variants in proto-oncogenes or chromosome abnormalities were identified in the patients in whom hematologic cancer developed, possibly related to the genetic instability caused by overexpression of *EVI1* or *PRDM16*. Therefore, vector insertions in *MECOM* were common, but they were not sufficient for the development of hematologic cancer, and clonal evolution with vector insertions into multiple genes, somatic abnormalities, or both were needed for progression to MDS or AML. However, no overt combinations of multiple integrations with *MECOM* appeared to confer a predisposition to the development of hematologic cancer.

Five of the seven patients (Patients 3, 33, 36, 46, and 61) with hematologic cancer had evidence of persistent oligoclonality between the time of eli-cel infusion and diagnosis. However, three patients (Patients 3, 44, and 54) did not have oligoclonality at their last insertion-site analysis before diagnosis, including two who had their last insertion-site analysis less than 6 months before receiving the diagnosis of cancer. Thus, although the testing was useful in detecting persistent oligoclonality, it was not reliably predictive of the transformation into cancer, which underscores the importance of monitoring the complete blood count. However, persistent insertions into oncogenes were more common in patients with hematologic cancer and may be a useful criterion to prompt bone marrow examination and genetic studies.

Among the patients with persistent oligoclonality, three (Patients 3, 33, and 61) also had persistent oligoclonal integrations in *SMG6* (frequently noted in the analysis of lentiviral vector trials) and two had integrations in *MIR100HG*. Analysis of the genes associated with persistent oligoclonality revealed that a majority of genes (69 of 74) with mapped insertion sites showed isolated instances of oligoclonality (i.e., one integration in one patient). In contrast, the most frequent sites associated with oligoclonality were found within *SMG6* and *MECOM*. This finding is also consistent with the finding that *SMG6* and *MECOM* were among the genes with the highest frequency of insertions in patients treated with eli-cel in general.

Clonal dynamics, however, differed markedly between *SMG6*-expanded and *MECOM*-expanded clones. Clones containing *SMG6* integrations showed transient expansion, whereas clones with *MECOM* integrations were stable or expanded over time (Figs. S10 and S11). The only marked difference in clonal dynamics of the MDS *MECOM* clones was the early onset of expansion in two patients; clones in Patients 46 and 36 were expanded at frequencies of more than 50% at the earliest analyses at 6 months after treatment with eli-cel (Fig. S10A and S10B). In contrast, other patients in the ALD-102 study had persistent oligoclonality associated with *MECOM* integrations without evidence of progression to hematologic cancer (e.g., Patients 10 and 29); these patients initially had very low clonal frequencies that subsequently expanded (Fig. S10C and S10D).

We hypothesize that the specific features of Lenti-D vector design, such as the presence of enhancer sequences, overexpression of the transgene *ABCD1* (which may affect the ability of cells to engraft in the administered product), aspects of the conditioning regimen for eli-cel, or G-CSF use, play a role in mediating insertional oncogenesis. The way these factors individually, in combination, or with other unidentified variables interact with one another to lead to genotoxicity is unclear. However, we believe that our results should inform future studies of gene therapy regarding the choice of promoter used when

designing lentiviral vectors. In addition, expression of the transgene should be high enough to arrest disease progression but also be lineage-restricted, if possible, and low enough to reduce the likelihood of genotoxicity and genomic instability. Our results suggest that patients who are considering lentiviral vector gene therapy should continue to be educated on the risk of hematologic cancer and, if treated, monitored closely. Finally, for patients treated with eli-cel, one intervention to reduce the risk of cancer may be to consider the use of busulfan–cyclophosphamide as the preparative regimen instead of busulfan–fludarabine.

The risk of oncogenesis with eli-cel[37] must be weighed against the severity and natural history of cerebral adrenoleukodystrophy as well as the availability of other treatments and their risks, including allogeneic HSCT. Overall survival was 77.8% among patients who had undergone allogeneic HSCT 4 years previously in a matched cohort study[38]; the cumulative incidence estimate for acute grade II to IV GVHD was 11.1%, and the estimate for GVHD overall was 17.2%.[38] Moreover, the availability of HLA highly matched donors is limited and is a crucial determinant of outcomes in patients undergoing allogeneic HSCT.[38] Most patients who received eli-cel in the ALD-102 and ALD-104 studies benefitted clinically, with 81% 4-year survival free of major functional disabilities and hematologic cancer, and without referral for allogeneic HSCT. Therefore, the probability and magnitude of benefit that gene therapy can offer in patients who do not have an appropriate donor must be considered. Because gene therapy is an evolving field, ongoing follow-up is critical to understanding the longer-term safety and efficacy of novel treatments like eli-cel.

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APPENDIX

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