Molecular Therapy
Commentary

# Balancing efficacy and safety in lentiviral vector-mediated hematopoietic stem cell gene therapy

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Hematopoietic stem cell (HSC) gene therapy has emerged as a transformative medical tool with the potential to cure previously untreatable diseases.[1,2] However, as with any intervention that affects the human genome, ensuring safety remains paramount. Although substantial progress has been made in mitigating the risks of insertional mutagenesis, including the use of self-inactivating (SIN) lentiviral vectors (LVs), studies reveal that these risks are not fully eliminated. This commentary examines the current safety profile of LVs in clinical use and the mechanisms underlying vector-mediated genotoxicity, outlining essential steps for advancing even safer, more effective gene therapies.

LVs with SIN long terminal repeats (LTRs) can transduce and stably integrate into the genome of both dividing and non-dividing cells, facilitating durable genetic modifications and long-lasting therapeutic effects. This integration capability renders LVs particularly suitable for HSC gene therapy, where stable transgene transmission across multiple cell lineages is crucial for treating genetic diseases. To date, over 500 patients have received LV-mediated HSC gene therapy for a range of genetic diseases, with significant therapeutic efficacy and clinically significant improvements in disease progression and quality of life.[1]

A recent comprehensive long-term clonal-tracking study of hematopoietic reconstitution post-LV-HSC gene therapy in 53 patients affected by β-thalassemia (β-thal, a hemoglobinopathy), Wiskott-Aldrich syndrome (WAS, an immunodeficiency), or metachromatic leukodystrophy (MLD, a neurodegenerative lysosomal storage disorder) showed highly polyclonal and stable grafts sustained by the LV-transduced HSCs. These HSCs contributed homogenously to hematopoietic output over up to 8 years, showing no signs of exhaustion or expansion while maintaining multipotency and providing durable therapeutic benefit.[3] Similarly, 62 patients with adenosine deaminase-severe combined immunodeficiency (ADA-SCID) treated with an SIN LV into autologous HSC have shown stable immune reconstitution without vector-related adverse events over 5–11 years from treatment.[4]

Despite these promising outcomes, insertional mutagenesis remains an important concern in certain contexts. Because LV integrates semi-randomly throughout the genome, insertions near genes with oncogenic potential upon dysregulation cannot be avoided. Moreover, the extremely high number of transduced cells present in typical cellular products for HSC gene therapy almost ensures saturation of the LV accessible genome. Thus, the critical issue is not whether some integrations occur near at-risk proto-oncogenes, as this can be taken for granted, but instead whether such integrations have a certain likelihood to dysregulate the expression of the flanking genes. A substantial number of pre-clinical and clinical studies have identified factors affecting such likelihood, which pertain primarily to gamma-retroviral vectors rather than SIN-LV, including the presence of transcriptionally active LTRs with strong enhancer/promoter elements, integration site selection bias favoring insertion near promoters, and the presence within the vector sequence of cryptic splice and polyadenylation sites that may cause truncation and premature termination of endogenous transcripts incorporating vector sequences.[5–7] All these conditions have been reported in pre-clinical models of experimental vector-driven oncogenesis, as well as in clonal expansion or full-blown malignancies in HSC gene therapy trials.

In addition to vector design, whether an at-risk insertion that may dysregulate a cancer-associated gene does trigger oncogenesis depends on the proliferative and self-renewing capacity of the transduced cells. Most insertions in a typical $CD34^+$ hematopoietic stem and progenitor cell (HSPC) product occur in cells that will not engraft, and among the few that do engraft, the majority are committed progenitors that only give rise to a short-term output. While gain of function induced by insertional mutagenesis may increase engraftment and clonogenic output of a hematopoietic progenitor, full transformation requires additional mutations that are more likely as its progeny expands over time. Indeed, insertional analyses and clonal abundance tracking of vectors posing a higher risk of insertional oncogenesis typically report an enrichment of insertions at proto-oncogenes in the human graft, with only a fraction of them showing progressive expansion over time and individual ones eventually progressing to leukemia/myelodysplastic syndrome (MDS) only after further acquisition of additional oncogenic events.

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The amount and quality of the infused cell product may further affect the likelihood of emergence and expansion of clones bearing gain-of-function insertional mutations. It is conceivable that the administered progenitors compete for successful engraftment and contribute to recipient hematopoietic repopulation, according to the total number infused and the extent that their fitness has been preserved or instead impacted by the *ex vivo* genetic manipulation. A product containing fewer or less-fit HSCs likely selects more efficiently for those rare cells with a gain-of-function insertional mutation and thus a growth advantage. However, abundant numbers of fit progenitors may effectively outcompete such rare mutants, especially if saturating the stem cell niches' capacity.

Disease background may also influence the selection of gain-of-function mutations. Diseased marrow environments can alter the fitness of harvested HSCs, potentially selecting for clones that expand during *ex vivo* manipulation and engraftment. For instance, HSPCs from patients with prior mutagen exposure or a pro-inflammatory marrow niche may harbor clonal hematopoiesis mutations, which could gain an advantage through the bottleneck imposed by the engraftment process. This might explain cases where malignancies in sickle cell disease patients post-HSC gene therapy were attributed to preexisting mutant clones further progressing to malignancy along the course of the treatment rather than to vector insertions.[8,9] Careful screening of patients is thus encouraged as gene therapy expands to broader patient populations.

Unfortunately, the occurrence of one or more of the above-described conditions augmenting the risk of insertional oncogenesis might explain the recently reported emergence of seven cases of hematologic malignancies among 67 cerebral adrenoleukodystrophy (CALD) patients treated by LV gene therapy (over 10% of those treated). CALD patients developed MDS or acute myeloid leukemia, with malignant clones harboring LV integrations near proto-oncogenes such as MECOM and PRDM16. These proto-oncogenes were upregulated by the vector's strong MND gamma-retroviral enhancer/promoter within the LV, adopted to drive high levels of expression of the peroxisomal transporter ABCD1 throughout hematopoietic progenitors and differentiated cells.[10] Longitudinal clonal tracking in nearly all treated patients showed enrichment for integrations near proto-oncogenes, oligoclonal expansions, documented truncation of oncogene transcripts, and accrual of additional genotoxic hits driving the transformation into full malignancy.[11,12] The frequent appearance of MECOM/EVI1 integrations in CALD patients, far more than in other LV-HSC gene therapy contexts, suggests that the MND promoter confers a unique proliferative advantage to cells carrying such insertions during engraftment. A clinical trial using a gamma-retroviral vector with the MND LTR driving the expression of ADA shows the presence of prominent MECOM-adjacent clones in several of the patients, which have been stable between 2 and 10 years after gene therapy.[13]

Other factors may have further contributed to the genotoxic risk of CALD gene therapy by favoring the selection and expansion of any given clone receiving an MECOM/EVI1 insertion. Delayed engraftment was observed in four of the five CALD patients developing MDS but not in other patients in good clinical condition, suggesting that cell products with fewer or less-robust progenitors might enable the expansion of gain-of-function mutants, increasing the risk of developing malignancy.

The choice of the MND promoter was justified by the need to achieve high levels of transgene expression to obtain therapeutic efficacy, and the same strategy has been adopted for a multi-center phase 1/2 gene therapy study for RAG1-deficient SCID (this study was registered at ClinicalTrials.gov: NCT04797260). Watchful monitoring of these patients will be crucial to detect early signs of clonal expansion at the molecular level. The MND promoter choice, although effective in increasing therapeutic expression, illustrates the trade-off between achieving therapeutic efficacy and minimizing genotoxic risks, especially when treating conditions that require high-level transgene expression, such as CALD and RAG1-SCID. In contrast, in other LV-based HSC gene therapies for MLD, WAS, X-linked SCID, ADA-SCID, Artemis SCID, leukocyte adhesion deficiency-1, and β-thal, partially reconstituted cellular promoters (eukaryotic translation elongation factor 1α, phosphoglycerate kinase, WAS, DNA cross-link repair 1C, and β-globin) have demonstrated efficacy with no evidence to date of emerging genotoxic risk. Thus, introduction of enhancer/promoter elements that are strongly active in HSPC should be avoided when designing new vectors. A safer approach to enhancing therapeutic expression involves increasing the amount of integrated vectors per cell but using cellular promoters, as in the MLD trial, where higher vector copy number (VCN) safely achieved supranormal therapeutic transgene expression without insertional mutagenesis over long-term follow-up.[3,14]

In summary, while HSC gene therapy has revolutionized the treatment prospects for severe diseases, recent cases of malignancy in the CALD trial underscore the importance of refining vector design and cell product quality. Using partially reconstituted cellular promoters or alternative VCN strategies may help balance therapeutic efficacy with safety. As gene therapy continues to advance its reach and ensure long-sought benefit to an increasing number of patients suffering from otherwise debilitating or lethal diseases, adherence to these safer design principles will be critical in realizing its full clinical potential while minimizing adverse outcomes.

## DECLARATION OF INTERESTS

L.N. is an inventor on patents on lentiviral vector technology filed by the Telethon Foundation and San Raffaele Scientific Institute and founder, equity holder, and consultant to Genenta Science and Genespire. A.A. is a principal investigator of clinical trials on LV gene therapy (sponsored by Orchard Therapeutics or Fondazione Telethon). D.B.K. is an inventor for the University of California Regents on an LV for ADA-SCID and founder, equity holder, and consultant for Rarity Public Benefit Corporation. C.B. is a principal investigator of clinical trials on LV gene therapy (sponsored by Rocket Pharma or Great Ormond Street Hospital).

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