RESEARCH LETTER

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TO THE EDITOR:

Molecular evaluation and vector integration analysis of HCC complicating AAV gene therapy for hemophilia B

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Genotoxicity remains an unknown safety concern of gene therapy. Molecular techniques for determining the frequency and genomic localization of vector integration are central to understanding primarily integrating viral vectors (ie, retrovirus and lentivirus). Unlike these vectors, recombinant adeno-associated virus (rAAV) vectors integrate into host genomes at low frequencies. Nevertheless, the integration of rAAV sequences in oncogenic hotspots could theoretically lead to hepatocellular carcinoma (HCC).[1] This report describes the molecular characterization of the first case of HCC complicating an rAAV gene therapy trial.

Studies using rAAV in mice reported low integration levels into host chromosomal sequences associated with HCC.[2,3] Investigations suggested that HCC was driven by *microRNA-341* dysregulation within the *Rian* locus, a hotspot for mouse genome integration. The *Rian* locus in mice has a human ortholog, the human long-coding RNA, *MEG8*, which is overexpressed in some HCCs and may interact with *microRNA-367-3p* in the pathogenesis and progression of some HCCs.[4] Nevertheless, the *microRNA-341* locus found to be susceptible to rAAV insertional mutagenesis in mice has no human homolog.[5]

Studies using mouse models have previously been performed, including work in tumor-prone mouse species and under experimental conditions that stimulate cell proliferation, which suggests specific components of vector design or administration may affect vector integration and infer greater risk of tumorigenesis (eg, use of chicken β-actin or thyroxine-binding globulin enhancer/promoter, treatment in the neonatal period, partial hepatectomy, and vector DNA–related contaminants).[5-7] In studies specific to hemophilia gene therapy, rAAV–factor IX (FIX) vectors in mice, dogs, and nonhuman primates showed no evidence of vector integration leading to HCC up to 8 years after treatment.[8-10] Recently, human biorepository analysis identified HCC with the insertion of wild-type AAV sequences, potentially implicating wild-type AAV in some cases of HCC (although not rAAV, which does not carry any viral genes).[11,12]

At least 39 human trials have used rAAV to target the liver to correct primary liver diseases, lysosomal storage disorders, liver protein synthetic deficiencies, and other metabolic disorders.[13] Seventeen studies of liver-directed rAAV hemophilia gene therapy have been published to date, comprising >320 patients followed up for 0.5 to 15 years.[14,15] Current clinical experience identifies no added oncogenic risk from liver-directed rAAV gene therapy. However, prior exposure to contaminating viruses in plasma-derived clotting factor concentrates leads to chronic hepatitis, meaning older patients with hemophilia have an increased incidence of HCC, which is a confounding factor.[16] Considering the conflicting data linking AAV specifically with HCC, the development of HCC in a recipient of liver-directed rAAV gene therapy is a highly anticipated event.

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The data that support the findings of this trial are available through the clinical trial registry platforms at www.clinicaltrials.gov (#NCT03569891) and www.clinicaltrialsregister.eu (2017-004305-40).

The full-text version of this article contains a data supplement.

We report, to the best of our knowledge, the first event of HCC in a recipient of rAAV gene therapy, including molecular characterization of the tumor to examine the biology of the event, generate 1 of the first human rAAV vector integration site (IS) profiles, and advance understanding of rAAV vector safety.

Etranacogene dezaparvovec, comprising a liver-directed rAAV5 vector containing a codon-optimized Padua-variant human FIX transgene and a liver-selective promoter, is the first systemically delivered AAV gene therapy approved to treat hemophilia B by the US Food and Drug Administration and the European Regulatory Authorities.

The phase 3 HOPE-B trial (NCT03569891) enrolled 54 adult males with hemophilia B (FIX $\leq$ 2%), including participants with prior hepatitis B virus (HBV; n = 9) and hepatitis C virus (HCV; n = 31) infection, and excluding participants with cirrhosis or advanced fibrosis.[17] Participants received a single intravenous dose of etranacogene dezaparvovec (2×10^{13} genome copies per kg). Liver-specific assessments included twice-yearly α-fetoprotein measurements and annual ultrasound over 5 years follow-up. The trial was conducted in accordance with the International Council for Harmonisation Good Clinical Practice guidelines and ethical principles originating in the Declaration of Helsinki. The protocol was approved by institutional review boards and independent ethics committees at each trial site.

Molecular analyses were conducted by GeneWerk GMBH (Heidelberg, Germany) independently from the sponsor and included the following experiments:

- Vector copy number measurement using quantitative polymerase chain reaction.
- Vector DNA IS analysis by shearing extension primer tag selection/ligation-mediated polymerase chain reaction (triplicate analyses: 3 × 500 ng DNA input).[18,19]
- Whole-genome sequencing (WGS) via Illumina next-generation sequencing platform (singlicate analysis: 1 µg DNA input).
- RNA sequencing transcriptome profiling, RNA fusion products, and differential RNA expression.

Detailed methodology and results are provided in the supplemental Material. Final interpretation of the results was evaluated by independent consultants experienced in clinical hepatology, hematology, and genetics, and by the HOPE-B trial independent data monitoring committee.

The patient who developed HCC received etranacogene dezaparvovec in October 2019. Medical history, prior treatments, and baseline characteristics are presented in Table 1. The patient's history of hepatitis virus infections and advanced age were HCC risk factors.

A chest computerized tomography with angiography including visualization of the upper abdominal organs and liver ultrasound performed 1 and 3 months after treatment revealed no liver abnormalities; α-fetoprotein levels remained within normal range. There were no notable changes in transgene (FIX) expression levels after the expected increase to steady-state.

The per-protocol, 1-year (day 365) postdose abdominal ultrasound revealed a subcapsular liver lesion. A subsequent abdominal computerized tomography revealed a single 3.1-cm lesion in segment 8. A needle biopsy (day 389) specimen revealed predominantly healthy liver tissue, with a single atypical focus consistent with HCC. Immunohistochemistry was positive for glutamine synthetase, HSP-70, and glypican-3, with increased capillarization (CD34). Histology of surrounding liver tissue showed steatosis grade 1, slight lobular infection, and periportal fibrosis (no certain bridging), consistent with nonalcoholic fatty liver disease.

Table 1. Medical history, prior treatments, and baseline characteristics

Parameter	
Age	69 y
Sex	Male
BMI	22 kg/m^2
Medical history	HBV Ab positive, HBV sAg negative (indicating cleared prior infection), first detected in 1980
	Non-A/B hepatitis since 1983, confirmed as HCV in 2003
	HCV genotype 1a, RNA PCR load: 1.34E + 006 IU/mL
	Minimal fibrosis: Fibroscan F0/F1 5.7 KPa obtained late 2015 as workup before DAA treatment for HCV eradication
Prior treatments	DAA treatment in early 2016, total course 3 months, as follows: Dasabuvir 250 mg twice daily Ribavirin 600 mg twice daily Ombitasvir/paritaprevir/ritonavir as 12.5/75/50 mg once daily
Family history	Diverse malignancies
Alcohol consumption	0–2 units per wk
Other risk factors for HCC	No diabetes
	No known NAFLD risk factors
	No evidence of significant fibrosis/cirrhosis or steatosis at screening or before treatment
Screening Fibroscan score	4.3 KPa (inclusion criterion, ≤9)
Steatosis grade (CAP)	186 dB

Ab, antibody; BMI, body mass index; CAP, controlled attenuation parameter; DAA, direct-acting antiviral; IU, international units; NAFLD, nonalcoholic fatty liver disease; PCR, polymerase chain reaction; sAg, surface antigen.

The patient underwent exploratory laparotomy for surgical excision (day 443). Intraoperative ultrasound revealed a secondary liver lesion 0.8 cm in diameter in segment 2/3; biopsy confirmed a 90% likelihood of HCC. Specimens from the resected secondary lesion and adjacent healthy liver tissue were analyzed for histopathology and molecular integration. The primary tumor was not excised because of the complex location, possible morbidity, and minimal impact on the multifocal HCC prognosis. Histopathology and immunohistopathology confirmed the occurance of moderately differentiated HCC. Results from adjacent tissue were consistent with the observations from the preoperative biopsy specimen.

The patient underwent transarterial chemoembolization for the lesion in segment 8 with doxorubicin (55 mg once, intra-arterial) 5 weeks after surgery. The patient subsequently received orthotopic liver transplantation on day 851 after treatment (February 2022) with a single preoperative bolus of FIX concentrate. The patient developed progressive transplant failure because of hepatic artery thrombosis

Table 2. WGS and RNA sequencing analyses

WGS results		
Expected findings if HCC was caused by rAAV direct integration	**Expected findings if HCC was not caused by rAAV direct integration**	**Observed findings**
Integration of vector sequences in or near known oncogenes.	Common HCC mutations (eg, *TP53* or *NFE2L2*).[21]	WGS provided a genome coverage of 120× and 107× for the HCC and HCC-adjacent sample, respectively.
	No AAV ISs near oncogenes.	WGS identified 3 additional ISs in the HCC and 2 in the HCC-adjacent sample.
		No IS was identified in >1 read, indicating a low IS rate in the liver and a lack of a dominant IS in the HCC sample.
		Independent of etranacogene dezaparvovec treatment there were the following mutations: Mutations in *TP53*, *NFE2L2*, and *PTPRK* Large chromosomal rearrangements in chromosomes 1, 8, and X, characteristic of HCC
		In addition, vector integration events occurred at a low rate and in genomic sites not known to be associated with HCC.
RNA sequencing transcriptome profiling		
Not described.	Differential expression of genes previously identified in HCC.	RNA transcripts identified that are among the most consistently differentially expressed in HCC (arising independent of AAV), including *COL1A1*, *LCN2*, *AEBP1*, and *CRP*.

AEBP1, Ae binding protein 1; COL1A1, α-1 type I collagen; CRP, C-reactive protein; LCN2, lipocalin-2.
For more detailed results, please see supplemental Material.

and, ultimately, had to undergo a second orthotopic liver transplant. Since then, the patient has been recovering well.

Molecular analysis of rAAV sequences in HCC and HCC-adjacent samples had 3.21 and 4.11 vector copies per cell, respectively. In both samples, many vector-vector fusion sequences were detected, demonstrating that most vector genomes were episomal, with ~1 in 10 000 integrated. There were 56 unique ISs in the HCC and 39 in the HCC-adjacent sample; <0.03% of cells (~60 of 250 000 cells) had AAV vector integration. In both samples, no dominant ISs were found; the highest shearing count detected was 2, indicating that the insertion occurred in only 2 cells. All the remaining IS detected had only 1 shearing count.

WGS detected a deletion on chromosome 8 (Table 2), which is present in 48% of HCCs arising outside of gene therapy.[20] Moreover, mutations within HCC-associated genes were apparent, including *TP53*, *NFE2L2,* and *PTPRK*. WGS and RNA sequencing of the HCC-adjacent sample revealed a premalignant genetic signature. Transcriptome profiling and analysis identified features and genes highly associated with HCC.

If HCC development was driven by AAV vector integration, frequent IS and a dominant IS would be expected; neither was observed in this patient's samples. Furthermore, WGS would show integration in/near known HCC oncogenes (eg, *TP53* and *NFE2L2*), which did not occur. Instead, mutations within these, and other, characteristically HCC-associated genes were observed independent of associated AAV integration.

The patient's prior history of hepatitis virus infection and advanced age were risk factors for HCC. Although most hepatitis-related HCCs occur in individuals with advanced fibrosis or cirrhosis,[22] up to 20% of HCCs occur in noncirrhotic livers.[23,24] Generally, the risk of HCC is higher in the population with hemophilia vs that in the general population, partly because patients with hemophilia have higher rates of hepatitis virus infection (with/without sustained viral response) and HIV infection.[25] HCV and HIV coinfection accelerates liver deterioration in patients with hemophilia.[26] Patients with hemophilia with HBV/HCV infection are generally also older than those with HBV/HCV infection but without hemophilia, with age-related HCC risk factors such as nonalcoholic fatty liver disease or nonalcoholic steatohepatitis, obesity, and excessive alcohol use.[25]

In conclusion, the molecular and vector integration analysis of the index case of HCC after liver-directed rAAV-based gene therapy established no relationship to rAAV administration, and provides a model for investigating neoplasms in future clinical application of gene therapy with integrating vectors. Collection of real-world data on HCC occurrence through gene therapy–specific registries will also be critical to understanding the long-term risk of malignancies associated with AAV vector integration.

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Manfred Schmidt died on 13 January 2022.

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