Low incidence of hepatocellular carcinoma in mice and cats treated with systemic adeno-associated viral vectors

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Adeno-associated viral (AAV) vectors have emerged as the preferred platform for in vivo gene transfer because of their combined efficacy and safety. However, insertional mutagenesis with the subsequent development of hepatocellular carcinomas (HCCs) has been recurrently noted in newborn mice treated with high doses of AAV, and more recently, the association of wild-type AAV integrations in a subset of human HCCs has been documented. Here, we address, in a comprehensive, prospective study, the long-term risk of tumorigenicity in young adult mice following delivery of single-stranded AAVs targeting liver. HCC incidence in mice treated with therapeutic and reporter AAVs was low, in contrast to what has been previously documented in mice treated as newborns with higher doses of AAV. Specifically, HCCs developed in 6 out 76 of AAV-treated mice, and a pathogenic integration of AAV was found in only one tumor. Also, no evidence of liver tumorigenesis was found in juvenile AAV-treated mucopolysaccharidosis type VI (MPS VI) cats followed as long as 8 years after vector administration. Together, our results support the low risk of tumorigenesis associated with AAV-mediated gene transfer targeting juvenile/young adult livers, although constant monitoring of subjects enrolled in AAV clinical trial is advisable.

INTRODUCTION

Adeno-associated viral (AAV) vectors are the most used viral vectors for in vivo gene transfer because of both their ability to ensure long-term transduction of a wide spectrum of tissues and their safety profile.1 Indeed, wild-type AAVs, from which AAV vectors derive, do not exhibit pathogenicity in humans as their lytic stage occurs only in the presence of a helper virus.7 In recombinant AAV vectors, viral sequences involved in replication, site-specific genome integration, and capsid production are replaced by a transgene expression cassette, which is flanked by the inverted terminal repeats (ITRs) that are necessary for DNA packaging. This makes conventional recombinant AAV vectors replication-defective, and, without a source of Rep, unable to integrate into AAVS1 or similar sites. Thus, recombinant AAVs (rAAVs) largely persist as episomes in the nucleus of transduced cells, which minimizes the risk of related genotoxicity. However, integration of recombinant AAV is well recognized and has been reported to occur with a low frequency and a preference for loci close to active genes or CpG islands.3,4

The universally accepted safety of AAV vectors has been recently questioned by several studies documenting insertional mutagenesis and hepatocellular carcinoma (HCC) formation following liver gene transfer in newborn mice.5,6 In HCCs, AAV was found integrated into the RNA imprinted and accumulated in nucleus (Rian) locus,7 which encodes for many regulatory non-coding RNAs, leading to deregulation of genes flanking the insertion site and promoting HCC development.7 Consistent with the importance of the Rian locus in hepatic biology, the overexpression of the human orthologs within the Rian locus, such as the delta-like homolog 1-deiodinase type 3 (DLK1-DIO3) gene, has been associated with poor survival in humans with hepatic carcinoma.8 However, the majority of AAV integrations into the Rian locus were located in Mir341, which does not have an ortholog in humans.9 Interestingly, Chandler et al.5 demonstrated that HCC development strongly correlates with AAV dosing and was influenced by the enhancer/promoter used; in particular, the chicken beta actin (CBA) and the thyroxine-binding globulin (TBG) promoters, but not the liver-specific promoter 1 (LP1), were able to induce the gene deregulation responsible for HCC development in mice. Also, genes known to have a high expression in the
neonatal liver, such as albumin (Alb), alpha-fetoprotein (Afp), and the Rian locus, were the most susceptible to AAV integration. This suggests that the different liver gene-expression pattern of neonatal mice compared to juvenile and adult mice may affect AAV vector integration, and thus may limit the relevance of AAV-HCC observations to neonatal liver gene transfer. In line with this, insertional mutagenesis studies of either the TBG (AAV2/8.TBG.hARSB, either 2 × 10^13 or 2 × 10^12 GC/kg), or the LP1 promoter, (AAV2/8.LP1.hARSB [2 × 10^13 GC/kg]) or encoding the enhanced green fluorescent protein (EGFP) under the control of TBG promoter (AAV2/8.TBG.EGFP [2 × 10^13 GC/kg]). The control mice received the excipient alone or were left untreated (Table 1). Mice were followed-up to 24 months post-injection, i.e., about 26 months of age, unless euthanasia was required because of poor health conditions as revealed by clinical observations, i.e., abdominal palpation and cage-side evaluation. Histopathology was performed on all livers and any other organs showing gross abnormalities at necropsy (Figure S1).

In parallel, the long-term safety of AAV2/8.TBG.hARSB was assessed in MPS VI cats dosed at post-natal day 50 with 2 × 10^{12} genome copies (GC)/kg, a dose that was subsequently used the clinical trial.

**RESULTS**

**Incidence of HCC and other tumors following AAV-mediated liver gene transfer in young adult mice**

Young adult C57BL/6 mice received a single intravenous administration of AAV2/8 vectors encoding the hARSB under the control of either the TBG (AAV2/8.TBG.hARSB, either 2 × 10^13 or 2 × 10^12 GC/kg), or the LP1 promoter, (AAV2/8.LP1.hARSB [2 × 10^13 GC/kg]) or encoding the enhanced green fluorescent protein (EGFP) under the control of TBG promoter (AAV2/8.TBG.EGFP [2 × 10^13 GC/kg]). The control mice received the excipient alone or were left untreated (Table 1). Mice were followed-up to 24 months post-injection, i.e., about 26 months of age, unless euthanasia was required because of poor health conditions as revealed by clinical observations, i.e., abdominal palpation and cage-side evaluation. Histopathology was performed on all livers and any other organs showing gross abnormalities at necropsy (Figure S1).

| CTR 1 | N/A | N/A |
| CTR 2 | N/A | N/A |
| CTR 3 | N/A | N/A |
| TEST 1 | N/A | N/A |
| TEST 2 | N/A | N/A |
| TEST 3 | N/A | N/A |
| TEST 4 | N/A | N/A |

| Groups | Number of animals | Treatment | Dose (GC/kg) |
|--------|------------------|-----------|--------------|
| CTR 1  | 10M + 10F        | None      | N/A          |
| CTR 2  | 10M + 10F        | Excipient 1 | N/A         |
| CTR 3  | 5M + 5F          | Excipient 2 | N/A         |
| TEST 1 | 10M + 10F        | AAV2/8.TBG.hARSB | 2 × 10^{13} |
| TEST 2 | 10M + 10F        | AAV2/8.TBG.hARSB | 2 × 10^{12} |
| TEST 3 | 10M + 10F        | AAV2/8.LP1.hARSB | 2 × 10^{13} |
| TEST 4 | 10M + 10F        | AAV2/8.TBG.EGFP  | 2 × 10^{13} |

CTR, control group; TEST, test group; M, male; F, female; Excipient 1, PBS/5% glycerol diluted 1:15 in saline solution NaCl 0.9%, as representative of vector formulation used in TEST 2 group; GC, genome copies; N/A, not applicable.

HCCs were not found in control mice (0 out of 46; 24 males and 22 females), while in mice receiving AAV2/8 vectors, HCCs were reported only in males (6 out of 39) with an overall incidence of 15.4% (p = 0.07) but not in females (0 out of 37).

Specifically, HCCs were found in 1 out of 10 (10%) male mice receiving 2 × 10^{13} GC/kg and 1 (which had both a hepatocellular adenoma [HCA] and an HCC) out 9 (11%) male mice receiving 2 × 10^{12} GC/kg of AAV2/8.TBG.hARSB (Figure 1C). No statistically significant differences were found between the two different dose cohorts (p = 1) and between each one of the cohorts and the controls (p = 0.3448 and 0.3913 for the dose of 2 × 10^{13} GC/kg and 2 × 10^{12} GC/kg, respectively), which suggests that the AAV dose in the ranges we tested did not affect HCC development in young adult AAV-treated C57BL/6 mice.

In the cohort of male mice receiving 2 × 10^{13} GC/Kg of AAV, HCCs incidence was similar (1 out 10, 10%) between mice receiving AAV2/8.TBG. and LP1.hARSB (p = 1; Figure 1C).

Overall, HCC incidence in male mice receiving AAV2/8.JhARSB vectors was close to the reported spontaneous HCC formation rate; that is, 8%–9% in C57BL/6 male mice aged 22–27 months and not significantly different than in control groups (p = 0.2425).
Conversely, HCCs were reported in 3 out of 10 male mice that received $2 \times 10^{13}$ GC/kg of AAV2/8.TBG.<sup>EGFP</sup>. Unlike the other experimental groups, the HCC incidence (30%) was significantly different compared to controls ($p = 0.03$; Figure 1C). The HCC incidence was higher, albeit not significantly ($p = 0.582$), than in male mice receiving the same dose of AAV2/8.TBG.<sup>hARSB</sup> (10%). Notably, 2 out of 10 male mice injected with $2 \times 10^{13}$ GC/kg of AAV2/8.TBG.<sup>EGFP</sup> developed hepatocellular adenomas, which increased the overall incidence of hepatic neoplasms in this experimental group to 50% ($p$ versus control $= 0.002$, $p$ versus AAV2/8.TBG.<sup>hARSB</sup> = 0.3498). Other tumors found in livers, as well as in other organs such as the intestine, pancreas, spleen, neck, and ovary, were classified as round cell neoplasms, which include lymphoma, histiocytic-associated lymphoma, or histiocytic sarcomas as possible cell types. No additional characterization of these tumors was performed. No increase in the incidence of these round cells neoplasms was found in AAV-injected groups compared to controls (29% versus 22%, respectively; $p = 0.40$), as these tumors are reported to spontaneously arise in C57BL/6 mice as they age. Elevated serum alpha-fetoprotein (AFP) has been reported associated with both human and murine HCC; moreover, AFP has been reported to remain below the threshold of 100 ng/mL in mice without hepatic tumors. Accordingly, we found that serum AFP progressively increased over time in mice bearing either HCA and/or HCC, while it remained unchanged in mice without tumors (Figure S2). Specifically, we found that serum AFP levels above 100 ng/mL strongly correlated with HCC ($p = 6.48e-10$). Similarly to what was observed in mice without tumors, we found no increase in AFP in mice bearing other solid tumors including round cell neoplasms found in livers, confirming these are not primary hepatic tumors and further supporting the concept that AFP can be considered as a marker of hepatocellular tumorigenesis in mice.

We also measured AAV GC in liver and serum ARSB activity in AAV-treated male and female mice. As expected, both GC and serum ARSB activity were significantly lower in mice receiving $2 \times 10^{12}$ GC/kg than in mice receiving $2 \times 10^{13}$ GC/kg (Figures 2A and 2B). In mice receiving $2 \times 10^{12}$ GC/kg of AAV2/8.TBG.<sup>hARSB</sup>, males showed about 7-fold higher levels of GC per molecule of diploid genome (mdg) than females ($0.13 \pm 0.03$ versus $0.02 \pm 0.03$ GC/mdg, respectively).
respectively, Figure 2A). This difference was not statistically significant when the Kruskal-Wallis test followed by the Dunn’s test was applied for multiple pairwise comparisons and became significant (p = 0.09) when direct comparison between males and females was performed by Wilcoxon rank sum test. As shown in Figure 2B, serum ARSB activity was higher, albeit not significantly, in males than in females (2,531 ± 544 versus 1,391 ± 256 pg/mL, respectively; p = 0.09; Wilcoxon test). Conversely, no gender-related differences were observed in mice treated with 2 × 10¹³ GC/kg of AAV2/8 vectors in terms of either liver AAV GC or serum ARSB activity.

Integration analysis

We extracted genomic DNA from mouse HCCs, as well as from healthy surrounding tissue, where available, prepared libraries that were then barcoded, amplified, and subjected to high throughput sequencing using the Illumina MiSeq platform. The grouped reads were aligned to the mouse genome using the Genomic Integration Site Tracker pipeline (https://github.com/mlafave/GeIST) with an additional step that discarded all reads that mapped to the vectors. Furthermore, in an effort to remove any possible protocol-induced artifacts, we also removed integration sites (ISs) that were seen in any other mouse AAV integration study previously performed using this pipeline (Wolfsberg et al., unpublished data).

We analyzed data from 6 animals. For three of the mice (81, 85, and 99), we studied both HCC and control samples, and we analyzed the HCC and control data separately. For another three mice (51, 94, and 95) we had only HCC samples, and we pooled these data together with the HCC data from the first three mice, using the pool of all control samples for comparison. The first step was to identify the number of ISs for each group. We report both the total number of ISs in each group, as well as the number of unique ISs if each genomic position is counted only once (Table S1).

Based on previous work, we only pursued integrations that were present in HCCs but not in controls and had high read counts, suggesting clonality. Only one integration site with a strikingly high read count (>40,000 reads) emerged after filtering based on read counts. This IS was recovered from an HCC sample derived from mouse 95 that had been injected with AAV2/8.TBG.EGFP. It accounted for 28% of all reads and mapped to the Rian locus, located in the vicinity of other Rian integrations both previously associated with HCC and arising from vectors with TBG promoters (Figure 3). We did not recover a corresponding integration on the other side of the Rian locus in this HCC.

As depicted in the sequence logo (Figure 4), the 150 nt of sequence surrounding the genotoxic integration in the Rian locus is fairly well conserved among 31 mammals. This conservation includes rodents, as well as primates, and widely encompasses the 5' and 3' flanking sequences of the integration event. This sequence was further inspected for homology to AAVS1 locus (chr19:50,900,000–58,617,616) and none was detected (data not shown).

No liver tumorigenesis in MPS VI cats following long-term AAV-mediated hepatic gene transfer

MPS VI cats received, on post-natal day 50 (p50), a single systemic administration of AAV2/8.TBG vector encoding the feline ARSB (AAV2/8.TBG.fARSB) at the dose of 2 × 10¹³ GC/kg and were followed-up long term. Above or close to normal serum ARSB activity...
was detected in all cats up to the end of the study, indicating that stable liver transduction was achieved following a single vector injection (Figure 5). Liver ultrasound was performed on MPS VI cats, without evidence of masses or other liver abnormalities (Table S2). Cat #7742 was followed up to about 8.5 years of age (3,065 days) when euthanasia was required because of limb paresis that was attributed to the presence of a focal meningioma, as revealed by microscopic evaluation. Liver histopathology did not reveal any sign of malignant transformation (Figure S3). Cats #8194 and 8197 were terminated when they were about 8 years old (2,878 days old). Liver histopathology was performed on both cats. No microscopic abnormalities were observed: liver was characterized by normal lobular architecture, portal tracts were within normal limits, and hepatocytes were not vacuolated (Figure S3). Thus, data obtained in MPS VI cats, together with those generated in mice, support the low risk of tumorigenicity associated with long-term AAV-mediated gene transfer to juvenile/young adult liver using doses of vectors between 10^{12} and 10^{13} GC/kg.

DISCUSSION

In the present study, we investigated the potential tumorigenic risk associated with long-term AAV-mediated liver gene transfer in young adult mice and juvenile cats after delivery of a variety of therapeutic and reporter AAV2/8 vectors, ranging in dose between 10^{12} to 2 \times 10^{13} GC/kg.

Most of the tumors found in livers were round cell neoplasms, which spontaneously arise in aging colonies16 and are not associated with the high (>100 ng/mL) AFP levels found in mice bearing hepatic tumors. This confirms the potential utility of serum AFP as a screening marker for mouse HCC as already reported for humans.27

Our data showed that HCCs were exclusively found in AAV-treated males at an incidence (15.4%) that was not statistically different than in control mice (0%, p = 0.07; Figure 1). Spontaneous HCC formation is reported in aging colonies of C57BL/6 mice.9,15,21 The absence of HCC in control mice may be explained by the lower number of animals in this group (n = 46) compared to the AAV-treated group (n = 76). Also, HCC were not found in female mice. We investigated if this finding could be associated to the higher AAV-mediated liver transduction in male than in female mice reported by previous studies.22,23 No gender-related differences in terms of both GC and serum ARSB activity were observed in mice treated with 2 \times 10^{13} GC/kg of AAV2/8 vectors (Figure 2), suggesting that this vector dose is possibly saturating and ultimately overcomes any sex-dependent difference in liver transduction. Therefore at least for this dose cohort, the different HCC incidence between males and females may be explained by the role of the androgen pathway in liver carcinogenesis,24,25 with the exception of the HCC in which a clear correlation with AAV integration in the Rian locus has been found.

Conversely, both AAV GC and serum ARSB activity were higher in male than in female mice treated with AAV2/8 at the dose of 2 \times 10^{12} GC/kg. It is therefore possible that in this group the higher HCC incidence is correlated with the higher AAV liver transduction levels observed in males than in females. On the other hand, the HCC incidence observed (1 out 10, 10%) is close to the spontaneous HCC formation rate reported in aging C57BL/6 male mice (8%–9%) and importantly no relevant AAV-ISs were identified in this HCC.9,15

Unlike what has been observed in mice treated as newborns,5,6,20,26 which were typically treated with higher AAV doses than those used here, the animals treated with similarly configured cassettes did not develop HCCs at high rates.

However, the recovery of a genotoxic integration from one HCC which developed in a liver of a mouse treated with an AAV2/8.TBG.EGFP, deserves further commentary. While male mice receiving AAV2/8.TBG.hARSB showed an overall incidence of HCC formation similar to that spontaneously observed in the aging colony of male C57BL/6 mice, i.e., about 8%–9%,9,15 HCC incidence was 30% in mice receiving the AAV2/8.TBG.EGFP vector (Figure 1). Although increasing the number of animals per experimental group may increase the chance to see additional HCCs even in other experimental groups, whether the expression of EGFP, which is a non-endogenous, potentially immunogenic protein,27 caused inflammation, and created an environment prone to tumor development remains a consideration. Indeed, the connection between inflammation and HCC development is well-known.28,29 A previous study from Bell et al.10 reported an increased incidence of HCC in mice treated with AAV vectors expressing the bacterial reporter LacZ compared to those receiving a therapeutic mammalian transgene. Furthermore, later studies on an HCC from a mouse treated with the AAV bearing
the therapeutic gene in this same study was found to harbor a genotoxic integration in Rian (Figure 3). For this reason, genomic analyses were conducted, and our results explain the formation of at least one AAV HCC in the AAV2/8.TBG.EGFP-treated mice.

Our results are consistent with previous studies showing no increased risk of tumor formation in large cohorts of adult mice receiving a variety of different AAV vectors for liver-directed gene transfer. However, the value of those previous studies, endorsing the low tumorigenesis risk associated with AAV-mediated liver gene transfer was limited by the short follow-up compared to the other studies in newborn mice, which indeed showed that in most cases HCC arose between 14 and 25 months of age. Also, where a long-term follow-up was performed, data were restricted to the combination of a self-complementary AAV with the liver-specific promoter 1 (LP1), which both showed no safety concerns in newborn rats and mice, respectively. Therefore, our study addresses with a 2-year prospective follow-up, the risk of tumorigenicity in young adult mice that received single-stranded, hepatotropic AAVs containing different liver-specific promoters and different transgenes. Our data suggest that the risk is low, although the doses used here are considerably lower than other studies that observed HCC in mice after neonatal AAV gene therapy. Neonatal gene transfer with traditional AAVs indeed relies on high dose of vectors (in the order of \(10^{14}\) GC/kg or higher) to counteract the dilution of episomal AAV DNA due to the hepatocyte proliferation. Conversely, our doses are similar to those (ranging from \(2 \times 10^{12}\) to \(4 \times 10^{13}\) GC/kg) used when adult mice were treated with AAV to assess genotoxicity. To the best of our knowledge, only Li et al. tested up to \(1 \times 10^{14}\) GC/kg in adult mice.

Doses spanning in the range of \(10^{14}\) GC/kg have been used in recent clinical trials (e.g., NCT03199469, X-linked myotubular myopathy), as well as for the approved Zolgensma, both targeting a very young population (infant-toddler). However, other gene therapy clinical trials use doses, which are in the range \((10^{12}–10^{13} \text{ GC/kg})\) tested in our study (NTC02991144, NTC04088734, NCT03001830, NCT02576795, NCT01620801), making our findings relevant and comforting in the field of gene therapy, further confirming that toxicity appears dose dependent.

Specifically, for the MPS VI clinical trial (NCT03173521), therapeutic doses have been widely investigated and validated in a pre-clinical setting in both feline and murine models of MPS VI. The vector doses at which we have observed a therapeutic effect ranged between \(2 \times 10^{11}\) and \(2 \times 10^{12}\) GC/kg. Therefore, the doses we tested in the current study, i.e., either \(2 \times 10^{12}\) or \(2 \times 10^{13}\) GC/kg, are in or well beyond (1 log), respectively, the range of therapeutically relevant doses defined in preclinical studies and translated to the clinic.

In addition, we show there are no liver cancers in the three MPS cats followed up to 8 years following vector administration. Future efforts

**Figure 4. Genomic conservation around the integration site in the mouse Rian locus**

The asterisk indicates the position of the integration site. This logo is a graphical representation of a multiple sequence alignment of genomic DNA derived from 31 mammals. The height of each letter indicates its frequency at that position. The overall height of the stack of letters depicts the information content (measured in bits) and reflects the sequence conservation at that position.
will focus on the development of an integration pipeline for the feline genome, with the subsequent capture and analysis of AAV integrations in the treated MPS VI cats as was done for the murine models. Although the low number of MPS VI cats makes the study purely observational, the data collected are consistent with other long-term studies in large animal models, such as dogs and non-human primates (NHPs), in which tumorigenicity associated with AAV hepatic gene transfer has not yet been observed. Also, serum ARSB levels in MPS VI cats were stable over time (Figure 5), suggesting that if integration had occurred, it was not associated with subsequent in vivo expansion.

The pattern of integrations we have observed after systemic AAV gene delivery with AAV2/8.TBG vectors in mice is worthy of commentary. As others have previously documented, the relative numbers of AAV integrations recovered in young adult mice after treatment with AAV is rather low, with only several hundred integrations recovered per mouse (Table S1). We took an additional measure in the pre-analysis step to mitigate any possible artifacts that could be derived from the pipeline, sample processing, and/or tissue contamination and removed any site that had the same coordinate as an IS recovered from any study we have conducted over the past 5 years (n = 5). Such filtering is conservative, as there is evidence for identical ISs being recovered by independent labs using different AAV vectors and different pipelines for the identification of integrations but affords more confidence in our final list of ISs (Table S1). In the future, a combination of tiling with LAM-PCR amplification and long read NGS may be needed to characterize AAV integrations to ensure that both sides of the event are captured for genomic analyses.

To further narrow our focus, we restricted our analysis to events that had relatively high read counts (>5,000), and then further, on only those seen in HCCs compared to controls, reasoning that those integrations may be causative under a model of insertional activation. After this analysis, only one locus emerged as a likely genotoxic integration. Mouse 95 had a single integration event that was confirmed after amplification with independent bar codes, and mapped very close to Mir341, within the Rian locus. In complete agreement with previous studies that have used AAV8.TBG vectors in mouse models of OTC deficiency and methylmalonic acidemia (MMA) and observed HCC formation afterward, the event we captured has only a single ITR detected, and is located very close to the Rtl1 gene (Figure 3), the suspected driver of HCC formation after AAV integration in mice. It should be noted that we were successful in recovering what we interpret as a clonal and pathogenic integration in only 1 of 6 HCCs. Whether the other HCCs we found in the course of our study were driven by AAV, or coincidental, remains unknown but highlights the need for future studies to include genome sequencing in the integration analysis pipeline, especially since the genotoxic event we recovered maps outside of the Mir341 gene, and in a region of the mouse genome that is widely conserved between mice and humans (Figure 4).

While the identification of at least one event that clearly suggests AAV-mediated genotoxicity has safety and monitoring ramifications, it should be noted that AAV integration has not yet been associated with hepatic genotoxicity in NHPs or noted in hemophilia B subjects as long as 10 years post-gene transfer, or in any of a dozen clinical trials based on AAVs, although an extended follow-up is certainly required.

In conclusion, our study suggests that exposure of the juvenile/young adult liver to doses of AAV up to 2 × 10^13 GC/kg, approximating what will be used in many clinical applications, carries a low tumor-genesis risk, likely because hepatocyte proliferation is reduced, which should enable long-term transgene expression by avoiding/minimizing the loss of episomal AAV genomes.

However, results from the integration analysis performed in our study suggest that clonal and pathogenic integrations may still occur and must be taken into consideration when monitoring subjects involved in clinical trials based on AAV-mediated liver gene transfer.

MATERIALS AND METHODS

Animal colonies

C57BL/6 mice were maintained at the IGB Animal House Facility (Naples, Italy). Animals were raised in accordance with the...
Institutional Animal Care and Use Committee (IACUC) guidelines for the care and use of animals in research. After treatment, health status was recorded twice a day, in the morning and at the end of the working day. Abdominal palpation to check for eventual hepatic tumors formation was performed from 9 months post-injection on a monthly schedule and from 21 months post-injection on a weekly schedule (Figure S1).

The feline model of MPS VI colony was maintained at the University of Pennsylvania, School of Veterinary Medicine. Animals were raised under National Institutes of Health (NIH) and U.S. Department of Agriculture (USDA) guidelines and in accordance with the Institutional Animal Care and Use Committee (IACUC) guideline for the care and use of animals in research. The genotype was determined by polymerase chain reaction (PCR). Daily observations were performed on treated MPS VI cats. All experiments performed in animals are conform to the relevant regulatory standards.

Vector production
The following AAV2/8 vectors were used in this study: AAV2/8 vectors encoding hARSB, the fARSB, and the EGFP under the control of the liver specific TBG promoter and the alpha-1-microglobulin/bikunin precursor, i.e., AAV2/8.TBG.hARSB, AAV2/8.TBG.fARSB, and AAV2/8.TBG.EGFP, respectively, and the vector encoding the hARSB under the control of the Lp1, i.e., AAV2/8.LP1.hARSB. All vectors were produced by the AAV Vector Core of Telethon Institute of Genetics and Medicine (TIGEM) (Pozzuoli, Italy), as previously described.

Vector Administration
Young adult (6–8 weeks old) C57BL/6 mice from Harlan Laboratories were injected in the retro-orbital sinus. 20 mice per test group, i.e., 10 males (M) and 10 females (F), were treated as follows (Table 1): 2 × 10¹³ GC/kg of AAV2/8.TBG.hARSB (TEST 1), 2 × 10¹² GC/kg of AAV2/8.TBG.fARSB (TEST 2), 2 × 10¹³ GC/kg of AAV2/8.LP1.hARSB (TEST 3), and 2 × 10¹³ GC/kg of AAV2/8.TBG.EGFP (TEST 4).

Liver ultrasound
Liver ultrasound was performed by manually restraining the cat in dorsal recumbency while the single organ scan is performed and interpreted by a board-certified radiologist. Scans were done on a GE Medical 9L machine using a 2.7–7.8 MHz linear transducer (GE Healthcare, Chicago, IL, USA).

DNA was obtained from tumor and matched control tissues using the DNeasy Tissue and Blood Kit (QIAGEN, Hilden, Germany), following the manufacturer’s instructions. Integration site recovery was performed as previously described. Briefly, DNA was digested independently with restriction enzymesMspI and Csp61. The independent digests for each sample were then pooled and barcoded. Following a nested PCR using primers specific to the barcoded linkers and AAV ITR-specific primers, the samples were then sequenced using the Illumina MiSeq platform. Unique ISs were deconvoluted after sequencing using the Genomic Integration Site Tracker pipeline (GeIST, https://github.com/mlafave/GeIST) and mapped to the mouse reference genome, with an additional step that discarded all reads that mapped to the vectors. The final step was to ensure that the resulting ISs were of high quality and did not result from any artifacts of our experimental or computational pipeline. We compared the recovered IS to the 26,498 unique IS that we have amassed from 5 independent analyses of mouse AAV ISs using the GeIST pipeline, and removed from this dataset any IS that we had detected previously (Wolfsberg et al., unpublished data). However, because
other studies have identified and validated IS in the Rian locus and/or delineated, in different studies, exactly the same ISs in the Alb gene,\textsuperscript{31} we retained all ISs near those loci, even if they were seen in any of the other five datasets.

**Comparative genomic analysis of the AAV8.TBG.EGFP Rian integration**

The conserved genomic sequences around the integration site in the mouse Rian locus (chr12:109607025 on GRCm38/mm10) were derived from the Conservation: Multiz Alignments of 60 Vertebrates track on the UCSC Genome Browser. We selected the region of this track that covered mouse chr12:109606956–109607087, and extracted the corresponding aligned sequences from the following 31 mammalian genomes: mouse, human, baboon, bush baby, cat, chimp, cow, dog, dolphin, elephant, gibbon, gorilla, guinea pig, horse, kangaroo rat, manatee, marmoset, megabat, microbat, mouse lemur, naked mole rat, orangutan, panda, rabbit, rat, rhesus, sheep, squirrel, squirrel monkey, tarsier, and tree shrew. For the gorilla genome, we substituted the sequence from genome assembly GorGor5, rather than the GorGor3 assembly used by Mutiz. Genome sequences were re-aligned using the MUSCLE sequence alignment tool (version 3.8, default parameters) at https://www.ebi.ac.uk/Tools/msa/muscle/. We then created a sequence logo of this alignment using the default parameters of WebLogo at http://weblogo.threeplusone.com/.

**ARSB activity and alpha-fetoprotein levels measurement**

Serum ARSB activity was measured by an immune capture assay based on the use of a specific custom-made anti-hARSB polyclonal antibody (Covalab, Villeurbanne, France), as previously described.\textsuperscript{32} As control, endogenous (murine) serum ARSB activity was measured on 100 ng of total DNA using a set of primers/probe specific for the bovine growth hormone (BGH) poly(A) in the viral genome and TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA), as previously described.\textsuperscript{33} Amplification was run on LightCycler 96 (Roche, Mannheim, Germany) with standard cycles. All the reactions were performed in triplicate.

**Quantification of AAV DNA in liver**

Genomic DNA was extracted from livers using a DNeasy Blood and Tissue Extraction kit (QIAGEN, Hilden, Germany), following the manufacturer’s instructions. Real-time qPCR analysis was performed on 100 ng of total DNA using a set of primers/probe specific for the bovine growth hormone (BGH) poly(A) in the viral genome and TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA), as previously described.\textsuperscript{33} Amplification was run on LightCycler 96 (Roche, Mannheim, Germany) with standard cycles. All the reactions were performed in triplicate.

**Statistical analysis**

The Fisher’s exact test was used to evaluate the incidence of HCCs (Figure 1) and other tumors found in livers and to assess whether AFP levels >100 ng/mL were predictive of hepatic pathology. All statistical computations were made by using the function fisher.test() of stats package of R software (R version 3.6.1).

AFP levels over time (Figure S1) are expressed as mean ± standard error (SE). A two-way ANOVA followed by the Tukey post hoc test was conducted to examine the effects of “group” and “time” on AFP score. There was a statistically significant interaction between “group” and “time” on AFP score (p = 3.44e-11 and eta-squared or effect size n2g = 0.2). Bonferroni adjustment was applied.

AAV liver DNA and serum ARSB activity (Figure 2) are expressed as mean ± SE. The Kruskal-Wallis test followed by the Dunn’s test was conducted to perform multiple pairwise comparisons between experimental groups. The Kruskal-Wallis p value is <0.001. Bonferroni adjustment was applied. The Wilcoxon rank sum test was used for direct comparisons between males and females for each group of treatment. All statistical computing was made by using rstatix package of R software (R version 3.6.1).

Statistical p values <0.05 were considered significant. More details about statistical analyses are reported in the Supplemental Methods section.

A prospective power analysis based on Fisher’s exact test was performed for sample size estimation, using the G * Power 3.1, software. We used data available for spontaneous HCC incidence and AAV-related HCC incidence.\textsuperscript{5,15} The minimum sample size ranged between 8 and 11 animals for each treatment group, using a significance level α = 0.05, a power (1 - β) = 0.80 and event rates p1 and p2 equal to 8% and 82% for control and AAV-treated males, respectively, and 7% and 69% for control and AAV-treated females, respectively.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.11.015.

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**AUTHOR CONTRIBUTIONS**

R.F. and A.A. conceived the study; A.-D.N. and Z.C. were involved in programming and software development; T.G.W. validated data; R.F., M.A., M.D., E.N., J.C., S.N.S., P.O., P.W., and C.V. conducted experiments, performed the investigation process, and collected data; J.C.,
DECLARATION OF INTERESTS
A.A. is founder and consultant of InnovaVector srl. The other authors have no competing interests to declare.

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