Adeno-Associated Virus Type 2 and Hepatocellular Carcinoma?

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IN A RECENT *Nature Genetics* letter, entitled “Recurrent AAV2-related insertional mutagenesis in human hepatocellular carcinomas,” Nault and colleagues¹ document that of 193 patients with hepatocellular carcinoma (HCC), 11 contained an integrated genome sequence of the wild-type adeno-associated virus 2 (AAV2), and suggest that AAV2 is associated with oncogenic insertional mutagenesis in human HCC.

Because AAV2 has long been known to be a nonpathogenic human parvovirus² and, in fact, has been shown to possess antitumor activity,³⁻⁶ it is critical that the scientific and clinical implications of these studies be rigorously assessed to justify their conclusions. We have carefully analyzed the data presented by Nault and colleagues¹ and reached a conclusion that is at variance with that of the authors.

The majority of the insertions contained the AAV2 poly(A) site. In the *TNFSF10* (TRAIL [tumor necrosis factor-related apoptosis-inducing ligand] gene) cases, AAV2 poly(A) increased TRAIL expression, which may be desirable because TRAIL, which selectively kills cancer cells, has been extensively investigated as a cancer therapy drug. For *CCNA2*, insertion in clones 2, 3, and 4 in fact generated transcripts that encode nonfunctional *CCNA2*. For the *KMT2B* case, the insertion of a 386-bp AAV2 3'-DNA fragment effectively introduced 5 in-frame stop codons in exon 3 of the gene's 37 exons, and thus inactivated this allele. Given

that in HCC, this gene is expressed at very high levels, its inactivation might be desirable as well. For one of the *CCNA2* insertions (CHC2128T), the authors did not present any RNA data, which makes it difficult to draw any definitive conclusion in this case.

The single case of *TERT* promoter insertion is also unconvincing. The promoter is taken out of the sequence context. The *TERT* promoter is usually extremely weak in normal cells, and the insertion is unlikely the cause of *TERT* activation. Also, the authors did not show how high the *TERT* activities (RNA counts) are in HCC cells without the AAV2 insertion; they may be on the same level regardless. The authors use DNA-mediated transfection assays in two established human hepatoma cell lines to document a modest 3- to 6-fold increase in *TERT* promoter activity to justify their claim that insertion of AAV2 sequences was responsible for the genesis of HCC. A more appropriate control would have been to use primary human hepatocytes to establish a causal relationship between AAV integration and *TERT* gene overexpression. The transfection experiments also did not include a reference reporter gene as an internal control for transfection errors.

PCR positivity of AAV2 sequences in only 7% of HCC tumors, while there was 21% in the adjacent normal liver tissues, further argues against the authors' conclusion, and can be further questioned by their deep sequencing data in only 7 tumors

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versus 20 normal tissues. Overall, our conclusion is that in the majority of the cases, AAV2 insertion may have either slowed tumor progression or had no effect.

It is important to emphasize that up to 90% of the human population is seropositive for AAV2,⁷ and this stands in stark contrast to an HCC prevalence of fewer than 10 cases of HCC per 100,000 in the United States, a rate that appears to be increasing coincident with the prevalence of hepatitis C virus infection.⁸ It is difficult to explain this if AAV infection truly represents a significant risk for HCC development. Moreover, one of the major limitations of the study by Nault and colleagues¹ is the complete absence of any serological data with reference to AAV2 antibody positivity among case subjects and control subjects. The final limitation is the absence of any information on the possible coinfection by adenovirus and/or herpesvirus in patients with HCC, both because these viruses serve as common natural helper viruses for AAV2 replication, and because both are known to be associated with tumorigenesis themselves. Furthermore, in contrast to established replication-competent oncogenic viruses (e.g. HPV, HCV), there is no evidence of insertion of full-length AAV genomes in AAV-positive HCC. Thus, the study by Nault and colleagues also fails to establish a causal relationship between replication-competent, fully functional AAV and HCC.

In general, AAV has been demonstrated to be an inhibitor rather than an enhancer of carcinogenesis caused by coinfecting viruses. AAV has been shown not only to induce selective apoptosis in cells that lack active p53, but also to inhibit tumor growth in mice.⁹ Furthermore, epidemiological data suggest a protective role for infection by AAV in patients with cervical carcinoma because only 14% of these patients were seropositive for AAV,^{10,11} and AAV has been shown to inhibit human papillomavirus type 16, a virus well known to be the etiologic agent of cervical cancer.¹²

Nault and colleagues¹ also mention a few examples of rodent models in which recombinant AAV vectors have been shown to induce HCC.^{13–15} However, none of the insertions described in their studies was found in previous studies with mice in which HCC was reported after AAV gene therapy with prohibitively high vector doses. Although this may be a reflection on interspecies differences, it could also be suggested that the risk of hitting these HCC driver genes is reduced with rAAV vectors as opposed to wild-type AAV. Furthermore, data from hundreds of normal mice treated with rAAV vectors showed no evidence of malignancy.^{16,17} Similarly, no evidence of

cancer has been detected in large animals, such as dogs¹⁸ and nonhuman primates,¹⁹ administered relatively high doses of rAAV vectors. Perhaps most importantly, in 117 phase I/II/III clinical trials carried out to date with rAAV vectors,²⁰ no adverse event, including cancer of any type, has ever been reported.

The conclusions drawn by Nault and colleagues¹ represent an enormous leap from their data. No comment is made about the possible influence of Rep, a key viral element not only for integration but also for its antioncogenic activity, on their findings. Also, it is important to learn where the integrations in normal tissues occurred relative to those seen in tumors. It would also be useful to know which other changes were seen in the sequences of the genes (introns) of the patients with HCC but without predisposing factors. Furthermore, natural infections with wild-type AAV2 have no demonstrable connection with administration of current rAAV vectors. The ~5% incidence of HCC associated with AAV2 integration appears to be a secondary event after the initiation of tumorigenesis.

In summary, it is difficult to draw any definitive conclusions from the data presented by Nault and colleagues,¹ given the complexity of the cause-and-effect relationship. Perhaps, time will tell. On the contrary, viewed in a more positive light, much like cervical carcinoma, AAV infection might indeed be a key factor in preventing HCC in humans.

NOTE ADDED IN PROOF

While our article was being reviewed, Büning and Manfred similarly challenged the conclusions by Nault and colleagues in an Editorial entitled: “Adeno-associated Vector Toxicity—To Be or Not to Be?”, which was published in the November 25, 2015 issue of *Molecular Therapy* (*Molecular Therapy*, 23: 1673–1675, 2015).

AUTHOR DISCLOSURE

B.J.B., W.W.H., and N.M. own equity in, and W.W.H. is a consultant for, Applied Genetic Technologies Corporation; T.R.F. is advisor to Dimension Therapeutics and Editas Medicine; G.G. is a founder of, and holds equity in, Voyager Therapeutics; and X.X. owns equity in Asklepios Bio-Pharmaceutical, Inc. These companies may in the future commercialize some aspects of AAV vector-mediated gene therapy. T.V. serves as key opinion leader for various companies with a vested interest in hemophilia gene therapy. The other authors declare no competing financial interests.

REFERENCES

1. Nault J-C, Datta S, Imbeaud S, et al. Recurrent AAV2-related insertional mutagenesis in human hepatocellular carcinomas. *Nat Genet* 2015;47:1187–1193.
2. Blacklow NR, Hoggan MD, Sereno MS, et al. A seroepidemiologic study of adenovirus-associated virus infection in infants and children. *Am J Epidemiol* 1971;94:359–366.
3. Mayor HD, Houlditch GS, and Mumford DM. Influence of adeno-associated satellite virus on adenovirus-induced tumours in hamsters. *Nat New Biol* 1973;241:44–46.
4. Cukor G, Blacklow NR, Kibrick S, et al. Effect of adeno-associated virus on cancer expression by herpesvirus-transformed hamster cells. *J Natl Cancer Inst* 1975;55:957–959.
5. Ostrove JM, Duckworth DH, and Berns KI. Inhibition of adenovirus-transformed cell oncogenicity by adeno-associated virus. *Virology* 1981;113:521–533.
6. Hermonat PL. Inhibition of H-ras expression by the adeno-associated virus Rep78 transformation suppressor gene product. *Cancer Res* 1991;51:3373–3377.
7. Erles K, Sebökova P, and Schlehofer JR. Update on the prevalence of serum antibodies (IgG and IgM) to adeno-associated virus (AAV). *J Med Virol* 1999;59:406–411.
8. de Martel C, Maucourt-Boulch D, Plummer M, et al. World-wide relative contribution of hepatitis B and C viruses in hepatocellular carcinoma. *Hepatology* 2015;62:1190–1200.
9. Raj K, Ogston P, and Beard P. Virus-mediated killing of cells that lack p53 activity. *Nature* 2001;412:914–917.
10. Georg-Fries B, Biederlack S, Wolf J, et al. Analysis of proteins, helper dependence, and seroepidemiology of a new human parvovirus. *Virology* 1984;134:64–71.
11. Mayor HD, Drake S, Stahmann J, et al. Antibodies to adeno-associated satellite virus and herpes simplex in sera from cancer patients and normal adults. *Am J Obstet Gynecol* 1976;126:100–104.
12. Hermonat PL. Adeno-associated virus inhibits human papillomavirus type 16: a viral interaction implicated in cervical cancer. *Cancer Res* 1994;54:2278–2281.
13. Donsante A, Miller DG, Li Y, et al. AAV vector integration sites in mouse hepatocellular carcinoma. *Science* 2007;317:477.
14. Rosas LE, Grieves JL, Zarsaspe K, et al. Patterns of scAAV vector insertion associated with oncogenic events in a mouse model for genotoxicity. *Mol Ther* 2012;20:2098–2110.
15. Chandler RJ, LaFave MC, Varshney GK, et al. Vector design influences hepatic genotoxicity after adeno-associated virus gene therapy. *J Clin Invest* 2015;125:870–880.
16. Bell P, Moscioni AD, McCarter RJ, et al. Analysis of tumors arising in male B6C3F1 mice with and without AAV vector delivery to liver. *Mol Ther* 2006;14:34–44.
17. Li H, Malani N, Hamilton SR, et al. Assessing the potential for AAV vector genotoxicity in a murine model. *Blood* 2011;117:3311–3319.
18. Nichols TC, Whitford MH, Arruda VR, et al. Translational data from adeno-associated virus-mediated gene therapy of hemophilia B in dogs. *Hum Gene Ther Clin Dev* 2015; 26:5–14.
19. Li S, Ling C, Zhong L, et al. Efficient and targeted transduction of nonhuman primate liver with systemically delivered optimized AAV3B vectors. *Mol Ther* 2015 [Epub ahead of print]; DOI: 10.1038/mt.2015.174.
20. Hastie E, and Samulski RJ. Adeno-associated virus at 50: a golden anniversary of discovery, research, and gene therapy success—a personal perspective. *Hum Gene Ther* 2015;26:257–265.