AAV Infection: Protection from Cancer

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There are conflicting reports that integration of the wild-type adeno-associated virus 2 (AAV2) genome is associated with induction of hepatocellular carcinoma (HCC) in a small subset of patients. However, there are several lines of evidence that contradict this assertion: (i) AAV2 has long been known to be a non-pathogenic virus, although ~90% of the human population is seropositive for AAV2 antibodies; (ii) AAV2 has been shown to possess anticancer activity; (iii) epidemiological evidence suggests that AAV2 infection plays a protective role against cervical carcinoma; and (iv) five different AAV serotype vectors (AAV1, AAV2, AAV5, AAV8, and AAV9) have been or are currently being used in 162 Phase I/II clinical trials and one Phase III clinical trial in humans to date, and no cancer of any type has ever been observed or reported. A brief historical account of the putative role of infection by AAV in the etiology of cancer, or lack thereof, is presented.

Keywords: wild-type AAV2, recombinant AAV vectors, DNA integration, liver cancer

In late 2015, Nault *et al.*[1] reported that of 193 patients with hepatocellular carcinoma (HCC), 11 (<6%) contained an integrated genome sequence of the wild type (wt) adeno-associated virus 2 (AAV2), and suggested that AAV2 is associated with oncogenic insertional mutagenesis in human HCC. Although this conclusion was questioned,[2,3] in more recent publications, Nault *et al.*[4–7] continue to insist that AAV2 is an oncogenic virus in initiating HCC. Interestingly, Park *et al.*[8] recently reported that following evaluation of a total of 289 unrelated patients with HCC, the presence of AAV2 DNA was detected in tumor tissues from only two (<1%) patients, and concluded that AAV2-mediated HCC is very rare in Korean patients. In view of these seemingly contradictory reports, this review provides a brief historical account of the putative role of AAV in the etiology of cancer, or lack thereof, and attempts to resolve some of this controversy.

WILD-TYPE AAV2 AND CANCER

AAV2 was discovered in 1965,[9] and for nearly half a century, it was not only considered to be a non-pathogenic human parvovirus,[10] but was shown to possess antitumor activity. For instance, very early studies showed that AAV particles could inhibit tumor formation by the type A adenoviruses, Ad12 and Ad31, in hamsters.[11,12] It was shown that this phenomenon did not require intact AAV genomes[13] and could be mediated by AAV particles that contained small defective-interfering (DI) genomes[14] that comprised only the AAV inverted terminal repeats (ITRs). Furthermore, tumor formation was also inhibited efficiently by purified DNA extracted from these particles. This activity of AAV2 is not limited to Ad12 viral-induced oncogenesis. Raj *et al.*[15] subsequently showed that AAV2 could selectively induce apoptosis and kill cells that lack p53 activity but had no effect on cells with normal p53. This effect appeared to be mediated by a DNA damage repair response induced by the AAV DNA ITR, and injection of a synthetic oligonucleotide containing the AAV2 ITR sequence mediated the same effect. The p53 tumor suppressor activity of p53 is lost in most human tumors,[16] and Raj *et al.*[15] also showed that injection of AAV2 could inhibit formation and subsequent growth of tumors mediated by a $p53^{-/-}$ cell line in athymic nude mice.

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AAV can inhibit cellular transformation and oncogenicity, and some of these effects may be mediated by the AAV rep gene. AAV1 inhibited transformation of hamster embryo cells by Ad12,[17] and infection of a HSV2-transformed hamster embryo cell line decreased its oncogenicity in syngeneic hamsters.[18] Also, the oncogenicity of H14b cells, an Ad5-transformed hamster cells line containing only the Ad5 E1 genes, in hamsters was substantially reduced by infection with AAV2.[19] Although AAV did not replicate in H14b cells, expression of AAV mRNA was detected, and there was a decrease in the expression of the Ad E1B 55K oncogene protein. This may have represented expression of the AAV rep gene. AAV2 infection of an NIH3T3 cell line transformed by the *ras* gene resulted in decrease growth efficiency *in vitro* and prolonged increase in the latent period for tumor appearance in nude mice.[20] The AAV rep78 gene can inhibit expression of the H-*ras* gene.[21] Also, *in vitro* transformation of mouse fibroblasts jointly by the adenovirus E1A gene and the Ha-*ras* gene was inhibited by expression of the AAV rep gene but not by the AAV DNA ITR sequences.[22]

An early report that examined the presence of AAV antibodies in sera from cervical cancer patients and normal individuals showed a negative correlation between the presence of AAV seropositivity and cervical carcinoma.[23] This suggested that AAV may have a protective effect against cervical carcinoma. Attempts to test this hypothesis using PCR assays to screen for AAV DNA sequences in normal and cervical tumor tissues yielded mixed and inconclusive results.[24–26] However, subsequent extensive studies by Hermonat *et al*. established the inhibitory effects of AAV2 and AAV2 Rep proteins on human and bovine papillomaviruses,[27–33] hepatitis B virus,[34] and human immunodeficiency virus 1 (HIV-1),[35,36] all associated with malignancy. Furthermore, these investigators also demonstrated the negative regulatory role of the AAV2 Rep proteins on expression from the promoters of the human c-Ha-*ras*, c-*fos*, and c-*myc* proto-oncogenes,[21,37,38] which have been implicated in the etiology of a wide variety of human cancers.

Thus, on the basis of all published studies to date, except for those reported by Nault *et al.*,[1] there is overwhelming, albeit circumstantial, evidence that infection by the wt AAV2 is not associated with cancer, and may in fact protect against cancer.

RECOMBINANT AAV2 AND CANCER

In contrast to the wt AAV2 genome, which has been shown to stably integrate site-specifically into human chromosome 19,[39–42] recombinant AAV (rAAV2) vectors were shown to lack this property.[43,44] It subsequently became clear that the lack of site-specific integration of rAAV2 genomes was due to the fact that the viral *rep* genes, which mediate site-specific integration, were deleted in all rAAV2 vectors. However, this property of rAAV2 vectors was deemed as an advantage, since the risk of insertional mutagenesis would be expected to be reduced, if not obviated. However, it subsequently became evident that rAAV vectors may integrate, albeit at extremely low frequencies,[45,46] and that these integrations occur preferentially into active genes, at least in mice.[47]

However, Donsante *et al.*[48] reported that rAAV2 vectors induced HCC in neonatal mice with mucopolysaccharidosis type VII. Similar observations were also made with normal mice,[49] in which four vector-chromosomal junctions were identified in the *Rian* locus, which contains the genes for several microRNAs. Several of these microRNAs were shown to be highly overexpressed in the tumors. Since the *Rian* locus is expressed highly in neonatal mice, compared with adult mice, rAAV genomes would be expected to undergo integration into this site at a higher frequency. Induction of HCC was also observed when AAV2 vector genomes were introduced into the *Rian* locus using homologous recombination.[50] Although a few additional studies further supported the observation that rAAV2 vectors induce HCC in mice,[51,52] it is important to note that the human genome lacks the *Rian* locus. Furthermore, whereas Rosas *et al.*[53] reported HCC induction in a mouse model using self-complementary AAV (scAAV) vectors, Gauttier *et al.*[54] found no tumor-initiating risk associated with scAAV vectors in newborn rat liver. In extensive studies with large numbers of mice, Bell *et al.*[55] and Li *et al.*[56] found no evidence for tumorigenesis induced by AAV vectors. Similarly, no evidence of tumor formation in the liver was observed following rAAV vector delivery in dogs and non-human primates.[57,58]

Kaeppel *et al.*[58] also reported a largely random pattern of AAV integration in patients with lipoprotein lipase deficiency following gene therapy, and concluded that AAV integration is potentially safe. More recently, Gil-Farina *et al.*[59] reported that rAAV integration is not associated with hepatotoxicity in humans or in non-human primates.

Thus, cumulative evidence to date suggests that despite the use of relatively large doses administered in large animal models as well as in humans, rAAV2 vectors are not associated with tumorigenesis.

OF MICE AND MEN

Despite their extensive use in biomedical research, it has become increasingly clear that for the most part, rodent models are poor surrogates for humans, as well as poor predictors for human diseases. The current controversy is an apt example to illustrate and emphasize this point further. For example, as stated above, the *Rian* locus in the mouse genome has been purported to play a critical role in the AAV vector–mediated induction of HCC, but there is no *Rian* ortholog in the human genome. Similarly, as reported by Chandler *et al.*[52] the rAAV genome was found to be integrated most commonly in the albumin gene in mouse livers, whereas no integration of the wt AAV genome was detected in this locus in the human genome in any of the HCC patients in the studies reported by Nault *et al.*[1]

Additional significant confounding factors between rodents and humans including species differences, average life expectancies, the inbred nature of most rodent models, susceptibility of infection with the wt AAV, and the relatively high rAAV vector dose administration, notwithstanding, the authors concur with Valdmanis *et al.*[60] in their conclusion that rAAV-mediated tumorigenesis still remains unresolved. The conclusion by Russell and Grompe[61] that AAV has found its disease might be deemed premature, and further corroborating evidence must be awaited in the human population.

EPILOGUE AND PROLOGUE

As stated above, on the basis of all published studies to date, except for those reported by Nault *et al.*,[1] there is overwhelming, albeit circumstantial, evidence that infection by the wt AAV2 is not associated with cancer, and may in fact protect against cancer, in view of the fact that the wt AAV possesses anti-tumorigenic properties. Additional epidemiological evidence, suggesting a protective role of AAV infection against cervical cancer notwithstanding, there is convincing evidence that the wt AAV2 negatively impacts various aspects of life cycles of several viruses known be associated with malignancies, such as adenoviruses, papillomaviruses, hepatitis B virus, and HIV-1, and that that AAV Rep proteins have anti-oncogenic activity and suppress expression of several human proto-oncogenes, such as c-Ha-*ras*, c-*fos*, and c-*myc*, which have been implicated in the etiology of a wide variety of human cancers.

The case for the association of rAAV vectors with cancer in humans is even less compelling, given that at least five different serotypes, AAV1, AAV2, AAV5, AAV8, and AAV9, have been, or are currently being used, in 162 Phase I/II, and one Phase III clinical trials in humans to date[62] (www.wiley.com/legacy/wileychi/genmed/clinical/), and no adverse events, much less cancer of any type, have ever been observed or reported. It should be emphasized that in all clinical trials performed to date, the vector doses administered are likely to be far higher than those encountered during a natural course of infection by the wt AAV. It is also important to note that for the most part, only adult patients have been enrolled in clinical trials with rAAV vectors, whereas infection with the wt AAV likely occurs during childhood. Thus, age may be an important consideration. In this context, it is of interest to note that detection rate of AAV2 sequences in Korean patients with HCC (2/289) was significantly lower than that in French patients with HCC (11/193). However, the Korean patients were younger than the French patients, with the median age of the 11 French patients being 55 years compared with the ages of the two Korean patients being 47 and 39 years. Considering that there is correlation between aging and cancer in general, this aspect warrants further studies, as many years must elapse before detectable tumors develop. It is also important, based on the observations of Kaeppel *et al.*,[58] to ascertain what role, if any, rAAV vector integration in the mitochondrial genome may play in humans.

In the unlikely event that rAAV vectors are found to induce neoplasia in a subset of patients due to random integration, additional future possibilities to increase the overall safety and efficacy further could be contemplated to include the development of rAAV vectors co-expressing the Rep proteins, which would not only mediate site-specific integration of the vector genomes but Rep-mediated suppression of oncogenicity as well. Although this would further limit the packaging capacity of rAAV vectors, it is not difficult to envisage the use a dual vector approach in which a therapeutic rAAV vector is ad-mixed with a Rep-expressing AAV vector. Indeed, such an approach has been proven to be successful.[63–67] Site-specific integration of the rAAV vector genome in the target cell would also ensure sustained, long-term transgene expression in post-mitotic as well as in proliferating cells, such as in the growing liver, which may be beneficial to pediatric patients in particular.

Finally, should high rAAV vector doses pose a potential oncogenic threat, efforts should also

continue toward the development of the next generation of rAAV vectors that are more efficient at significantly reduced doses.[68–70]

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AUTHOR DISCLOSURE

A.S. holds issued patents related to AAV vectors that have been licensed to various AAV gene therapy companies. No competing financial interests exist for B.J.C.

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