Molecular Therapy
Editorial

Liver gene therapy and hepatocellular carcinoma: A complex web

Genetic correction of inborn errors recently became a reality with the first gene therapies being approved by regulatory authorities. Several inborn errors affecting the liver have been at the forefront of gene therapy research, which often utilizes adeno-associated viral (AAV) vectors for hepatic gene transfer. Controversial evidence has been provided in humans and animal studies that wild-type AAV or, in some cases, AAV vectors can contribute to the formation of hepatocellular carcinoma (HCC). This question is further complicated in humans who may have a history of infection with hepatitis viruses or developed non-alcoholic fatty liver disease (NAFLD), which increase the risk for HCC. These findings suggest that proactive HCC screening is warranted in subjects undergoing hepatic AAV gene therapy and more research is needed to understand potential interactions between these factors and gene transfer.

In the liver, hepatocytes synthesize a large number of proteins required throughout the body, including blood clotting factors. Two common bleeding disorders, hemophilia A and B, are the result of inborn insufficiencies of clotting factors VIII (FVIII) and IX (FIX) in sinusoidal endothelial cells and hepatocytes, respectively, that cause recurrent bleeding events and long-term morbidity. Historically, hemophilias were treated with repeated administrations of plasma products, resulting in widespread iatrogenic hepatitis B (HBV), hepatitis C (HCV), and human immunodeficiency virus (HIV) infections. More recently, recombinant FVIII and FIX and other treatments have replaced plasma-based therapies, protecting the majority of hemophilia patients under 40 from exposure to these viruses. A number of gene therapy strategies are being explored to correct hemophilia, with AAV vectors having advanced into late-phase clinical development. Infectious AAVs are small DNA parvoviruses that cause minimal symptoms and are naturally replication deficient. While a majority of humans are AAV seropositive, most notably to AAV2, no clinical diseases have convincingly been associated with these infections. AAV vectors aim to exploit some of these stealth properties to deliver a cargo gene (e.g., FIX) in hepatocytes, while minimizing immune activation. After entry, AAV-FIX vectors translocate to the nucleus, where they are maintained primarily as an episome from which the FIX transcript is expressed using hepatocyte-specific promoters. Because of their low basal proliferation rate in healthy human livers, hepatocytes transduced with AAV vectors are believed to stably express the transgene for years. The therapeutic efficacy of this approach was recently shown in late-phase AAV-FIX clinical trials, and therapeutic expression has been demonstrated in patients for over a decade.[1]

In addition to forming an episome, AAV vectors have long been known to integrate into the host genome at a low rate. In mouse livers, AAV occasionally caused HCC, a primary liver cancer.[2] Several factors, including relatively high vector dose, were shown to affect this association between AAV integration and murine HCC, yet the relevance to human liver biology remained unclear. Six years ago, Nault et al.[3] described 11 HCCs in patients without the typical risk factors for this type of cancer, namely cirrhosis from chronic liver disease. The tumors contained chromosomal wild-type AAV2 3′ end sequences integrated in oncogenes that could plausibly have contributed to HCC formation, albeit that the rep gene (which directs site-specific integration and has anti-proliferative and anti-oncogenic properties) was consistently missing from the integrants. Since then, wild-type AAV integration in oncogenes has been confirmed in cohorts around the world, accounting for a small fraction of HCC. Whether AAV gene therapy vectors (which lack rep) can integrate into oncogenes in human hepatocytes remains unknown.

Recently, two dogs with hemophilia A that received AAV gene therapy with canine *F8* displayed rising plasma FVIII activity starting 4 years after vector administration.[4] Although no HCCs were detected, integration events were enriched in or near genes previously associated with cell growth, yet notably distinct from the oncogenes affected by AAV2 in humans. Whether rising canine FVIII activity could have resulted from the expansion of hepatocyte clones with functional integrants is unclear, since most of the integrants they found comprised promoters only and lacked F8 cDNA sequences. These observations of infectious AAV integration in HCC and long-term AAV vector causing FVIII rises in dogs raise concerns about AAV vector integrants affecting hepatocyte proliferation. One study followed a small number of hemophilia B patients up to 15 years after liver-directed AAV2-FIX gene transfer and found no evidence of tumor formation.[1]

Viral integration is not unique to AAV. HBV is another small stealthy DNA virus that infects hepatocytes and integrates into its genome. Chronic HBV infection is the leading cause of HCC worldwide. Contrary to most other HCC etiologies, which develop overwhelmingly in cirrhotic livers, approximately 20% of HBV tumors develop without cirrhosis. This has sparked widespread research into whether HBV integration drives HCC formation. After entry into a hepatocyte, HBV is converted into an episome in the nucleus in the form of covalently closed circular (ccc)DNA. Viral transcripts originate from cccDNA, including pregenomic RNA, which is the template for reverse transcription by viral polymerase. After completing the first DNA strand, polymerase can form the second strand in two directions, depending on where its RNA primer binds. This results in about 90% circular DNA, the precursor of infectious virus, and 10% double-stranded linear DNA. The latter can integrate into chromosomal breaks, which happens early in the infection. Despite its ability to integrate into hepatocytes, only a modest percentage of HBV-associated HCC contain viral integrants in oncogenes. Intriguingly, some

putative oncogenes found in HBV tumors overlap with the AAV2 integration loci described by Nault et al.[3] How infectious AAV2 integrates and whether AAV vectors themselves or defective genomes created during production use identical integration mechanisms requires further investigations. Somewhat reassuring is that only small fractions of HCC carry plausible tumor-causing AAV2 or HBV integrants. The widespread AAV2 seroprevalence, with a fraction showing liver replication,[5] and the many decades between HBV infection and HCC formation both suggest that viral integrants infrequently cause HCC.

In December 2020, a participant in an AAV-FIX clinical trial, who had received vector 1 year earlier, was diagnosed with HCC, which resulted in the pausing of the trial. Limited publicly available data indicate that this participant also had HBV, HCV, and NAFLD. All three conditions are independently associated with increased HCC risk, albeit mostly in livers with advanced fibrosis or cirrhosis. Chronic HCV was the leading etiology of advanced liver disease and HCC in many countries, including the US. Seven years ago, its dire prognosis changed with the approval of direct acting antivirals (DAAs). These medications are easily tolerated and highly effective at curing HCV, including in patients with hemophilia. After curing HCV with DAAs, many patients with advanced fibrosis or early cirrhosis regress by non-invasive parameters, such as elastography. And while curing HCV lowers HCC risk starting 1–2 years after treatment, individuals with cirrhosis remain at elevated risk for at least 10 years compared to those without advanced fibrosis.[6] So, while DAAs have relegated HCV to a problem of the past for individuals with hemophilia, residual HCC risk may remain elevated based on past advanced fibrosis or cirrhosis. Whether HBV exposure without detectable replication in peripheral blood further increases this risk remains controversial. In addition to HBV and HCV, the trial participant with HCC was also reported to have NAFLD. This recently recognized liver disease is associated with the obesity epidemic, already affecting 25% of the world population and continuing to rise. In the US, NAFLD is modeled to become the leading etiology of advanced liver disease later this decade. While individuals with hemophilia are probably at similar risk for NAFLD as the general population, its high prevalence will add yet another unknown for a subset of individuals undergoing liver-directed gene therapy. In addition to further research, one immediate concern is whether more proactive HCC screening is warranted in people undergoing liver-directed AAV gene therapy without a history of current or past advanced fibrosis. HCC screening for liver disease combinations without advanced fibrosis is not commonly practiced nor recommended by current liver society guidelines. Given the likely very small but possibly real risk of AAV vector integration contributing to HCC formation, liver diseases from the past and future warrant careful scrutiny in individuals undergoing liver-directed AAV gene therapies.

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