Integration/Insertion Considerations for AAV-based Gene Therapy Vectors

ASGCT Recommendations based on the Society's AAV Integration Roundtable event

ASGCT-FDA Liaison Meeting
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ASGCT AAV Integration Roundtable

- ASGCT Convened **a roundtable of multi-stakeholder experts** in the field on August 18, 2021 to discuss AAV integration, findings from non-clinical research, and implications for drug development and clinical trials.

ASGCT AAV Integration Roundtable Experts	
Kevin Eggan, Ph.D., BioMarin Pharmaceutical	**Markus Grompe, M.D, Oregon Health & Science University**
Randy Chandler, Ph.D., NIH, NHGRI	Mark Kay, MD, Ph.D, Stanford University School of Medicine
Ronald Crystal, M.D., Weill Cornell Medicine	David Lillicrap, M.D, Queens University
Ricardo Dolmetsch, Ph.D., UniQure	Eugenio Montini, Ph.D., San Raffaele Telethon Institute for Gene Therapy
Guangping Gao, Ph.D., Umass Medical School	Denise Sabatino, Ph.D., Children's Hospital of Philadelphia
Irene Gil-Farina, Ph.D, GeneWerk	Dinah Sah, Ph.D., Voyager Therapeutics
Fred Bushman, Ph.D, University of Pennsylvania School of Medicine	Jing Yuan, Ph.D., Pfizer

Discussion from FDA's September 3, 2021, CTGTAC meeting on AAV toxicity-integration was considered in preparation for this liaison meeting.

Learnings and recommendations regarding AAV integration will be compiled in a **whitepaper issued by ASGCT in collaboration with multi-stakeholder experts**.

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rAAV Integration Background and Current Thinking

rAAV Integration Mechanism

Takeaways:

- AAV genomes uncoat within the nucleus and present as linear DNA molecules with hairpin inverted terminal repeat (ITR) structures.
- Free DNA ends are recognized by DNA damage repair pathways leading to conversion to double-stranded DNA (dsDNA) and ligation of free ends to form circles, concatemers, and chromosomally integrated forms.
- The repair processes are frequently imprecise, leading to aberrant junctions and deletions and rearrangements within the vector genome and/or the chromosome.
- We do not yet have a full understanding of the factors that determine the distribution of species that result from repair processes in mammalian cells, or how they might be influenced by parameters of the design and production of the vector and the tissue being transduced.

ASGCT Position

- As a field, we need to further our understanding of the molecular fate of recombinant AAV (rAAV) DNA and the factors that influence these outcomes.

rAAV Integration Frequencies

Takeaways

- rAAV integration is generally random in terms of DNA sequence, but favored in actively transcribed regions, DNA hairpin structures (or other features that are prone to dsDNA breaks), or ribosomal RNA genes in those AAV serotypes that traffic to the nucleolus.*
- In rodents, these processes are largely complete within 4-6 weeks post-administration in liver and muscle, and the likelihood of integration after that point is greatly reduced.**
- Estimates of rAAV integration rates in hepatocytes range from 0.1 to 10% of vector genomes. Recent estimates in humanized mouse models suggest 1 to 3%. Recent analysis of patient samples from UniQure suggest 0.01 to 0.1%.
    - The integration rate is likely dependent on the quantity of rAAV entering the nucleus.
    - rAAV vectors bearing significant homology to chromosomal sequences have been shown to lead to targeted recombination at those loci, but the relative frequency of these events are difficult to predict.

ASGCT Position

- Estimates of integration frequencies remain highly variable, illustrating a need for better assays to quantitate and characterize integration events in nonclinical and clinical samples.

*Nakai et al., AAV serotype 2 vectors preferentially integrate into active genes in mice. Nat Genet. 2003 Jul;34(3):297-302. [PMID 12778174]
**Nakai et al., Extrachromosomal recombinant adeno-associated virus vector genomes are primarily responsible for stable liver transduction in vivo. J Virol. 2001 Aug;75(15):6969-76 [PMID: 11435577]

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Impact of Vector Product Quality

Takeaways

- Known features of rAAV vector design including potential hairpin structures, repetitive and GC rich regions, and excessive genome size can contribute to aberrantly packaged genomes.
- The process whereby rAAV products are manufactured may have an influence on the quantity and characteristics of sequence contaminants and the form of vector genome termini.

ASGCT Position

- The scientific community must remain committed to understanding the integrated vector forms associated with HCC and how vector design and quality attributes contribute
- Additional research in the field is needed to determine whether AAV vectors without termination signals pose an increased risk and also to further our understanding of what design features are needed to mitigate.

Risk Assessment of rAAV Integration

Theoretical Risk of Oncogenesis Associated with rAAV Chromosomal Integration

Takeaways:

- To date, the only observed cancer associated with recombinant AAV (rAAV) integration has been hepatocellular carcinoma (HCC) in mice. Transduction in muscle, CNS, pancreas and other organs does not reach the copy number achieved in the liver and has not led to measurable tumor incidence in mouse models.
    - Systemically administered products with transduction in liver may have different considerations than locally administered products, e.g., in the eye.
- Given the large number of hepatocytes transduced with rAAV in many gene therapy applications, even a small percentage of integrated genomes would result in a large number of integration events.
- Random integration patterns could result in integration events near or within proto-oncogenes.
- Although rAAV chromosomal integration is likely to be complete within weeks of vector administration, longer-term data will inform any impact of integration events in humans. Humans treated with AAV and followed for up to 8 years have shown no signs of oncogenesis.

ASGCT Position

- The risk of oncogenesis associated with rAAV chromosomal integration in humans is theoretical based on current data and information.

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Applicability of Animal Models to Predicting Risk in Humans

Takeaways:

- Although rAAV has unequivocally enhanced occurrence of HCC in laboratory mice, several aspects of these models suggest that they have enhanced sensitivity to these events relative to humans:
    - HCC occurs as a background tumor in most mouse strains
    - Mice have longer telomeres, which allows increased proliferation prior to crisis and associated apoptotic events that prevent tumor formation
- Vector designs that reduce HCC risk in mice (e.g., lacking strong enhancer/promoters that are active in the liver), would be likely to reduce the risk of HCC in humans.
- Large animal models such as canines and NHP may more accurately predict oncogenic risk in humans, although frank tumor formation may require one to two decades.

ASGCT Position and/or Recommendations

- Additional studies assessing the oncogenesis of strongly transactivating vectors in large animals could be investigated through public/private partnerships to address these issues but are likely impractical as a near term solution for individual program development as this may require one to two decades of research.
- Basic AAV vector platform research may find use of sensitive mouse models of oncogenesis to provide information about the relative risk of different vector designs, however, the translatability of any murine oncogenic events to humans remains unclear.

Canine Studies

Takeaways

- In canines treated with rAAV-FVIII, there was evidence of clonal expansion of hepatocytes with integrated rAAV approximately 4-9 years after vector administration, associated with increased expression of FVIII, but without histological evidence of HCC or other cancerous lesions.
    - Clonal expansion of hepatocytes containing integrated rAAV was confirmed through sequencing.
    - The significance of this clonal expansion with regard to interactions with integrated rAAV is difficult to interpret due to a general lack of knowledge regarding clonal expansion with age in normal liver tissue in canines.
- Observation of high variability in measured rAAV copy numbers within single liver tissue samples suggest that data from liver biopsies may not be representative of the liver as a whole.
- **Findings are variable** - In one study there was modest enrichment for integrations near oncogenes or growth control genes among clonal expansions. In the other study clustering of integrations in 3 specific chromosomal regions were not associated with proto-oncogenes, though some integration sites associated with suspected proto-oncogenes were identified.
    - Some of the observed differences in integration patterns noted between the canine studies may be due to methodologies including sequencing methods and genome annotation.

ASGCT Position

- Recent observations of clonal expansions in canine hepatocytes in the absence of tumor formation warrant additional studies but may reflect natural clonal dynamics in aging animals.

Methodologies for Assessing rAAV Integration

Takeaways

- Newer sequencing methods are improving our ability to sensitively detect rAAV DNA integration events and may reveal that integration frequencies of rAAV vector genomic DNA is higher than previously determined.
- Sequencing methodologies that incorporate steps to minimize interference from episomal rAAV genomes should be emphasized, although separate measurement of episomal copy number provides a critical perspective for integration rates.
- Differences between methodologies for sequencing methods make standardization and rigorous quantitation challenging.

ASGCT Position

- Need to improve sequencing methods for integrated rAAV genomes and genome fragments.

In Vitro Models

Takeaways

- **Features needed in cell cultures to reflect patterns of rAAV integration in vitro:**
    1. Non-dividing or slowly dividing cultures that are dominated by non-homologous end-joining pathways for DNA repair
    2. Transcription/expression patterns that are representative of hepatocytes or other target tissues. Tissue architecture may also be important
    3. Sufficiently infectable with relevant AAV serotypes to achieve vector genome copy numbers approximating those observed in vivo, recapitulating concatemeric populations (this has been difficult to achieve with liver organelle models)
    4. The ability to measure clonal expansion over time
    5. The ability to detect signals of transformation or interactions with proto-oncogenes
    6. Without the ability to report signals of transformation, in vitro models are unlikely to reveal anything beyond the typical quasi random integration patterns

ASGCT Position

- Further research is needed on development and potential predictability of in vitro models on integration and associated risks; however, limitations in primary hepatocyte culture are currently a substantial barrier to the development of such models.

Clinical Considerations

Takeaways

- No clinical adverse events related to integration have been observed thus far in the many patients treated with AAV vector-based gene therapies followed for up to 8 years to date,* however, it was noted that the overall clinical database is small, both in size and in duration.

ASGCT Position and/or Recommendations

- While there is a theoretical risk that rAAV integration could lead to insertional oncogenesis in patients, the paucity of rAAV-associated HCC in large animals and humans suggests that this risk is small and does not likely outweigh the potential benefit from AAV vector-based gene therapies, especially for serious and life-threatening conditions.
- As with other classes of therapeutics, it is important to communicate the relative risk benefit equation to patients and their physicians; specifically highlighting what we know and emphasize the theoretical versus observed / documented risks (e.g., risk demonstrated pre-clinically vs. those observed in clinic). Product labeling and risk management plans will be a critical element of communicating risks.
- It is view of ASGCT that given the current diseases targeted with AAV gene therapy, and the actual documented risks, the risk/benefit ratio would suggest that trials should be able to proceed in parallel with additional investigation, and in the post-market setting. This is particularly true for disorders in which the liver is not the target.

*Nathwani et al., Adeno-associated mediated gene transfer for hemophilia B: 8 year follow up and impact of removing “empty viral particles” on safety and efficacy of gene transfer. Blood. 2018 Nov;312(Suppliment 1):491. [doi: 10.1182/blood-2018-99-118334]

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Long-Term Follow-Up Monitoring of Subjects

Takeaways

- Long term monitoring for development of HCC will likely be a key component of clinical plans, particularly for therapies with liver-directed expression, constitutive expression, or strong liver enhancers.
- Routine biopsies for monitoring/follow-up after administration are invasive and of limited utility. Given the heterogeneity within liver samples noted in the canine studies, and the low probability of sampling an incipient clonal expansion, biopsies are likely to reflect random rAAV integration patterns.
- Cell-free DNA (cfDNA) could be a potential safety biomarker that may allow for early identification of expanding clones growing in solid organs, but further research and validation is needed.

ASGCT Recommendations

- Duration of long-term follow-up monitoring should be risk-based and depend on the product-specific risk factors (e.g., promoter, route of administration, dose) and patient population's background risk.
- Monitoring should focus on non-invasive methods such as ultrasound, liquid biopsies, biomarkers through blood work, etc.
- Biopsies of neoplastic nodules and surrounding non-tumor tissue should be conducted to further investigate any positive signal picked up in non-invasive methods.
- Cell-free DNA (cfDNA) should be further researched and validated in the field as a potential safety biomarker.

ASGCT Recommendations

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Summary: ASGCT Recommendations

- **Theoretical Risk**
  - The risk of oncogenesis associated with rAAV chromosomal integration in humans is theoretical based on current data and information.
  - While the risk of leading to insertional oncogenesis in patients is theoretical, the paucity of rAAV-associated HCC in large animals and humans suggests that it is small and does not likely outweigh the potential benefit from AAV vector-based gene therapies, especially for serious and life-threatening conditions.
  - Given the current diseases targeted with AAV gene therapy, and the actual documented risks, the risk/benefit ratio would suggest that trials should be able to proceed in parallel with additional investigation
- **Animal Models**
  - The use of sensitive mouse models of oncogenesis may provide information about the relative risk of different vector designs; however, translatability of any murine oncogenic events to humans remains unclear.
    - If longer term PD studies are conducted, the background rates of HCC must be taken into account and risks must be understood.
  - While additional studies assessing the oncogenesis of strongly transactivating vectors in large animals will be needed to better understand these issues, these studies are impractical as a near-term solution for specific development programs and may require one to two decades of research.
  - Recent observations of clonal expansions in canine hepatocytes in the absence of tumor formation warrant additional studies in the field more generally; however they may reflect natural clonal dynamics in aging animals

Summary: ASGCT Recommendations

- **Methods for Assessment**
  - There is value today in performing integration-site analysis with existing methods on tumor tissue samples from patients, and WGS on tumor samples from non-clinical and clinical studies should they occur. ISA, WGS and gene expression analysis of any malignant tumors should be performed.
    - Limitations to these methods may be overcome by rapid and ongoing improvements in sequencing technologies, such as target enrichment and long-read sequencing methods. These methods may be used as a secondary screening method, and are likely to provide greater insight into concatemeric and highly rearranged integrated vector genomes.
  - ASGCT proposes an expert group be convened to make recommendations regarding the shortcomings of these existing methods used to assess oncogenic events when detected, and assess methods that would overcome them, to enable better understanding of the relationship between AAV integration and HCC in humans.
  - Cell-free DNA (cfDNA) should be further researched and validated in the field as a potential safety biomarker.
- **Clinical Assessment**
  - Biopsies of neoplastic nodules and surrounding non-tumor tissue should be conducted to further investigate any positive signal picked up in non-invasive methods.
  - Duration of long-term follow-up monitoring should be risk-based and depend on the product-specific risk factors (e.g., promoter, route of administration, dose) and patient population's background risk, including age and presence of chronic liver regeneration.

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Thank you