Directed evolution of adeno-associated virus 5 capsid enables specific liver tropism

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Impressive achievements in clinical trials to treat hemophilia establish a milestone in the development of gene therapy. It highlights the significance of AAV-mediated gene delivery to liver. AA5 is a unique serotype featured by low neutralizing antibody prevalence. Nevertheless, its liver infectivity is relatively weak. Consequently, it is vital to exploit novel AA5 capsid mutants with robust liver tropism. To this aim, we performed AA5-NNK library and barcode screening in mice, from which we identified one capsid variant, called AA5zk2. AA5zk2 displayed a similar yield but divergent post-translational modification sites compared with wild-type serotypes. Mice intravenously injected with AA5zk2 demonstrated a stronger liver transduction than AA5, roughly comparable with AA8 and AA9, with undetectable transduction of other tissues or organs such as heart, lung, spleen, kidney, brain, and skeletal muscle, indicating a liver-specific tropism. Further studies showed a superior human hepatocellular transduction of AA5zk2 to AA5, AA8 and AA9, whereas the seroreactivity of AA5zk2 was as low as AA5. Overall, we provide a novel AA5 serotype that facilitates a robust and specific liver gene delivery to a large population, especially those unable to be treated by AA8 and AA9.

INTRODUCTION

Applications of adeno-associated virus (AAV) vectors in the treatment of liver diseases have drawn enormous attention in recent years, as featured by the impressive success in several clinical trials for hemophilia A and B.1–5 Fortunately, AAV administration at moderate doses is sufficient to restore blood coagulation safely in these cases,3,5 However, extensive usage of AAV beyond hemophilia may require more robust gene delivery into hepatocyte and less off-target tissue/organ transduction. Moreover, preexisting neutralizing antibodies (NAbs) against the AAV particles also set barriers to the application of gene therapy in a broader range of population.6,7 NAbs in the circulation are able to shield the receptor binding sites exposed on the capsid protein surface and nullify the therapeutic effect of the AAV vectors.9,10 Therefore, developing novel AAV serotypes with superior hepatocyte transduction efficiency is an interesting direction to take. During the past decade, many studies have tried various methods such as DNA shuffling, error-prone PCR, and directed point mutations to evolve natural capsid proteins.11–14 Although improved liver transduction has been reported among their engineered AAV particles in primary human hepatocytes and human hepatocellular xenograft mouse models, preexisting NAbs still block the activity of these AAV serotypes.15 It is probably associated with the conserved epitope sequences from the natural AAV capsids, which is hard to eradicate. AAV5 is a distinct serotype due to its relatively low amino acid identity compared with others, unique Rep protein cleavage site, divergent crystal structure, and its preference for the herpes simplex virus (HSV) as a helper in nature.16–20 Interaction with 2,3-N-linked sialic acid containing glycans is a prerequisite for the entry of AA5 into neuronal and airway epithelial cells, together with platelet-derived growth factor receptor (PDGFR) as a co-receptor.21–24 The top edge of AA5 is the low preexisting NAbs levels among multiple groups of people, ranging from European to Asian and from healthy to hemophilia and HIV-infected patients.25–28 In addition, in some clinical trials, sustained systemic Factor VIII levels were detected even in hemophilia patients with preexisting anti-AA5 NAbs,29,30 implicating the potential ability of AA5 to circumvent circulatory neutralizing factors, although the detailed mechanisms remain obscure. Therefore, despite the inferior transduction efficiency of murine and human hepatocytes to AA5, AA8, or AA9,13,14 AA5 is a promising backbone to exploit desired serotypes that are suitable for a large number of subjects. We previously combined error-prone PCR and shuffling to engineer AA5.11 Whereas the directed evolution generated capsid mutants exhibiting profound transduction capability in Huh7 cells and human primary hepatocytes without impeding the advantage of mild seroreactivity, most of them displayed higher lung infectivity compared with AA5, suggesting the lack of liver specificity.

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Oligopeptide-insert library and barcode selections stand out with the advantage of high-throughput screening of more than $1 \times 10^6$ capsid variants both in vitro and in vivo. These methods have been successful in developing novel AAV serotypes with more splendid tissue and organ tropisms toward the brain, skeletal muscle, and retina as compared with wild-type AAVs. To generate novel AAV5 capsid mutants with better liver specificity while retaining low seroreactivity and NAb prevalence, we tried the above methods with minor modifications. After 2 rounds of library screening and another single round of barcode selection in mice, we picked up a candidate with a 7-mer oligopeptide "FAPTPGP" after the amino acid Q574 on the variable region VIII of the AAV5 capsid (referred to as AAVzk2 hereafter), which was demonstrated to have no negative impact on virus production. However, mass spectrum analysis revealed significant differences in multiple post-translational modification (PTM) patterns between AAV5 and AAVzk2, implying distinct biological properties. Furthermore, the intravenous administration of the novel AAV vector in mice that expressed green fluorescent protein (GFP) enabled a robust and specific gene delivery in the liver, but not other tissues and organs such as the heart, lung, spleen, kidney, brain, and skeletal muscle. Gender differences had little effect on the biodistribution of AAVzk2. Additional study on human hepatocellular carcinoma and normal liver cell lines revealed a relatively higher transduction of the engineered serotype than AAV5, AAV8, and AAV9. Concomitantly, the prevalence of NAbs against the novel serotype was comparable to AAV5, significantly lower than AAV8 and AAV9, illustrating its potential usage in a large population group that is not able to be treated with AAV8 and AAV9. Overall, this engineered AAVzk2 possesses specific liver tropism with diminished off-target tissue/organ transduction and seroreactivity. It may pave the way for the extensive application of gene therapy in more liver diseases.

RESULTS

Selection of liver tropic AAV5 capsid variant

To develop novel AAV capsids, we generated an AAV5-NNK library by inserting the coding sequence of a 7- to 8-amino acid (aa) oligopeptide library after Q574 on the variable region VIII of the AAV5 capsid (referred to as AAVzk2 hereafter), which was demonstrated to have no negative impact on virus production. However, mass spectrum analysis revealed significant differences in multiple post-translational modification (PTM) patterns between AAV5 and AAVzk2, implying distinct biological properties. Furthermore, the intravenous administration of the novel AAV vector in mice that expressed green fluorescent protein (GFP) enabled a robust and specific gene delivery in the liver, but not other tissues and organs such as the heart, lung, spleen, kidney, brain, and skeletal muscle. Gender differences had little effect on the biodistribution of AAVzk2. Additional study on human hepatocellular carcinoma and normal liver cell lines revealed a relatively higher transduction of the engineered serotype than AAV5, AAV8, and AAV9. Concomitantly, the prevalence of NAbs against the novel serotype was comparable to AAV5, significantly lower than AAV8 and AAV9, illustrating its potential usage in a large population group that is not able to be treated with AAV8 and AAV9. Overall, this engineered AAVzk2 possesses specific liver tropism with diminished off-target tissue/organ transduction and seroreactivity. It may pave the way for the extensive application of gene therapy in more liver diseases.
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fect of the inserted oligopeptide on viral production.

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ally, such as acetylation site K232 on AAVzk2 and methylation sites
spite of a vast majority of conserved sites on both, we identi-

We then packaged a self-complementary GFP transgene cassette
controlled by the chicken β-actin (CB) promoter (hybrid of cytomeg-

Discrepancies in post-translational modification sites between
AAVzk2 and AAV5
During vector production, AAV capsids acquire PTMs, which play
a critical role in immunogenicity, targeting, functional activity,
and stability. To determine whether the oligopeptide insertion could
alter the capsid PTM patterns, we established a liquid chromatog-
raphy-tandem mass spectrometry (LC-MS/MS)-based workflow
(Figure 2A). After digestion by chymotrypsin or trypsin/Glu-C, 6
types of PTMs, including acetylation, methylation, phosphoryla-
tion, ubiquitination, deamidation, and O-glycosylation, were
analyzed by LC-MS/MS based on the b/y ion fragments. PTMs
were distributed along the capsid proteins of the AAV5 and
AAVzk2, and more PTMs were identified on the VP3 (Figure 2B).
We identified protein N-terminal acetylation on both AAV5 and
AAVzk2 capsids, which was similar to other AAV serotypes.35 In
spite of a vast majority of conserved sites on both, we identified
many unique PTMs only on AAV5 or AAVzk2 capsids individu-
al, such as acetylation site K232 on AAVzk2 and methylation sites
R437 and R456 on AAV5 (Figures 2C, S2A, and S2B). We also
observed that residues S77, S108, and S487 were only phosphory-
lated on the AAVzk2. Residue K451 was only ubiquitinated on
AAV5 capsids. Intriguingly, we failed to identify any ubiquitination
site on the AAVzk2 capsids (Figures 2C, S2C, and S2D), which are
widely known to degrade capsid proteins and impair transgene de-

Specific liver tropism of AAVzk2 in mice
To study global transduction in vivo comprehensively, we treated
male mice with the indicated AAV encoding GFP via tail vein injec-
tion at a dosage of 1 × 1013 vg/kg. Three weeks post-administration,
we found that 92.25% hepatocytes were GFP positive in the AAVzk2-
treated group, dramatically superior to the AAV5-treated liver—only
22.64% expressed GFP. This level was even comparable with the
AAV8- (85.37%) and AAV9- (88.11%) treated groups (Figure 3A).
Nevertheless, unlike AAV8 and AAV9 demonstrating splendid trans-
duction in the heart and mild to moderate infectivity of the lung,
spleen, and kidney, there were few GFP-positive cells in those tis-
uues/organs of the AAVzk2-treated mice, implicating the specificity
of liver transduction. Thus, we quantified the relative tropisms be-
tween the liver and the heart, lung, spleen, and kidney by calculating
the ratios of GFP-positive cells in the liver versus other tissues/organs.
Intriguingly, the relative tropisms of liver versus heart, lung, spleen,
and kidney of AAVzk2 were 4- to 11-fold higher than AAV5,
AAV8, and AAV9 (Figures 3B–3E). Furthermore, AAVzk2 exhibited
little brain infectivity at dosages up to 5 × 1013 vg/kg (Figures 3F
and 3G). A discrepancy has been observed between genders regarding
AAV transduction of murine livers.36,37 The copy number of vgs
delivered by AAV into male livers could be several-fold higher than
those into female livers, probably associated with the androgen levels.
To illustrate whether the liver transduction of AAVzk2 would be
diverse in females, we intravenously injected the female mice with
the indicated AAV particles and found that 68.4% ± 8.6% of the he-
patocytes were transduced by AAVzk2 (Figure S3A). Although
the level was lower than AAV8 (78.4% ± 3.6%) and AAV9 (82.6% ±
3.1%), few cells in the heart, lung, spleen, kidney, and brain were
infected by AAVzk2 as opposed to AAV8 and AAV9 (Figures S3B–
S3G), consolidating the specific liver transduction of AAVzk2 in
females.

Figure 2. AAV5 and AAVzk2 capsids were post-translationally modified and exhibited differential PTM patterns
(A) A schematic workflow for mass spectrometry-based PTM analysis of AAV5 and AAVzk2 capsids. (B) PTM residue positions along the capsid sequences of the AAV5 and
AAVzk2 from the N to the C terminus. Different colors represent different PTMs (red, acetylation; blue, methylation; orange, ubiquitination; purple, phosphorylation; light blue,
deamidation; violet, O-glycosylation). The inserted sequence FAPTPGP in AAVzk2 capsid is highlighted in the gray box. (C) Common and unique capsid PTMs for AAV5 and
AAVzk2 capsids. The color and its corresponding PTMs were the same as those in (B). (D) The number of the individual PTM site detected in AAV5 and AAVzk2 capsids.

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We also tested the GFP expression in the tissues and organs of the AAV-treated male mice. Compared with AAV5, the GFP mRNA levels were 14.7 ± 5.7-fold higher in AAVzk2, 13.3 ± 3.1-fold higher in AAV8 and 19.98 ± 1.4-fold higher in AAV9-treated livers (Figure 4A). In contrast, the GFP mRNA levels in the AAVzk2-treated heart, lung, spleen, kidney, and brain were similar to or less than AAV5, significantly lower than AAV8 and AAV9 groups. Moreover, a western blot showed similar GFP protein levels among AAV8-, AAV9-, and zk2-treated livers, whereas remarkable GFP expression was observed only in AAV8 and AAV9 groups. Moreover, a western blot showed similar GFP protein levels among AAV8-, AAV9-, and zk2-treated hearts (Figures 4B and 4C). The trend of biodistribution was recapitulated in female mice (Figure S4). Altogether, the results suggest a specific tropism of AAVzk2 toward the liver, but not the heart, lung, spleen, kidney, and brain for both genders.

### Dimmed skeletal muscle transduction of AAVzk2

Skeletal muscle is the largest tissue, accounting for 35%–40% of total body weight. Metabolic and functional issues of the muscle cells trigger multiple disorders such as muscular dystrophies and congenital myasthenic syndromes. The current application of AAV5 in the treatment of hemophilia has exhibited a relatively high dose, ranging from $2 \times 10^{13}$ to $6 \times 10^{13}$ vg/kg, implying the potential risk of off-target gene delivery into muscle cells. To investigate whether AAVzk2 could exert any impact on skeletal muscles via systemic administration, we treated the mice with a relatively high dose ($5 \times 10^{13}$ vg/kg) of the indicated AAVs. Muscles from multiple regions of the body, including the hindlimb (gastrocnemius, quadriceps, soleus), the back (longissimus thoracis), the forelimb (triceps), and the neck (sternocleidomastoid), were stained with GFP. As shown in Figures 5A and 5B, AAV9 displayed the best transduction efficiency, with ~60% of the gastrocnemius, quadriceps, and triceps muscle cells and 30%–40% soleus and longissimus thoracis muscle cells successfully expressing GFP; AAV8 ranked second, with ~15%–40% gastrocnemius, quadriceps, and longissimus muscle cells effectively transduced. Even in AAV5-treated mice, the percentage of GFP-positive quadriceps muscle cells was 19.19%. In contrast, in all of the muscles of the AAVzk2-treated group, negligible numbers of muscle fibers were transduced. To further evaluate GFP expression,
we performed western blot to test the GFP protein levels in gastrocnemius, quadriceps, and triceps, the three types of skeletal muscles that AAV transduced more efficiently (Figure 5A). Consistently, GFP protein bands were hardly visible in AAV5- and AAVzk2-treated gastrocnemius, quadriceps, and triceps, as opposed to the AAV8- and AAV9-treated muscles (Figures 5C and 5D), suggesting little GFP protein expression in the muscle cells faced with circulatory AAV5 and AAVzk2. These observations further consolidate the specific liver transduction property of AAVzk2.

**Robust transduction of human liver cell lines by AAVzk2**

To further test the transduction efficiency of AAVzk2 in human liver cells, we selected a human hepatocellular carcinoma cell line, Huh7, and a normal liver cell line, L02, that have been frequently applied to studies on liver drug delivery.11,40–43 As shown in Figures 6A and 6B, the GFP-positive cell ratio of the AAVzk2 group, which refers to the GFP signal area versus total cell area, was modestly more than the AAV8 and AAV9 groups, but dramatically (21.51-fold for Huh7 cells and 20.83-fold for L02 cells) higher than the AAV5-treated cells at the multiplicity of infection (MOI) of $1 \times 10^5$ vg/cell. When the MOI declined to $1 \times 10^4$ vg/cell, the percentages of Huh7 and L02 cells transduced by AAVzk2 were 25.51% and 21.98%, respectively, which is still significantly higher than the wild-type AAV groups (0.84% and 0.74% for AAV5-, 19.96% and 15.07% for AAV8-, 19.35% and 16.27% for AAV9-treated Huh7 and L02 cells). Similar results were observed at the protein level, while the total GFP protein delivered by AAVzk2 was 4.68- and 3.61-fold higher than AAV5 into Huh7 and L02 cells, respectively (Figures 6C and 6D). Furthermore, the cell binding and internalization assays revealed significantly more AAVzk2 viral particles bound to and internalized by the Huh7 cells than AAV5, AAV8, and AAV9 (Figures S5A and S5B), suggesting cellular mechanisms underlying the superior hepatocyte transduction of AAVzk2. To further investigate the physicochemical properties of AAVzk2, the purified viral particles underwent a gradual increase in temperature from 30°C to 100°C, when the ratio of fluorescent signals at 350 nm versus 330 nm was measured to

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**Figure 4. Selective GFP mRNA and protein expression in the livers of AAVzk2-treated mice**

(A) Male C57BL/6 mice were injected via tail vein with the indicated AAV serotypes encapsulating GFP transgene at a dose of $1 \times 10^{13}$ vg/kg, and liver, heart, lung, spleen, kidney, and brain were subjected to qRT-PCR to detect GFP expression, in which the GFP mRNA levels of the AAV8, AAV9, and AAVzk2 groups were normalized to the AAV5 group. n = 4 mice per group. (B) Western blot to detect GFP protein expression in the livers and hearts from the mice treated with the indicated AAVs. (C) Quantification of GFP protein levels in the liver relative to the heart. n = 4 male and 4 female mice per group. *p < 0.05, **p < 0.01, ***p < 0.001, One-way ANOVA.
reflect conformational changes and the denaturation of capsid proteins, an index of thermostability. F350/F330 displayed a sharp increase since 70°C for AAVv2, and the melting temperature (Tm) value was 75.39°C (Figure S5C), which was close to AAV8 and AAV9 (79.59°C and 77.9°C, respectively) rather than AAV5 (88.5°C), implicating a drastic alteration in thermostability stemming from oligopeptide insertion.

**Low seroreactivity retained by oligopeptide insertion**

The preexisting NAb s in serum have elicited some major concerns regarding the deactivation of AAV vectors used for gene therapy. AAV5 stands out with the lowest circulatory NAb levels, with a prevalence of 3% in certain population groups. To investigate the effect of oligopeptide insertion on the capsid seroreactivity, we collected sera samples from 10 hemophilia B patients, a representative disease requiring liver gene delivery by AAV vectors. These sera samples were diluted at a 1:2 ratio and mixed with the indicated AAV vectors encoding luciferase, followed by the treatment of Huh7 cells with the virus-serum mixture. The highest serum dilution ratio that inhibits luciferase activity by at least 50% was defined as the NAb titer. As shown in Table 1, we found that 4/10 of the patients demonstrated NAb titers higher than 1:4 against AAV8 and 9, which was determined as NAb positive. However, none of the patients were NAb positive against AAV5 and AAVzk2. Moreover, we also checked the NAb prevalence in 21 healthy rhesus monkeys. There were 3/21 monkeys NAb positive against AAV5, 4/21 monkeys NAb positive against AAV8, 9/21 monkeys NAb positive against AAV9, and 1/21 monkey NAb positive against AAVzk2 (Table S1). These results illustrate little effect of the inserted oligopeptide FAPTPGP on the capsid seroreactivity. AAVzk2 retains low NAb prevalence similar to AAV5.

**DISCUSSION**

In this study, we performed library screening in mice and identified a novel AAV5 capsid mutant by inserting a 7-aa-oligopeptide FAPTPGP on the variable region VIII after Q574, called AAVzk2. The production of this engineered serotype in suspension HEK293 cells was comparable with wild-type AAV serotypes such as AAV2, AAV5, AAV8, and AAV9. Systemic administration revealed a robust liver transduction of AAVzk2 in both male and female mice, whereas its infectivities of heart, lung, spleen, kidney, brain, and skeletal muscle were rarely observed. In addition, the capability of AAVzk2 to transduce human hepatocellular cell lines overwhelmed AAV5, AAV8, and AAV9, further supported by better cell binding and uptake. Concomitantly, the seroreactivities of AAVzk2 in human and non-human primates were at low levels comparable with AAV5. These results implicate the potential application of AAVzk2 in a broad range of population groups to safely and effectively treat liver diseases, particularly for those patients unable to receive AAV8 or AAV9, probably due to high NAb titers. Moreover, the LC-MS/MS detected great diversity in acetylation, methylation,
phosphorylation, ubiquitination, deamidation, and O-glycosylation sites on the individual capsid proteins between AAV5 and AAVzk2. The thermostability of AAVzk2 was also lower than that of AAV5. These data indicate that the PTM patterns and physicochemical properties of AAVzk2 differ from its parental AAV5.

There has been remarkable progress in inventing novel capsids that target the skeletal muscle, retina, and central nervous system exceptionally well in the insertion of peptides on the variable region VIII of many AAV serotypes, such as AAV2 and AAV9.\textsuperscript{31,34,44,45} However, sporadic successes have been achieved in the evolution of the AAV5 capsid, probably due to the sequence and structure diversities between AAV5 and other serotypes.\textsuperscript{18,20,46} Khabou and colleagues tried to transfer the 7m8 oligopeptide, which enhanced the retinal transduction of AAV2 in several species, onto the AAV5 capsid, probably due to the sequence and structure divergences between AAV5 and other serotypes.\textsuperscript{18,20,46} An approach for future study either to manipulate AAV5 for extensive utilization or to delve into the nature of this unique serotype.

PTMs contribute vitally to AAV transduction, stability, and tissue tropism.\textsuperscript{30–32} For example, AAV3 S663V + T492V and AAV1 S669A mutations could enhance human and murine hepatocyte transduction, while AAV5 S485A mutation leads to a switch from

Figure 6. Robust human hepatocellular transduction property of AAVzk2

(A) Human hepatocellular carcinoma cell Huh7 and normal liver cell L02 were infected by the indicated AAV particles encapsulating the GFP gene at a multiplicity of infection (MOI) of 1 × 10\(^5\) and 1 × 10\(^4\) vg/cell, respectively. Images were captured 48 h post-virus treatment. Upper panel, GFP signal; lower panel, bright field. Scale bar, 100 μm. (B) Quantification of GFP-positive cell percentage. The ratios of GFP signal area of Huh7 and L02 cells to the whole bright-field area were quantified at an MOI of 1 × 10\(^5\) and 1 × 10\(^4\) vg/cell, respectively. *p < 0.05, **p < 0.01, ***p < 0.001. n = 6 wells of cells. One-way ANOVA. (C) Lysates of Huh7 and L02 cells treated with the indicated AAV particles at an MOI of 1 × 10\(^5\) vg/cell were subjected to immunoblotting for GFP. Three lanes were performed for each AAV-treated group. (D) Quantification of relative GFP protein levels in (C). *p < 0.05, **p < 0.01, ***p < 0.001. n = 4 wells of cells. One-way ANOVA.
Table 1. Anti-AAV neutralizing antibody titers in hemophilia B patients’ sera

| Patient ID | Anti-AAV5 | Anti-AAV8 | Anti-AAV9 | Anti-AAVzk2 |
|------------|-----------|-----------|-----------|-------------|
| 1          | <1:1      | 1:16      | 1:16      | 1:2         |
| 2          | <1:1      | <1:1      | <1:1      | <1:1        |
| 3          | <1:1      | <1:1      | <1:1      | <1:1        |
| 4          | <1:1      | 1:2       | <1:1      | <1:1        |
| 5          | <1:1      | <1:1      | <1:1      | <1:1        |
| 6          | <1:1      | 1:8       | 1:16      | <1:1        |
| 7          | <1:1      | <1:1      | <1:1      | <1:1        |
| 8          | 1:1       | 1:32      | 1:32      | 1:4         |
| 9          | <1:1      | 1:32      | 1:32      | 1:1         |
| 10         | <1:1      | <1:1      | <1:1      | <1:1        |

The samples with neutralizing antibody titers >1:4 (NAb positive) are indicated in boldface type.

Our LC-MS/MS dataset showed that both AAV5 and AAVzk2 capsids were post-translationally modified, and the PTM patterns differed from one another (including acetylation, methylation, ubiquitination, phosphorylation, deamidation, and O-glycosylation; Figures 2 and S2). We observed that residues S77 and S108 were only phosphorylated on the N terminus of VP1 in AAVzk2; residue K451 was ubiquitinated only on the AAV5 capsid, while no ubiquitination pattern was detected on the AAVzk2 capsid. As to glycosylation sites, although common sites such as T550 were observed on both AAV5 and AAVzk2 capsids, residues T370 and S553 were glycosylated only on AAV5 capsids, and T223, T472, T538, and S712 glycosylations were solely present on AAVT36 capsids. Nevertheless, the most abundant PTM observed was deamidation, accounting for ~70% of total PTMs (Figures 2C and 2D). It has been reported that multiple asparagine to aspartic acid mutations that mimic deamidation could affect the liver transduction efficiency of AAV8 in vitro and in vivo. Consequently, the large variety of different deamidation sites, together with other PTM patterns, between AAV5 and AAVzk2, may explain at least to some extent the improved liver tropism of AAVzk2. Although it is time intensive to fully figure out which distinct PTM or the combination of several on the AAVzk2 capsid contribute to the enhanced murine liver transduction efficiency, current PTM results may still provide valuable clues to illuminate the nature of AAV5, expand our knowledge about the field, and guide future studies.

MATERIALS AND METHODS
Reagents and antibodies and AAV serotypes

Unless otherwise indicated, the chemicals mentioned here were purchased from Sigma-Aldrich (St. Louis, MO, USA). The antibodies used here were as follows: GFP (11814460001, 1:2,000 for western blot and 1:500 for staining) from Sigma-Aldrich; β-tubulin (sc101527, 1:3,000 for western blot) from Santa Cruz Biotechnology (Dallas, TX, USA); Alexa Fluor 488 goat anti-rabbit immunoglobulin G (IgG), Alexa Fluor 488 goat anti-mouse IgG (1:1,000 for immunofluorescence), horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, and goat anti-mouse IgG antibodies (1:4,000 for western blot) were as previously described. The accession numbers of the AAV sequences were from the NCBI database as follows: AAV2 (NC_001401.2), AAV5 (NC_006152.1), AAV8 (NC_006261.1), and AAV9 (AY530579.1).

AAV production and quantification

AAV particles were produced by means of triple-plasmid transfection in the suspension HEK293 cell line and purified by iodixanol gradient ultracentrifugation, as previously described. Briefly, the transgene plasmid (scCB-GFP construct, as previously reported), Rep/Cap plasmid and AdHelper plasmid were transfected at the concentrations of 1 μg/mL, 1 μg/mL, and 2 μg/mL, respectively, when the HEK293 cell density reached 5 × 10⁶ cells/mL. Cell lysates plus medium were subjected to benzonase (E8263, Sigma-Aldrich) digestion 72 h post-transfection and then loaded onto iodixanol gradients, followed by ultracentrifugation at 68,000 rpm for 2 h. AAV samples were recovered from the 40% iodixanol layer and then resolved to another round of iodixanol gradient ultracentrifugation. Finally, the AAV particles were switched to phosphate-buffered saline (PBS) containing 0.001% poloxamer 188 via Amicon 15-100,000 molecular weight cutoff (MWCO) concentration unit to a final volume of ~1 mL.

The purified AAV samples underwent DNase I (10104159001, Roche, Basel, Switzerland) digestion for 30 min at 37°C. Then, the vg was quantified by qPCR with the GFP primers (F: 5′-CGACCTACACGACACACC-3′, R: 5′-CGAATCCAGGAGACATTG-3′) based on the standard curve made from a serial dilution of linearized CB-GFP plasmid samples. The amount of vg capsid protein was determined by the Pierce Silver Stain Kit (Cat#24612, Thermo Scientific, Waltham, MA, USA) based on the standard curve made from a serial dilution of standard bovine serum albumin (BSA) protein. Unless otherwise indicated, VP3 was quantified on behalf of all of the capsid proteins.

Systemic administration of AAV vectors in mice

C57BL/6 mice (006644, Jackson Laboratory, Bar Harbor, ME, USA) of both genders were housed in a room with a 12-h light/dark cycle and ad libitum access to water and rodent chow diet (Diet 7097,
Harlan Teklad, Madison, USA). The animal protocols were approved by the institutional animal care and use committee (IACUC) of the Department of Ophthalmology, Daping Hospital, Chongqing, China. Mice 6–8 weeks old were injected via the tail vein with the indicated AAV vectors. Insulin syringes (REF328421, BD, Franklin Lakes, NJ, USA) were used for injection. Unless otherwise indicated, the mice were sacrificed 21 days after AAV treatment to collect tissues and organs for the experiments that followed.

**Generation and screening of AAV5 capsid variant library**

The capsid library screening process was generally based on previous studies.31–33 Briefly, the AAV5-NNK library plasmid contains part of a CB promoter (568-bp sequence of CMV enhancer and CB promoter) followed by the p40 region from Rep 2 to drive the expression of capsid variants. To construct the library, the inserted oligopeptide coding sequences were synthesized as NNK primers by GenScript (F: 5′-GGTGGANNKNNKNNKNNKNNKNNKCTGCCCGCCGAC-3′, R: 5′-CGCGGGGGGCACTGGTGGAANNKNNKNNKNNKNNKNNKNNKNNKTTGTCTGCCGAC-3′), amplified by PCR (F plus R primers) and ligated to the plasmid backbone by the Gibson assembly, which was digested in advance by restriction enzymes EcoRV and AfeI. To generate the 3-stop plasmid, 3 stop codons were introduced into the AAV5 capsid nucleotide sequence at 36 bp after VP1, 15 bp after VP2, and 42 bp after VP3 translation start codons, respectively, to annihilate the nucleotide sequence at 36 bp after VP1, 15 bp after VP2, and 42 bp after VP3 translation start codons, respectively, to annihilate the expression of the VP1, VP2, and VP3 proteins. Afterward, HEK293 cells were transfected with 16 μg AdHelper plasmid, 8 μg 3-stop plasmid, 8 μg pUC19 plasmid, and 10 ng AAV5-NNK library plasmid per 2 × 10⁷ cells. The AAV library was then purified by iodixanol gradient ultracentrifugation 72 h post-transfection.

To screen the AAV5-NNK library for liver-tropic candidates, 3 mice were injected intravenously with 1 × 10¹³ vg/kg the purified AAV library. Forty days post-viral treatment, mRNAs in the livers were extracted by the RNAprep Pure Tissue Kit (DP431, TIANGEN BIOTECH, Beijing, China) and the TIANSeq mRNA Capture Kit (NR105, TIANGEN) and reverse-transcribed to cDNA. The sequences of AAV5 capsid variants in the cDNA were then amplified by PCR to produce a 160-bp fragment for NGS sequencing, which was followed by dehyd- ination in 30% sucrose solution at 4°C overnight. Tissue samples were sliced into 25-μm sections on a cryostat (HM550; Thermo Scientific) at −22°C, which were then blocked and penetrated with the blocking buffer (5% BSA, 2% Triton X-100, and 5% goat serum in PBS) for 1.5 h at room temperature. Afterward, they were incubated with primary antibodies in antibody dilution buffer (5% BSA, 0.5% Triton X-100, and 5% goat serum in PBS) at 4°C overnight. After washing with 0.5% Triton X-100 in PBS 3 times, 10 min each at room temperature (25°C), samples were incubated with Alexa Fluor-tagged secondary antibodies for 3 h at room temperature and mounted with Vectashield mounting medium. Images were collected with a Zeiss confocal laser scanning microscope (LSM 700) and analyzed by ImageJ (National Institutes of Health, Bethesda, MD, USA).

**Western blot**

Western blot was performed as previously reported.54,69 Briefly, cells and tissues were homogenized and lysed in the lysis buffer with ingredients as follows: 150 mM NaCl, 2.5 mM EDTA, 50 mM Tris-HCl (pH 7.4), 1% SDS, 20% glycerol, 1% PMSF, and 1% protease inhibitor cocktail (no. 04693159001, Sigma-Aldrich). The debris was removed by centrifugation at 12,000 × g for 10 min at 4°C, while supernatants (referred to as lysates) were collected and diluted with 4× loading buffer containing 20% Tris-HCl (pH, 8.8), 8% SDS, 8% β-mercaptoethanol, 0.04% bromophenol blue, and 40% glycerol, and boiled at 100°C for 10 min. Samples were resolved to SDS-PAGE running and transferred to a nitrocellulose membrane (cat. no. 1620112, Bio-Rad, Hercules, CA, USA). The membrane was blocked in 5% skim milk for 1 h and incubated in primary antibodies overnight at 4°C. After washing 3 times with 0.1% Tween 20 in PBS in a span of 10 min, membranes were incubated in HRP-conjugated secondary antibodies for 1 h at room temperature. Protein bands were visualized by the Pierce enhanced chemiluminescence kit.

**Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay**

Total messenger RNA (mRNA) was extracted from the indicated tissues/organs with Trizol (Invitrogen, Waltham, MA, USA) reagent and reverse -transcribed to cDNAs with GoScript reverse transcription kit (Promega, Madison, WI, USA). cDNAs were used as templates for qPCR in a 20-μl reaction system containing SYBR GreenER qPCR mix with gene-specific primers. The PCR reaction was initiated with a denaturation step at 95°C (3 min), followed by 40 cycles consisting of denaturation at 95°C (15 s), annealing, and extension at 60°C (60 s). The GFP primers were mentioned above and the internal control, Gapdh primers, were as follows: F: 5′-AAGGTGTCATCCAGAGCTGAA-3′, R: 5′-CTGCTTCACCCCTTCA-3′).

**AAV neutralization assays**

Human sera samples were collected from 10 hemophilia B patients at the Institute of Hematology & Blood Disease Hospital, Chinese Academy of Medical Sciences and Pekin Union Medical College (Tianjin,
China), which was approved by the ethics review board. Sera samples from rhesus monkeys were obtained from Greentech Bioscience (Sichuan, China). NAb levels were detected following our previous protocols, with minor modifications.\(^\text{42,70}\) Briefly, the rationale of the ratio to mix AAV and sera (or sera dilutions) was grossly based on an intravenous administration at a dose of \(1 \times 10^{13}\) vg/kg, namely \(2 \times 10^{9}\) vg vps per 1 \(\mu\)L serum. Huh7 cells were cultured at the density of \(1 \times 10^5\) cells/well in a 48-well plate. A serial 2-fold dilution of sera samples of 5 \(\mu\)L in volume each were incubated with \(1 \times 10^6\) vg AAV particles for 2 h at 4°C, followed by treatment of the Huh7 cells with the AAV-sera mixture. The activity of luciferase delivered by AAV was measured by the Wallac-1420 Victor 2 plate reader (PerkinElmer, Waltham, MA, USA) 48 h post-AAV treatment. NAb titers were defined as the largest number of dilution ratios able to reduce the luciferase activity by half compared with the AAV-PBS mixture group. A serum sample was considered NAb positive when the titer was higher than 1:4.

**AAV cell binding and internalization assay**

These 2 experiments were slightly modified from our previous paper.\(^\text{41}\) Briefly, for the binding assay, the indicated AAV vectors encapsulating dsCB-GFP transgene were incubated with pre-cooled Huh7 cells at 4°C for 30 min. After washing with PBS 3 times to rinse un-bound AAV particles, the cells were harvested for DNA extraction; for the internalization assay, the AAV-treated cells were incubated at 37°C for 1 h to allow internalization of the bound virus by Huh7 cell. The cells were then digested by trypsin for 10 min at 37°C to remove the un-internalized AAV. After washing 3 times in PBS, the cells were subjected to DNA extraction. Viral genomic DNA was extracted by the DNeasy Blood & Tissue Kit (Cat#69506, Qiagen, Hilden, Germany) and quantified by qPCR with the GAPDH gene copy number as the internal control. The relative vg numbers of the other AAV serotype groups were normalized to AAV5.

**Preparation of AAV capsid samples for LC-MS/MS analysis**

AAV capsid samples were lysed in SDT lysis buffer (4% SDS, 100 mM dithiothreitol [DTT], 100 mM Tris-HCl, pH 7.6), heated at 100°C for 5 min and centrifuged at 12,000 \(\times g\) for 10 min. The supernatants were collected from which protein samples were precipitated with a 5-fold acetic acid/ethanol buffer (ice-cold 50% acetic acid, 50% ethanol, 0.1% acetic acid) at \(-20°C\) overnight. Precipitated proteins were centrifuged at 16,000 \(\times g\) for 25 min at 4°C and subsequently washed twice with ice-cold acetone. The extracted proteins were dissolved in 50 mM ammonium bicarbonate (ABC) buffer. After reduction with 10 mM DTT for 30 min at 56°C and alkylation with 20 mM iodoacetamide (IAA) for 30 min at room temperature in the dark, the AAV capsid samples were equally divided into 2 parts. One aliquot was treated with chymotrypsin (Promega) in an enzyme protein ratio of 1:50 (w/w) and incubated at 25°C for 18 h with an agitation speed of 1,200 rpm. The other aliquot was treated with trypsin and Glu-C (Promega) in an enzyme protein ratio of 1:50 (w/w) and incubated at 37°C for 18 h with an agitation speed of 1,200 rpm. The digested samples were subjected to vacuum centrifugation and the C18 solid-phase (3M Empore, Oxford, PA, USA) desalting process. The desalted peptides were evaporated using vacuum centrifugation again and stored at \(-80°C\) until further processing.

**Nano-LC-MS/MS data acquisition and analysis**

Data acquisition and analysis were technically supported by the Institutional Technology Service Center of the Shanghai Institute of Materia Medica. To obtain raw files for acetylation, methylation, ubiquitination, phosphorylation, and deamidation, the dried peptides (~1 \(\mu\)g each sample) were resolved using 0.1% formic acid (FA) and separated on an analytical column (75 \(\mu\)m \(\times\) 200 mm) packed with reverse-phase (RP) beads (3 \(\mu\)m ReproSil-Pur C18 beads, 120-Å pore size; Dr. Maisch GmbH, Ammerbuch, Germany) on a nanoflow HPLC Easy-nLC 1000 system (Thermo Scientific) with a 120-min gradient at a flow rate of 300 nL/min. Buffer A consisted of 0.1% (v/v) FA in water and buffer B consisted of 0.1% (v/v) FA in acetonitrile (ACN). The gradient was set as follows: 2%–5% B in 1 min, 5%–25% B in 100 min, 25%–35% B in 12 min, 35%–100% B in 1 min, and 100% B in 5 min. An Orbitrap Fusion Tridrib mass spectrometer (Thermo Scientific) is integrated with the LC system. The spray voltage was set at 2,500 V in positive ion mode and the ion transfer tube temperature was set at 275°C. MS acquisition was programmed in data-dependent acquisition (DDA) mode, in which 1 full MS scan (350–1,500 m/z) was performed at 60,000 resolution, with a \(4 \times 10^5\) automatic gain control (AGC) target and a 50-ms maximum injection time. Then, MS2 scans were generated by higher energy collisional dissociation (HCD) fragmentation in a 3-s cycle time and 45-s dynamic exclusion. MS/MS acquisition was performed at 15,000 resolution, with a \(5 \times 10^5\) AGC target and a 100-ms maximum injection time. Precursor fragmentation was performed with a 1.6-m/z isolation window and 27% normalized collision energy (NCE). Precursors with charge 2–6 were selected for MS2 analysis.

To acquire raw files for O-glycosylation, the resolved peptides (~1 \(\mu\)g each sample) were separated using an 80-min gradient at 300 nL/min. The RP chromatography column, buffer A, and buffer B were the same as above. The gradient was set as follows: 2% B in 5 min, 2%–35% B in 60 min, 35%–50% B in 7 min, 50%–90% B in 10 s, 90% B in 2 min and 50 s, and 90%–2% B in 5 min. An Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific) is integrated with the LC system. The spray voltage was set at 2,500 V in positive ion mode and the ion transfer tube temperature was set at 275°C. MS acquisition was programmed in DDA mode, in which 1 full MS scan (350–1,800 m/z) was first performed at 120,000 resolution, with a \(4 \times 10^5\) AGC target and a 50-ms maximum injection time. Then, MS2 scans were generated in a 3-s cycle time and a 15-s dynamic exclusion. HCD was performed at 15,000 resolution, with a 100-ms maximum injection time. Precursor fragmentation was performed with a 1.6-m/z isolation window and 30% NCE. Precursors with charge 2–7 were selected for MS2 analysis. Electron-transfer/higher energy collision dissociation (EThCd) was performed after HCD, under the condition that one of the glyco-fingerprint ions (126.055, 129.1023, 138.0545, 144.07, 168.065, 186.0762, 204.0867, 274.0866, 292.103, and 366.1396) had to be present in the HCD spectrum.
with a 15-ppm mass accuracy. Calibrated charge-dependent electron transfer dissociation (ETD) parameters were used.

Raw files of acetylation, methylation, ubiquitination, phosphorylation, and deamidation were further analyzed by MaxQuant version 2.0.1.0 and O-GlcNAcylation raw files were analyzed by pGlyco, as previously described. All PTM mass spectra were manually validated and the data have been deposited to the integrated proteome resources (iProX) (https://www.iprox.cn/) via the PRIDE partner repository with the identifier IPX0003661000.

Statistical analysis
Data were analyzed via GraphPad Prism 7 (GraphPad, San Diego, CA, USA) by unpaired t test and 1-way ANOVA (with Tukey’s multiple comparison test). Unless otherwise indicated, the data were shown as means ± SDs. Statistical difference was considered when p < 0.05. The sample size (n) was based on the literature.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2022.03.017.

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AUTHOR CONTRIBUTIONS
X.X. and K.Z. designed the project and drafted the manuscript. H.H., C.Y., and K.Z. constructed the AAV5 capsid NNK library. K.Z., Y.W., C.Y., and K.Z. designed the project and drafted the manuscript. H.H., R.D., and Z.D. provided the platforms for the rodent and non-human primate experiments and AAV production, respectively.

DECLARATION OF INTERESTS
X.X., K.Z., C.Y., H.H., and Z.D. are co-inventors of a patent about the novel AAV serotype generated in this paper.

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