Superior human hepatocyte transduction with Adeno-associated Virus Vector Serotype 7

Wenwei Shao[1], **Xiaolei Pei**[1,2], **Caibin Cui**[3], **Charles Askew**[1], **Amanda Dobbins**[1], **Xiaojing Chen**[1], **Yasmina L. Abajas**[4], **David A. Gerber**[3], **R. Jude Samulski**[1,5], **Timothy C. Nichols**[6], **Chengwen Li**[1,4,7,*]

[1]Gene Therapy Center, University of North Carolina at Chapel Hill, NC 27599

[2]Chinese Academy of Medical Sciences and Peking Union Medical College, Institute of Hematology and Blood Diseases Hospital, University of North Carolina at Chapel Hill, NC 27599

[3]Department of Surgery, University of North Carolina at Chapel Hill, NC 27599

[4]Department of Pediatrics, University of North Carolina at Chapel Hill, NC 27599

[5]Department of Pharmacology, University of North Carolina at Chapel Hill, NC 27599

[6]Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, NC 27599

[7]Carolina Institute for Developmental Disabilities, University of North Carolina at Chapel Hill, NC 27599

Abstract

Although therapeutic outcomes have been achieved in hemophilia patients after delivery of clotting factor genes to the liver using adeno-associated virus (AAV) vectors, it is well known that the pre-clinical results generated from hemophilia animal models have not been directly predictive of successful translation in humans. To address this discrepancy humanized mouse models have recently been used to predict AAV transduction efficiency for human hepatocytes. In this study we evaluated AAV vector transduction from several serotypes in human liver hepatocytes xenografted into chimeric mice. After systemic administration of AAV vectors encoding a GFP transgene in humanized mice, the liver was harvested for either immunohistochemistry staining or flow cytometry assay for AAV human hepatocyte transduction analysis. We observed that AAV7 consistently transduced human hepatocytes more efficiently than other serotypes in both immunohistochemistry assay and flow cytometry analysis. To better assess the future application of AAV7 for systemic administration in the treatment of hemophilia or other liver diseases, we

*To whom correspondence should be addressed: Gene Therapy Center, 7007 Thurston-Bowles, CB 7352, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, Phone: (919)-962-7624, Fax: (919)-966-0907, chengwen@med.unc.edu.

analyzed the prevalence of neutralizing antibodies (NAbs) to AAV7 in sera from healthy subjects and patients with hemophilia. In the general population, the prevalence of NAbs to AAV7 was lower than that of AAV2 or AAV3B. However, a higher prevalence of AAV7 NAbs was found in patients with hemophilia. In summary, results from this study suggest that AAV7 vectors should be considered as an effective vehicle for human liver targeting in future clinical trials.

Keywords

AAV; hemophilia; human hepatocytes; humanized mouse model; neutralizing antibody

Introduction

The liver is a common target for gene transfer and hepatocytes are the key cell type affected in a variety of inherited diseases, such as hemophilia and alpha-1 antitrypsin deficiency. Although several different gene delivery vectors have been evaluated in liver-targeted gene therapy, adeno-associated virus (AAV) is one of the most promising vectors. At present, efficient expression of liver-derived transgenes have been documented in several clinical trials (1–3).

AAV is a single-stranded DNA virus that is commonly used as a non-pathogenic recombinant vector for gene delivery. During the last two decades, AAV based gene therapy has been rapidly developed for a number of planed or ongoing clinical trials (4–6). Twelve AAV serotypes and over 100 variants, including numerous genetically engineered mutants, have been characterized and used as gene delivery vehicles (7, 8). These AAV vectors have been extensively studied in several animal models such as rodents, dogs, and nonhuman primates. Different AAV serotypes show distinct tropism for tissues and organs in animal model studies (9, 10). It is essential to identify suitable AAV vectors that target specific tissues or cells for successful gene therapy.

Currently, the common method to select AAV vectors for clinical trials is to test transduction efficiency in animal models. However, due to species differences the results derived from animal models are not always well translated in human clinical trials. In a mouse model, administration of self-complementary AAV8 (scAAV8) vector encoding the human clotting factor IX (hFIX) transgene resulted in stable and therapeutic levels of hFIX at a low relative AAV vector dose to weight ratio. However, similar therapeutic levels of hFIX in the rhesus macaque model required administration of scAAV8/hFIX vector at more than a 10-fold higher dose to weight ratio. In clinical trials, even a 100-fold higher dose to weight ratio of scAAV8/hFIX was needed to achieve the therapeutic effect in humans compared to mice (11) (12, 13). In the canine model, Wang et al., 2005 reported that hemophilia dogs receiving a single stranded AAV2/8 vector (at the dose of 5.25×10^{12} vg/kg) can achieve sustained expression of 10%-26% of normal FIX values(14). However, in a human hemophilia B clinical trial, only a limited increase of FIX (1%-6% of the normal level) was achieved in patients who had received a dose of 2×10^{12} vg/kg scAAV2/8 vector administration(2, 3). In other liver studies, it has also been reported that animal models do not accurately predict human clinical trial outcomes and the transduction efficiency is inconsistent between

different species (3, 15–17). In murine models and nonhuman primates, AAV8 has induced much higher transgene expression levels than that of AAV2 in liver (3, 11, 13, 18). In humans, the outcome is different from pre-clinical animal models. It has been observed that the FIX transgene level of AAV2 treated hemophilia patients was similar with that of the AAV8 treated group(16). This finding of less efficient AAV transgene expression was confirmed from another study reported by Nietupski et al., where they found that levels of α-galactosidase A were 1–2 logs lower in nonhuman primates than that in male mice at the same vector dose by weight[17]. Despite a similar vector genome copy number detected in primate and mouse liver, the expression of α-galactosidase A at the mRNA level was much lower in primates. Markusic et al. directly compared the transduction efficiency between mouse and dog models with hemophilia and demonstrated that while some AAV vectors transduced mouse liver very efficiently, results were not the same in dogs(19). These studies implicate species-specific differences in AAV transduction efficiency as an explanation for why results in mouse liver studies may not be predictive of other species or humans. Therefore, alternative models need to be explored to better examine AAV vector transduction efficiency in human hepatocytes.

In recent years, a novel model based on an immunodeficient mouse xenografted with human cells was developed, and this model has been used to improve prediction of AAV transduction of human hepatocytes[33]. The common model used is the FRG mouse (Fah$^{-/-}$, Rag2$^{-/-}$ and Ilr2g$^{-/-}$) xenografted with human hepatocytes. The FRG model is generated from a fumarylacetoacetate hydrolase (Fah) mutant mouse with severe immunodeficiency (T, B and NK cell deficient). Induction of cell autonomous hepatotoxicity in the absence of the drug Nitisinone (NTBC) in mouse hepatocytes can enable human hepatocytes to repopulate the mouse liver after hepatocyte xenograft(20). This human liver chimeric mouse model has been popular to study human hepatocyte toxicity in animal models. The chimeric mouse model has been utilized to develop AAV vectors and test AAV transduction in human hepatocytes.

In clinical trials, especially when systemic administration of AAV vectors is needed, humoral immunity against the capsid poses another important limitation for effective gene therapies. The high prevalence of human neutralizing antibodies (NAbs) directed against the AAV capsid plays an important role in limiting the broad use of AAV vectors for clinical applications. In the general population, about 50% of subjects are seropositive for AAV NAbs. The percentage of pre-existing NAbs varies depending on which AAV serotype is assayed. For example, the prevalence of anti-AAV2 IgG can reach up to 72%, which is higher than that of anti-AAV8 IgG (38%)(21). To screen for anti-AAV NAbs in human serum samples, a cell-based *in vitro* assay is widely used(21–25). It has been reported that the presence of NAbs to AAV vectors was associated with the lack of transduction efficacy, even at a low titer, in preclinical and clinical studies(15, 26–29).

In this study we assayed the transduction efficiency of different AAV serotypes for human hepatocytes in chimeric mice using multiple methods of comparison, and found that AAV7 was superior to other serotypes for human hepatocyte transduction. Additionally, we screened human serum samples from healthy subjects and hemophilia patients for the prevalence of NAbs against different AAV vector candidates.

Material and Methods

Mice and human hepatocytes:

Female FRG (Fah$^{-/-}$, Rag2$^{-/-}$ and Ilr2g$^{-/-}$) mice xenografted with human hepatocytes were used for AAV transduction experiments. Briefly, human chimeric xenografted mice with 70% human hepatocyte repopulation were purchased from Yecuris (Yecuris, Tualatin, OR). FRG mice were maintained with Nitisinone in a specific pathogen-free facility at the University of North Carolina at Chapel Hill. All procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee under protocol IACUD ID# 15-294. Human hepatocytes were supplied by 4 separate donors and comparisons of AAV transduction efficiency between serotypes was performed using the same hepatocyte donor tissue. The scAAV/GFP vectors in 100 μl were administered via retro-orbital vein injection.

Cell lines:

HEK-293 cells and Huh7 cells were grown in Dulbecco's Modified Eagle's Medium with 10% FBS and 1% penicillin–streptomycin at 37 °C in 5% CO_2. It is negative when cells were tested for mycoplasma contamination.

AAV vector production and purification:

AAV virus production was performed as previously described using the triple plasmid transfection (30). Briefly, HEK-293 cells were transfected with an AAV transgene plasmid (single-stranded (ss) pTR-CBA-Luciferase, or self-complementary (sc) pTR-CBh-GFP), a Rep and Cap AAV helper plasmid, and the adenovirus helper plasmid pXX6-80. 48 hours post-transfection, cells were harvested, lysed, and AAV vectors were purified by cesium chloride (CsCl) gradient density centrifugation. The virus titer was determined by Q-PCR with ITR primers using the vector genome as template and is reported as viral genomes (vg).

Liver cell isolation and flow cytometry:

Mouse liver was perfused with 2% fetal bovine serum in phosphate buffered saline. Freshly harvested mouse liver tissue (about 0.5cm*0.5cm*0.3cm) was ground gently in PBS buffer containing 150 U/mlDNase I. Single-cell suspensions were generated by 100 μm cell strainer filtration. After centrifuge, cells were resuspended with 2% fetal bovine serum in PBS and was ready to use. Cells were then fixed and permeabilized by adding 500 μ;L of Fixation/Permeabilization solution (BD) and incubated at 20°C for 20 minutes. After washing the cells by adding 2 mL BD Perm/Wash buffer for two buffer exchanges, the cell pellet was resuspended in 100 μL BD Perm/Wash buffer containing goat anti human albumin primary antibody (Bethyl, A80-129A). Cells were immunostained with antibody for 30 minutes. After washing by adding 2 mL BD Perm/Wash buffer, the cell pellet was resuspended in 100 μL BD Perm/Wash buffer containing Allophycocyanin (APC) conjugated donkey anti goat IgG secondary antibody (R&D system). Cells were immunostained for 30 minutes at 20°C in the dark. After washing by PBS, cells were analyzed by flow cytometry.

Immunostaining:

First, mice livers were harvested, coarsely sectioned, drop fixed in 10% formalin, and then embedded in paraffin. The paraffin sections were rehydrated by ethanol with the concentrations of 100%, 95%, 90%, 80%, and 75% after deparaffinization. Antigen retrieval was performed in Lab Vision™ HIER Buffer L (ThermoFisher). To block the non-specific staining, the slide was blocked by Protein Block Serum-Free Buffer (DAKO). Slides were incubated with primary antibody, goat anti-human albumin (Bethyl, A80-129A) or goat anti-mouse albumin (Bethyl, A90-134A) at 4°C for overnight in a humidified chamber. After washing, the sections were incubated with anti-goat Alexa Flour 488-conjugated secondary antibodies (Invitrogen) at 20°C for 2hrs. After washing with PBS, the sections were blocked again. Then sections were stained with chicken anti-GFP antibody (Aves labs, GFP-1020) at 4°C overnight, followed by anti-chicken Alexa Flour 594-conjugated secondary antibody (Molecular Probes). Finally, sections were stained with DAPI and washed prior to mounting on glass slides. Mean and standard deviation were determined from 5 number of liver pictures from each mouse.

Positive staining cell count in IHC

After immunostaining, we used confocal images to count and quantify the positive staining cells. First, we selected five areas under the microscope randomly for image capture. Then we scored the single cell staining condition under the selected area as negative, low, medium, or high staining according to fluoresce density. Medium and high staining cells were counted as positive cells. Positively labeled green staining hepatocytes were counted by Image J. Double positive cells, which are golden, were counted manually using the cell counter function.

Human sera:

Normal human serum from individuals was purchased from Valley Biomedical (Winchester, VA). The sera from hemophilia patients were collected in The Hemophilia Treatment Center of School of Medicine at UNC-Chapel Hill (UNC IRB#15-2126).

Neutralizing antibody analysis:

Huh7 cells were seeded in a 48-well plate at 1×10^5 cells/well, and cultured with 1×10^8 particles of AAV-Luciferase vectors that had been pre-incubated with a serial dilution of normal human serum and hemophilia patients' serum for 2 hours at 4°C. First, we obtained the NAb range using a 10-fold dilution series, with undiluted sera as the start concentration. Then a 2-fold dilution series within the NAb range was used to determine the actual NAb titer. Luciferase activity in the cell lysate was measured with a Wallac-1420 Victor 2 automated plate reader 48 hours post AAV transduction. NAb titers were defined as the highest dilution of serum that reduced luciferase activity by 50% in comparison to that in cells transduced with AAV-Luciferase vectors that had been pre-incubated with phosphate-buffered saline as control.

Statistical analysis:

All statistical calculations were performed using a statistical software (GraphPad Prism 7.0 software). Data are presented as mean± SD. Data from single comparisons were evaluated by the paired Student's t test. Differences between different groups were considered to be statistically significant when P values were <0.05.

Results

Human hepatocyte transduction by different AAV serotypes in humanized chimeric mice

Different AAV serotypes have distinct tropisms for mouse and human hepatocytes. In this study, we screened human hepatocyte transduction in xenografted mice using several AAV serotypes to assay which ones are able to induce higher human liver transduction. In the first experiment, AAV2, AAV8, or AAV9 were tested in mice xenografted with hepatocytes from human donor #1. Three weeks after systemic injection of 3×10^{11}vg/mouse of scAAV-GFP vectors, mice were perfused with phosphate buffered saline (PBS) and their liver was collected for sectioning. The AAV transduction of human or mouse hepatocytes was analyzed by double staining of human serum albumin or mouse albumin and GFP. Wild type mouse liver tissue without xenograft was used as a negative control (Supplementary Figure 1). As shown in Figure 1 **and** Table 1, AAV9 induced slightly higher human hepatocyte transduction (17.9% and 16.7% in two mice) than AAV2 (17.7% and 9.5%) or AAV8 (11.9% and 13.7%). Consistent with published literature, AAV8 induced greater transduction in mouse hepatocytes (51.2% and 38.6%) than that in human hepatocytes.

Additional serotypes were screened in a second experiment, where we tested AAV3B, AAV6, AAV7, AAV8 or AAV9. A lower dose of 1×10^{11}vg/mouse of scAAV-GFP vectors were injected into mice xenografted with donor #2 human hepatocytes. At 3 weeks after gene delivery, mouse livers were harvested for IHC staining and flow cytometry analysis. For flow cytometry analysis, liver cells were isolated and stained with an anti-human albumin antibody to differentiate human from mouse hepatocytes and to analyze GFP transgene expression in these cell populations. As shown in Figure 2, similar transduction levels in human hepatocytes were observed in the AAV7 and AAV8 groups, which were greater than for AAV3B, AAV6 or AAV9 (Table 1). Based on immunohistochemistry images, AAV3B (6.7% and 6.5%) induced lower human hepatocyte transduction than AAV8 (20.8% and 18.9%). AAV7 (20.5% and 12.4%) had similar transduction in human hepatocytes to that of AAV8 group, while lower mouse hepatocyte transduction was observed for AAV7 (27.8% and 28.2%) when compared to AAV8 (53.7% and 62.5%).

Consistent with the immunohistochemistry data, flow cytometry analysis showed similar trends in human hepatocyte transduction efficiency among the serotypes. AAV7 (27.6% and 32.9%) induced highest human hepatocytes transduction followed by AAV8 (20.0% and 30.1%). Moreover, the Mean Fluorescence Intensity (MFI) of GFP expressed cells in AAV7 human hepatocytes (43.2 and 60.5) was a little higher than that with AAV8 (20.9 and 45.3). These results indicate that AAV7 has higher human liver tropism in xenograft mice when compared to AAV8 and other serotypes.

AAV7 induced higher human hepatocytes transduction compared to AAV3B or AAV8

In order to confirm the findings observed above, we performed 2 more experiments with different human hepatocytes donors. In the experiment with human liver donor #3, the AAV7 group was injected with 1×10^{11}vg of AAV vector, while the dose of AAV3B and AAV8 group was 3×10^{11}vg/mouse. Under this condition, as shown in Figure 3A **and** Table 2, AAV7 (7.2% and 10.2%) induced similar human hepatocytes transduction as AAV8 (9.7% and 7.7%), which was used as 3-fold greater dose than that in AAV7. Despite a 3-fold greater dose, AAV3B transduction remained low in human hepatocytes (6.4% and 5.2%) when compared to AAV7. Consistently, AAV8 still led to higher transduction in mouse hepatocytes (13.2% and 27.7%). In another experiment with human liver donor #4, a dose of 1×10^{11}vg vector per mouse was used for each serotype. As shown in Figure 3B **and** Table 2, based on immunohistological staining, the highest human hepatocyte transduction efficiency was observed in AAV7-injected mice (16.4% and 11.7%), compared to that in AAV3B (5.7% and 5.4%) and AAV8 (7.4% and 7.5%) groups. Similarly, flow cytometry data showed that AAV7 transduced human hepatocytes most efficiently (15.0% and 17.7%) when compared to AAV3B (5.5% and 6.9%) and AAV8 (15.6% and 4.5%). In addition, the intensity of GFP expression in AAV7 transduced human hepatocytes (MFI: 28.3 and 35.0) was also higher than that in AAV8 group (MFI: 26.1 and 27.1) based on flow analysis.

These results obtained in xenografted mice from a total of 3 different human donor further support that AAV7 can induce higher human hepatocyte transduction than AAV3B and AAV8. A total summary statistics analysis was performed (Supplementary Figure 2). This is the first report that AAV7 is able to efficiently transduce human hepatocytes.

Prevalence of anti-AAV NAbs in normal human serum and hemophilia B patient serum

Antibody-mediated inhibition is one of the potential impediments to successful AAV liver targeting gene therapy. NAbs are induced by prior natural AAV infections or a previous AAV treatment. According to our NAb assay protocol, the neutralizing titer is determined as the serum dilution at which 50% of AAV reporter gene expression is permitted, compared to no serum controls. We have demonstrated that among the different serotypes AAV7 is able to efficiently transduce human hepatocytes as described above. Next, we would like to study the prevalence of AAV7 Nabs in the general human population and patients with hemophilia. We first surveyed NAbs to AAV2, AAV3B, AAV7, AAV8 or AAV9 and determined the titer in sera of 20 healthy subjects (Fig. 4A, Supplementary Table 1). Generally, a similar pattern was observed for AAV2 and AAV3B, which demonstrated the highest prevalence of NAbs. This was followed by a lower prevalence for AAV7 and AAV8, and the lowest prevalence was found for NAbs to AAV9 in the general population. No NAbs were detected for AAV2 or AAV3B in 2 and 1 subjects, respectively. No NAbs to AAV7 or AAV8 were detected in 4 and 5 subjects, respectively. Eight subjects demonstrated no detectable NAbs to AAV9. Similar to NAb prevalence, the NAb titers to AAV2 and AAV3B were usually higher than that for AAV7, AAV8 or AAV9. Among 20 subjects, 6-7 individuals presented with a relatively high (NAb >100) neutralizing antibody titer to AAV2 and AAV3B. The number of subjects with high NAb titers to AAV7, AAV8 and AAV9 were 3, 5 and 2, respectively (Fig. 4A, Supplementary Table 1).

AAV vectors have been used successfully to treat patients with hemophilia. To achieve therapeutic effect, it is necessary to systemically deliver AAV vectors for liver targeting. Thus, the existence of Nabs in blood is the biggest challenge for AAV gene delivery in hemophilia patients. Next, we screened the Nabs in 26 serum samples from hemophilia patients (Fig. 4B, Supplementary Table 2). To compare the neutralizing antibodies present in hemophilia patients with those in healthy human subjects, the Nab titers to different AAV serotypes are listed for both groups and divided into low (0-10), medium (10-100), and high (100-1000) titer groups. NAbs were detected in all patients against AAV2, AAV3B and AAV7. No NAbs were detected to AAV8 and AAV9 in 6 and 8 patients, respectively. Similar to the general human population, the NAb titer difference between AAV2 and AAV3B in most hemophilia patients was within a 2-fold difference. Six patients had much higher NAb titers (over 4-fold) to AAV2 than that to AAV3B. Both the prevalence and titers of NAbs to AAV8 and AAV9 were lower than that to AAV2. Although Nabs against AAV7 were detected in all patients, there were 6 less case in medium and high titer group when NAbs against AAV7 were compared to AAV2. There were 5 more case in medium and high titer group when NAbs against AAV7 were compared to AAV3B.

Discussion

Several AAV serotypes have been applied in clinical trials to target hepatocytes. Although therapeutic effect has been achieved, their transduction efficiency for human hepatocytes is still lacking when compared to preclinical animal models. In this study, we systematically compared the human hepatocyte transduction from different AAV serotypes in a humanized mouse model and found that AAV7 is able to induce higher human hepatocyte transduction than the currently clinically used serotypes of 2, 5, 6 and 8. Additionally we investigated the prevalence of NAbs against AAV7 in healthy human subjects and hemophilia patients. The prevalence of AAV7 NAbs was similar to that of AAV8 and 9, and much lower than AAV2 and AAV3B in healthy subjects. However, all hemophilia patients had detectable NAbs to AAV7, which was similar to AAV2 and AAV3B. Surprisingly, the titers of AAV7 NAbs were generally higher in some hemophilia patients when compared to that for AAV2 and AAV3B.

Animal models have been widely used to verify the therapeutic efficacy of gene therapy. It is well known that results from mouse model studies rarely translate well into larger animals and humans in hemophilia AAV gene therapy due to differential hepatocyte tropisms in different species. To develop liver targeting AAV vectors for human clinical trials, more authentic animal models are greatly needed. Humanized chimeric mouse models engrafted with normal human liver cells have recently provided a more faithful tool for this purpose. Compared to other strains of mice, NRG mice with fumarylacetoacetate hydrolase deficiency (FRG) have demonstrated high efficiency of human liver cell repopulation, no limitation of age for xenograft, and easy breeding. The FRG mice xenografted with human hepatocytes have been widely used to evaluate AAV transduction efficiency in human hepatocytes or isolate human hepatocyte tropic AAV variants (31–35). Leszek et al. first used the FRG model to select AAV vectors from an AAV shuffling library and found that one AAV variant called AAV-LK03, which is closely related to AAV3B, has ten times higher transduction efficiency than AAV8 in human hepatocytes(31). Since then, several studies

have compared human hepatocyte transduction efficiency from different AAV serotypes or mutants in humanized mice, but results have been inconsistent. One study showed that AAV9 and AAVrh10 were the most efficient vectors to transduce human hepatocytes (34). However, Wang et al. demonstrated that AAV3B, AAV8, AAV-LK03 and AAVrh10 all had similar transduction efficiency in human hepatocytes in xenograft mice(32). Li et al. confirmed that AAV8 and AAV3B displayed similar human hepatocyte transduction efficiency in a human liver xenograft NSG-PiZ mouse model (33). Each of these studies are in disagreement with what Leszek et al. initially reported (31–33). In the latest published study using xenografted mice, an AAV3 capsid tyrosine mutant showed ~10 times higher efficiency than AAV8(35). Despite inconsistencies in the currently published data, our results demonstrate that gene transfer using AAV8 in human hepatocytes of xenograft mice was much lower than in mouse hepatocytes, which is consistent with the previous animal model and clinical trial results. In this study, we examined human hepatocyte transduction efficiency from AAV2, AAV3B, AAV6, AAV7, AAV8 and AAV9 in FRG mice xenografted with human hepatocytes from different donors. Consistently, AAV8 displayed high human hepatocyte transduction when compared to other tested serotypes except for AAV7. In our studies, AAV7 induced a similar or greater degree of human hepatocyte transduction than AAV8 in multiple experiments using xenografted mice with hepatocytes from different human donors.

AAV7 was first isolated along with AAV8 from rhesus monkey tissues in 2002(36). The capsid homology between AAV7 and AAV8 is 88%, which is highest compared to that between AAV7 and other serotypes. AAV7 also shows a high degree of homology to AAV9 (85%)(37). It has been reported that AAV7 vector induces efficient transduction in the liver, heart and retina in mouse as well as liver of non-human primate. However AAV7 is less efficient when compared to the transduction of AAV8 or AAV9 in mouse (9, 38–42). The transduction efficiency of AAV7 in human hepatocytes has not been previously reported. This study is the first to show that AAV7 transduces human hepatocytes in human xenograft mice better than previously tested serotypes.

At present, our data and the previously published data indicate variability of AAV transduction in human hepatocytes in xenograft mice. Several factors may influence AAV transduction efficiency. The first of all, different human hepatocyte donors are used in these reports(31, 32). In clinical trials, the transgene expression level varies in different patients after AAV vector mediated gene delivery even though the same dose per weight ratio was used(2). Secondly, timing post-injection to evaluate transduction differs between these reports. Some data was obtained ~14 days after gene transfer (31, 34, 35), whereas the two other groups waited for 3 weeks or longer to measure transduction. Both studies that waited 3 weeks found that AAV8 was similar to AAV3B for human hepatocyte transduction (32, 33). Thirdly, virus purity and the approaches for purification can also influence hepatocyte transduction efficacy. The most common methods of AAV purification were ultracentrifugation using Cesium Chloride (CsCl) and iodixanol-based gradient purification. Viruses purified from a CsCl gradient are usually contaminated with other cellular proteins. The iodixanol-based purification strategy can separate the empty virions from full particles, and empty particles have been shown to impact transduction (43, 44). Fourthly, the determination of transduction efficiency can be influenced by evaluation methods. In prior

studies, transduction efficiency has been evaluated most often by immunohistochemistry or FACS. Slightly different results were observed with these two methods(43). Fifthly, the relative ratio of human hepatocytes to mouse hepatocytes can vary between different mice, resulting in different relative transduction efficiencies. For example, AAV8 induces much higher transduction efficiency in mouse hepatocytes(31, 32, 35). If the remaining mouse hepatocytes make up too much of the liver volume after xenograft of human hepatocytes, it may influence the transduction in human hepatocytes. Lastly, the condition of the mice during the AAV administration may induce different AAV transduction efficacies. NTBC is a drug required to reduce hepatotoxicity in Fah$^{-/-}$ mice and is usually used to maintain the health of FRG mice even after xenograft with human hepatocytes. It is unclear whether NBTC has any effect on AAV transduction. Detailed information was not provided in prior studies as to whether NTBC was used during the time that AAV vectors were administered. In our study, two of 7 tested xenograft mice injected with AAV3B vectors (mouse ID 754 and 755) showed higher transduction efficiency (36.4% and 16.6%) in mouse hepatocytes, which were inconsistent with others and published data. There are several potential factors that may contribute to this difference. Firstly, the function of hepatocytes from FRG mice are not normal, which may impact AAV transduction efficiency from different AAV serotypes. Secondly, the relative ratio of human hepatocytes to mouse hepatocytes and the number of remaining mouse hepatocytes after xenograft of human hepatocytes may causes different transduction efficiencies from AAV serotypes. Thirdly, the variation of AAV3B transduction in mouse hepatocytes of different xenograft mice was also reported by Wang et al. (1-12% transduction for mouse hepatocytes from three mice based on immunohistochemistry staining) (32).

Although the human xenograft mouse model may help to predict human outcomes better than mice or large animal models including primates (31, 32), it remains still unclear how well the human xenograft model could predict human transduction efficiency. To address this concern, the best approach would be to test AAV transduction efficiency in mice xenografted with hepatocytes from other species, such as primates or dogs, and then independently corroborate the results in these native animals.

The existence of neutralizing antibodies is one of the most significant challenges to successful AAV liver gene therapy. NAbs can be induced by natural AAV infections or a previous AAV treatment. Due to natural exposure to wild type AAV, NAbs against the AAV virus capsid can be developed in human body as early as 2 years after birth (22, 45, 46). Furthermore, humans can be immunized to develop long-term persistent high titers of NAbs against the AAV capsid after exposure during AAV vector administration (47). In this study, we found that AAV7 was able to efficiently transduce human hepatocytes. The level of pre-existing AAV7 NAbs in the relevant patient population is also crucial to the successful application of AAV7 vectors in future clinical studies. It is surprising to note that a higher NAb prevalence to AAV7 was observed in patients with hemophilia than that in healthy human subjects, which is different from the NAb profiles observed for AAV8 and AAV9 between these two groups. The precise mechanism of such a phenomenon is unknown.

Collectively, this study revealed that AAV7 was able to transduce human hepatocytes in xenografted mice at a greater level than the other tested serotypes. Furthermore, a

surprisingly high prevalence of serum NAbs to AAV7 exists in the hemophilia patients that we assayed. This observation may restrict the broad application of AAV7 vectors for AAV liver targeting gene therapy in future clinical trials. Therefore it is necessary to expand testing of NAb prevalence against AAV7 in a larger human population and other types of patients that could benefit from AAV7 liver targeting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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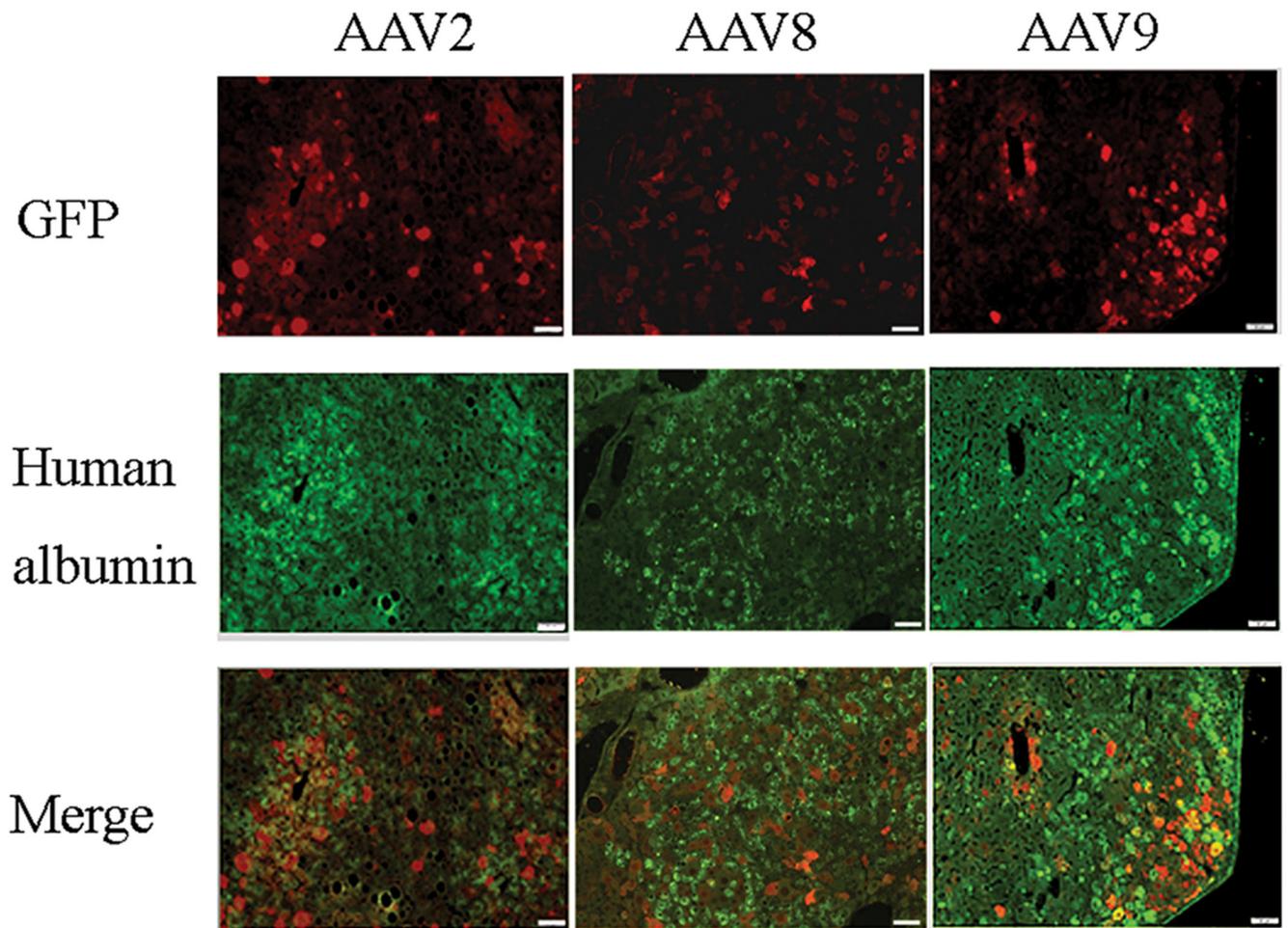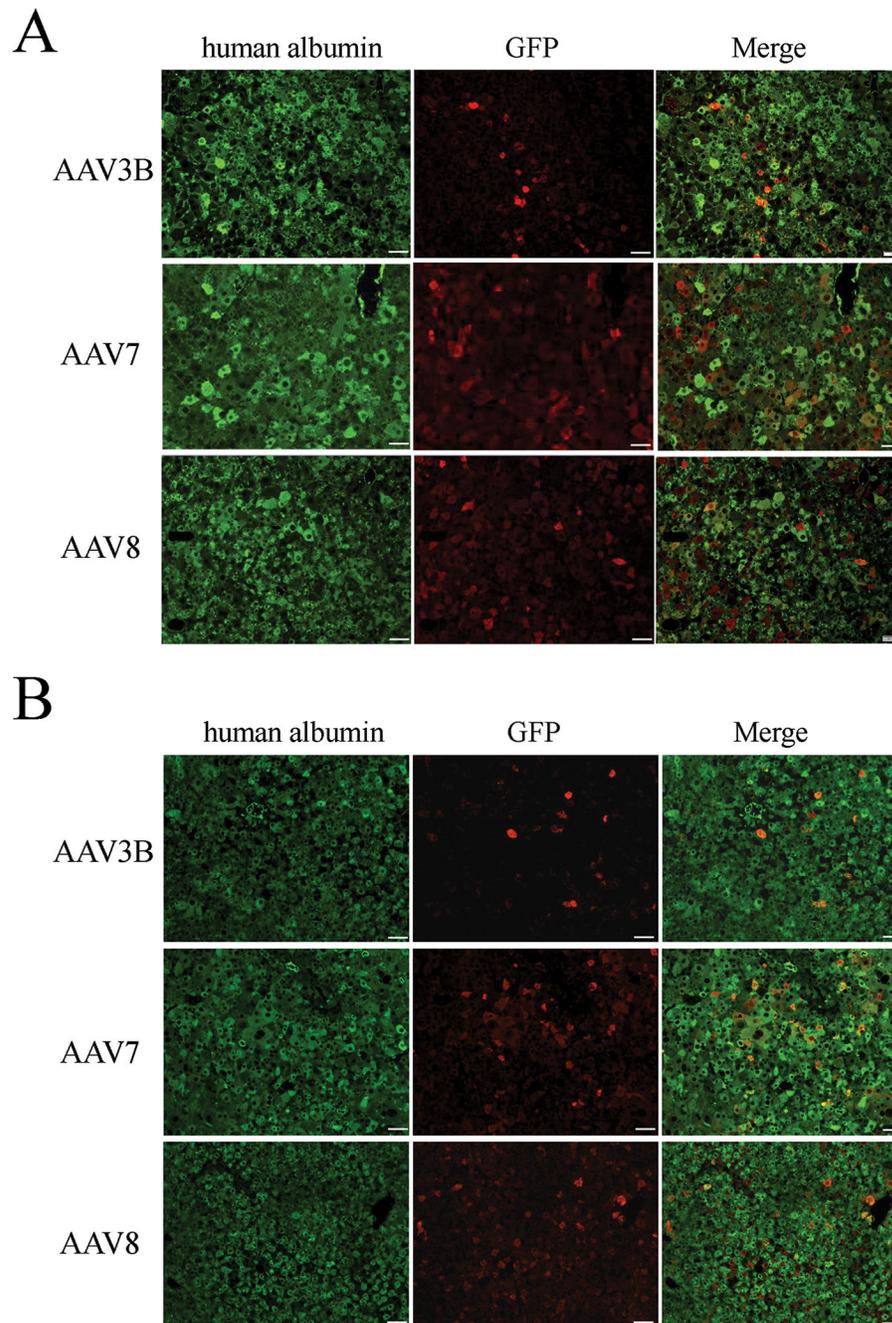
Figure 1. AAV transduction efficiency of human hepatocytes in xenograft mice.
AAV2, AAV8, or AAV9 vectors containing a GFP transgene were injected to human hepatocyte xenograft mice by intravenous injection at dose of 3×10^{11} vg/mouse. Three weeks later, mouse livers were harvested. Immunofluorescence was used to detect immunostained GFP protein (red) expression in human hepatocytes immunostained for human serum albumin (green). Representative images are shown and the scale bar represents 50μm.
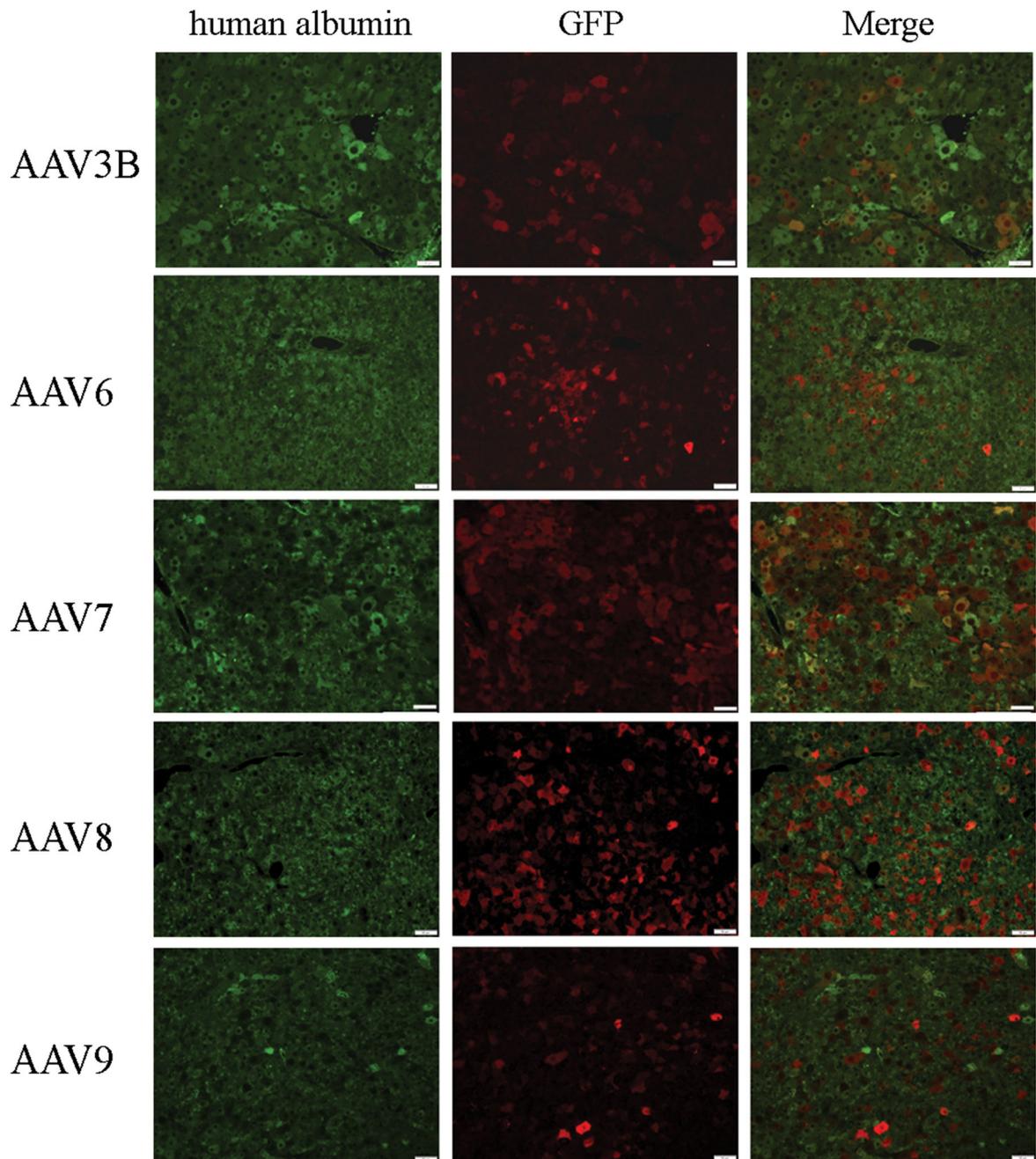
Figure 2. AAV7 induces higher human hepatocytes transduction in xenograft mice.
AAV vectors serotypes 3B, 6, 7, 8, or 9 containing a GFP transgene were injected to human
hepatocyte xenograft mice by intravenous injection at dose of 1×10^{11}vg/mouse. Three weeks
later, mouse livers were harvested. Immunofluorescence was used to detect immunostained
GFP protein (red) expression in human hepatocytes immunostained for human serum
albumin (green). Representative images are shown and the scale bar represents 50μm.

Figure 3. AAV7 induces higher human hepatocyte transduction efficiency compared to AAV3B or AAV8 in xenograft mice from additional human donors.

AAV3B, AAV7, or AAV8 vectors containing a GFP transgene were injected at a dose of 1×10^{11} vg/mouse into human hepatocyte xenograft mice by intravenous injection. Three weeks later, mouse livers were harvested. Immunofluorescence was used to detect immunostained GFP (red) expression in human hepatocytes immunostained for human serum albumin (green). **A,** AAV3B, AAV7 or AAV8 vector transduction efficiency was assayed in xenograft mice from donor #3. **B,** AAV3B, AAV7 or AAV8 vector transduction
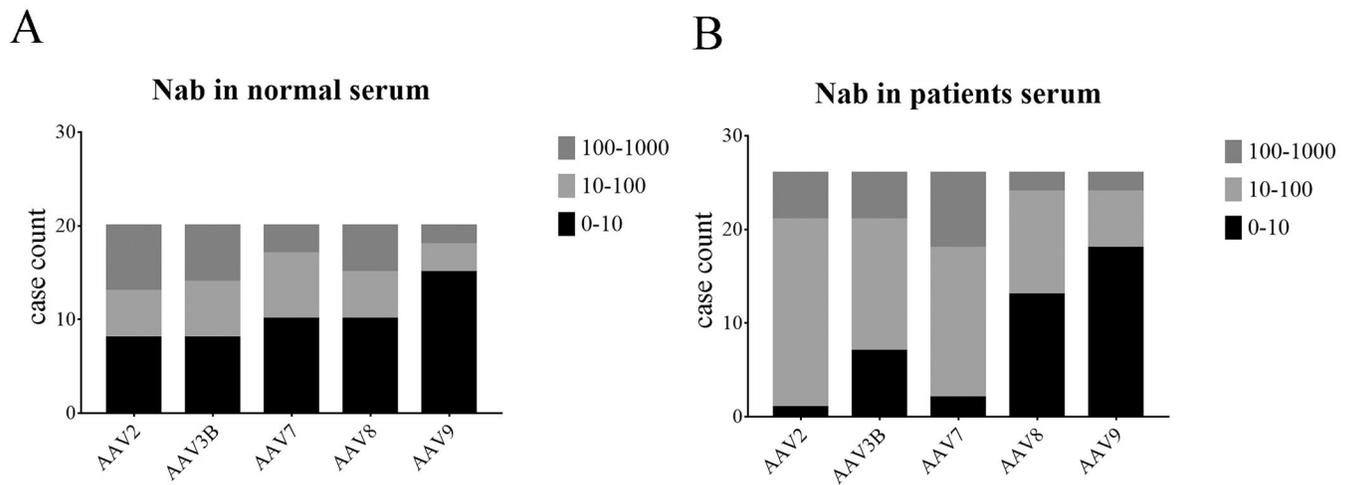
efficiency was assayed in xenograft mice from donor #4. Representative images are shown and the scale bar represents 50μm.

A

Nab in normal serum

B

Nab in patients serum

Figure 4. Nab assay analysis in normal human serum and hemophilia B patients' serum.
The distribution of different AAV serotype NAb titers in individuals is depicted and grouped into either low (0-10), medium (10-100), or high (100-1000) titers in healthy subjects (**A**) and hemophilia patients (**B**)

Table 1

Transduction of human and mouse hepatocytes by different AAV vectors in xenograft mice

mouse ID	Donor#	AAV serotype	Vector genome/mouse	IHC method transduction %(mean±SD)		Flow method	
				human	mouse	human transduction	human MFI
3858	1	2	$3×10^{11}$	17.7±6.2	26.8±10.0	n.d.	n.d.
2296	1	2	$3×10^{11}$	9.5±4.6	23.6±13.6	n.d.	n.d.
3862	1	8	$3×10^{11}$	11.9±2.8	51.2±4.5	n.d.	n.d.
3863	1	8	$3×10^{11}$	13.7±10.7	38.6±13.2	n.d.	n.d.
3864	1	9	$3×10^{11}$	17.9±3.9	41.2±12.8	n.d.	n.d.
2297	1	9	$3×10^{11}$	16.7±3.2	26.9±4.6	n.d.	n.d.
754	2	3B	$1×10^{11}$	6.7±2.2	36.4±12.2	7.1	23.3
755	2	3B	$1×10^{11}$	6.5±4.1	16.6±8.3	2.5	47.4
758	2	6	$1×10^{11}$	7.4±4.5	32.0±15.8	4.9	50.4
759	2	6	$1×10^{11}$	9.8±6.2	11.8±8.2	2.3	44.8
760	2	7	$1×10^{11}$	20.5±6.6	27.8±12.8	27.6	43.2
761	2	7	$1×10^{11}$	12.4±6.3	28.2±9.6	32.9	60.5
764	2	8	$1×10^{11}$	20.8±3.6	53.7±13.7	20.0	20.9
765	2	8	$1×10^{11}$	18.9±7.4	62.5±10.9	30.1	45.3
762	2	9	$1×10^{11}$	6.1±4.6	16.1±8.7	5.8	23.6
763	2	9	$1×10^{11}$	8.4±3.3	19.9±20.0	4.4	48.9

n.d. not done

Table 2

Transduction of human and mouse hepatocytes by different AAV vectors in xenograft mice

mouse ID	Donor#	AAV serotype	Vector genome/mouse	IHC method transduction %(mean±SD)		Flow method	
				human	mouse	human transduction	human MFI
2901	3	3B	3×10^{11}	6.4±2.6	7.6±13.7	n.d.	n.d.
2902	3	3B	3×10^{11}	5.2±2.5	1.3±0.6	n.d.	n.d.
2909	3	7	1×10^{11}	7.2±3.2	7.7±1.6	n.d.	n.d.
2910	3	7	1×10^{11}	10.2±5.1	11.6±6.6	n.d.	n.d.
2905	3	8	3×10^{11}	9.7±2.7	13.2±4.8	n.d.	n.d.
2906	3	8	3×10^{11}	7.7±2.7	27.7±13.3	n.d.	n.d.
39896	4	3B	1×10^{11}	7.0±2.1	4.4±1.5	5.0	24.3
39974	4	3B	1×10^{11}	5.7±1.8	1.6±0.4	5.5	21.1
39975	4	3B	1×10^{11}	5.4±1.8	4.1±1.2	6.9	32.2
39868	4	7	1×10^{11}	16.4±5.0	6.7±8.2	15.0	28.3
39869	4	7	1×10^{11}	11.7±3.2	19.4±5.8	17.7	35.0
39864	4	8	1×10^{11}	7.4±2.7	12.7±5.1	15.6	26.1
39976	4	8	1×10^{11}	7.5±2.0	8.6±2.2	4.5	27.1

n.d. not done