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Original Article

Gene therapy with covalently closed-end AAV vector for spinal muscular atrophy

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Covalently closed-end adeno-associated virus (cceAAV) vector is a new generation of self-complementary adeno-associated virus (scAAV) vector that does not utilize a mutant inverted terminal repeat (ITR) for vector production. Importantly, packaged genomes of these cceAAV vectors are markedly more intact than traditional scAAVs, which typically contain a large fraction of incomplete genomes, including many that lost their self-complementary configuration. Here, we report the first in-human experience with a cceAAV vector. High quality clinical grade cceAAV vector based on AAV serotype 9 (AAV9) was produced in 200 L of suspension 293 cells with a total yield of 4.3×10^{16} vector genomes (vg). Clinical trial in two spinal muscular atrophy (SMA) patients via intravenous injection at 12–24 months of age revealed no treatment-associated severe adverse events with a dose ranging from 6×10^{13} to 1.2×10^{14} vg/kg. Both patients showed rapid improvements in motor capabilities after gene therapy, as evidenced by substantial gains in motor function and electrophysiological parameters and capacity for independent mobility. Our strategy enabled us to perform gene therapy in older SMA patients who had received initial treatment with RNA-splicing modifying drug during infancy. These early data provide preliminary evidence for clinical use of cceAAV vectors, though further validation in larger cohorts is warranted.

INTRODUCTION

Spinal muscular atrophy (SMA) is a hereditary neuromuscular disorder characterized by the degeneration of motor neurons, resulting in hypotonia, muscle weakness, and atrophy.[1,2] Most cases are caused by deletions or mutations in the survival motor neuron (SMN) gene located on chromosome 5q.[1,3] There have been three life-changing targeted therapies of 5q-SMA, which have been rapidly integrated into clinical practice: an antisense oligonucleotide (nusinersen),[1,4,5] a gene transfer therapy (onasemnogene abeparvovec),[6–9] and a small molecule that corrects SMN2 splicing (risdiplam).[10–12] Recent clinical trials have shown a dramatic increase in survival and function for SMA patients, with these findings confirmed in long-term follow-up studies of the initial trials and supported by real-world data.[1,6,9–17] While these therapies are transforming the lives of people with SMA, several questions remain unanswered, and some needs are still unmet. One important question is whether combination therapies that increase SMN expression are more effective than single therapies. Combination treatments are theoretically promising, and clinical trials involving onasemnogene abeparvovec combined with nusinersen have been conducted, as well as real-world data supporting this.[15,18–21] But clinical trials have strict inclusion criteria that make it difficult to apply these protocols to older patients in real-world settings. Recent real-world data from a large cohort suggest that the use of onasemnogene abeparvovec in older patients leads to more limited improvements compared to those seen in infants treated before 6 months of age.[18] Additionally, prior exposure to nusinersen and older age may increase the risk of elevated transaminases.[18,19]

Onasemnogene abeparvovec is based on a recombinant adeno-associated virus (AAV) vector that include an AAV serotype 9 (AAV9) capsid, a functional SMN gene controlled by a cytomegalovirus enhancer/chicken-β-actin hybrid promoter.[22] It employs self-complementary adeno-associated virus (scAAV) vectors, which featured a modified inverted terminal repeat (mITR). This mITR design makes it possible to package a complementary-stranded DNA into the AAV capsid, allowing for rapid expression of the transgene.[22,23] However, inefficient resolution at the mITR may lead to the packaging of monomeric or subgenomic AAV genomes.[23–26] These non-canonical particles may diminish transgene expression and potentially compromise the safety of gene transfer.[23–25] Recently, we developed a new class of AAV9-based self-complementary vectors known as covalently closed-end double-stranded AAV (cceAAV) vectors, which eliminates the mITR resolution step during production.[23] Instead of using a mITR, a 56-bp sequence recognized by protelomerase (TelN) was used to covalently link the top and bottom

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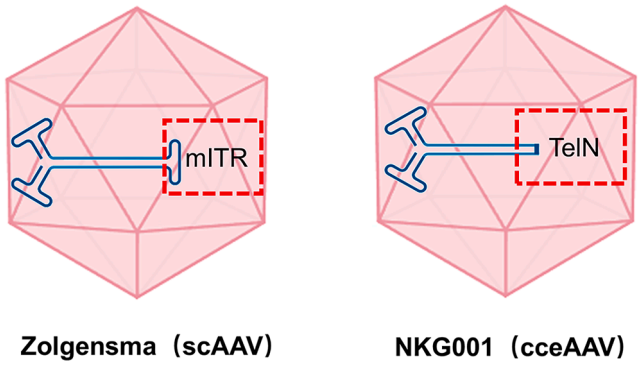

Figure 1. The diagram of scAAV vectors and cceAAV vectors
Zolgensma uses scAAV (mITR) for self-complementary DNA packaging, while NKG001 employs cceAAV with TelN-replaced mITR for closed-ended genomes.

strands, allowing the vector to be produced with a single ITR. This modification resulted in enhanced transgene expression compared to traditional self-complementary AAV (scAAV) vectors in animal studies. In the hemophia B mouse model, cceAAV-FIX (factor IX) led to significantly improved long-term expression of FIX as compared to scAAV.[23] To explore the utility of cceAAV vectors, we developed cceAAV vector carrying SMN1 gene for the gene therapy of SMA. Since this is the first time cceAAV is tested in a clinical setting, we choose a cceAAV vector construct that shares the identical genetic sequence as onasemnogene abeparvovec, since this expression cassette has been tested extensively in human patients. After the clinical vectors were produced, we performed clinical trials using cceAAV-SMN1 in two patients. These results highlight the promising nature of cceAAV-SMN1 gene therapy for SMA.

RESULTS

Rational design of cceAAV-SMN1 for human clinical trial

cceAAV is a new class of self-complementary AAV vector that employs an *in vitro* enzymatic conversion step to generate self-complementary DNA termini before production procedures. Since these structures have never been tested in human, we elected to use an identical expression construct that has been tested in human patients such as Zolgensma. The illustration of cceAAV-SMN1 is presented in Figure 1. Apart from the special non-AAV ITR termini, the rest of the cceAAV-SMN1 sequence—including the promoter, cDNA, and polyA—is identical to the previously published scAAV-SMN1 construct.[22]

Production of the clinical batch of cceAAV-SMN1 (NKG001)

In order to produce adequate vectors for testing cceAAV-SMN1 clinically, we have developed a process for manufacturing cceAAV-SMN1 vectors with suspension 293 cells. The vector DNA (pAdΔF6, pRC29, and pAAV-SMN1) were pre-digested by TelN enzyme, confirmed for completed digestion, and purified by anion exchange chromatography before they were used for transfection to the human embryonic kidney (HEK) 293 cells. The critical parameter of DNA-transfection reagent formation time is optimized and highly controlled to make sure the transfection efficiency of triple linearized plasmids is higher than 90%. The downstream processes were based on affinity and anion exchange chromatography. The final total yield was 4.3×10^{16} vector genomes (vg) from 200 L of suspension HEK 293 cells. The releasing criterion is presented in Table 1. The endotoxin level is 0.0464 EU/1 $\times$ 10^{13} vg. The empty particle is low at 4.2%. The host cell DNA and other key parameters all met the requirement for use in patient.

The therapeutic efficacy and tolerability of NKG001 are promising in SMA patients

Case presentation

Patient 1 is an SMA female infant diagnosed at 63 days of age with two copies of SMN2. Following her diagnosis, she initiated treatment with nusinersen, which resulted in modest clinical improvements. However, at 95 days of age, she developed a severe pulmonary infection requiring enteral feeding support. In response to her deteriorating condition, risdiplam was subsequently incorporated into her therapeutic regimen, along with an extensive rehabilitation program aimed at enhancing motor function. At 8 months of age, she demonstrated the ability to achieve head control in an upright position but was unable to lift her head when placed in a prone position. By 13 months, she attained the ability to sit independently; however, she faced significant challenges in transitioning between sitting and supine positions. At 20 months, she was capable of standing with bilateral support for one minute while wearing orthotic footwear. Although she achieved two developmental milestones relative to the natural progression of type 1 SMA, her caregivers expressed ongoing concerns regarding her risk of falls during prolonged sitting and her inability to regain an upright position independently. To further improve her motor capabilities and overall quality of life, she received a dose of NKG001 at 6.0×10^{13} vg/kg via intravenous infusion two days prior to her second birthday. On day 14 following treatment, she achieved standing with assistance, a gross motor milestone according to World Health Organization (WHO) Motor Milestones and the Hammersmith Infant Neurological Examination, 2nd Edition (HINE-2), exhibiting static weight-bearing in an upright stance for 10 s using only unilateral hand support and trunk clearance. By day 98, she progressed to ambulating 5 steps with bilateral support, and by day 170, she successfully ambulated 20 steps with assistance. Follow-up assessments on day 198 indicated that she could ambulate nearly 100 meters while using one hand for support and transition seamlessly between sitting and lying positions. By day 261, she was able to stand independently for 45 s. On day 289, she was capable of crawling with assistance from her hands and knees, completing approximately 20 movements consecutively. Consequently, her caregivers no longer needed to be concerned about falls; she exhibited the ability for independent mobility within her environment, with an expanded range of free movement at home, significantly improving the quality of life for both herself and her caregivers (Figure 2).

Patient 2 is a 2-year-old male who exhibited developmental delays in motor skills by 4 months of age, characterized by unsteady head control and restricted limb movement. By 6 months, he experienced

Table 1. The releasing criterion of the clinical batch of cceAAV-SMN1 (NKG001)

Category	Attributes	Assay	Lot-release criteria	Results
Quantity	vector genome copy	ddPCR method	>2 × 10^{13} vg/mL	2.69 × 10^{13} vg/mL
	vector particles number	ELISA	report result	2.68 × 10^{13} capsids/mL
	infectious particle titer	TCID50	report result	6.99 × 10^{9} IU/mL
Identity	sequencing	Sanger sequence	conform to theory	conform
	protein expression	western blotting	report result	positive
	vector particle	CE-LIF	report result	conform
Process related impurities	rHCP	ELISA	<100 ng/1 × 10^{13} vg	<0.743 ng/1 × 10^{13} vg
	rHCD	qPCR	≤500 ng/1 × 10^{13} vg	67.1 ng/1 × 10^{13} vg
	residual benzonase	ELISA	≤10.0 ng/mL	<0.100 ng/mL
	residual plasmid	qPCR	<2.0%	0.34%
	transfection reagents	HPLC	<2.0 ppm	<2.0 ppm
	residual E1 DNA	qPCR	<1.00 × 10^{7} copies/1 × 10^{13} vg	<3.03 × 10^{5} copies/1 × 10^{13} vg
	residual affinity ligands	ELISA	<0.8 ng/mL	<0.8 ng/mL
	purity of particle	CE-LIF	VP1+VP2+VP3 ≥ 90.0%	99.6%
	aggregation	SEC-HPLC	≥90.0%	99.6%
	empty ratio	AUC	<20.0%	empty ratio 4.2%; full ratio 67.8%
	size distribution	DLS	27.00–32.00 nm	29.18 nm
	residual Tween 20	HPLC	<2 μg/mL	<2 μg/mL
	concentration of P188	HPLC	0.00250%–0.00750%	0.00572%
Microbiological contamination	sterility test	pharmacopeial methods	negative	negative
	endotoxin level	pharmacopeial methods	<0.25 EU/1 × 10^{13} vg	<0.0464 EU/1 × 10^{13} vg
	mycoplasma	pharmacopeial methods	negative	negative
	abnormal toxicity	pharmacopeial methods	undetectable	undetectable

ddPCR, droplet digital polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; EU, endotoxin unit; TCID50, tissue culture infectious dose 50; CE-LIF, capillary electrophoresis with laser-induced fluorescence detection; qPCR, quantitative polymerase chain reaction; rHCD, recombinant host cell DNA; rHCP, recombinant host cell protein; HPLC, high-performance liquid chromatography; SEC-HPLC, size exclusion high-performance liquid chromatography; AUC, analytical ultracentrifugation; DLS, dynamic light scattering.

regression in motor development, leading to a diagnosis of SMA confirmed by genetic testing, which revealed three copies of the SMN2 gene. At 8 months, he commenced treatment with risdiplam and engaged in supportive rehabilitation therapy. Following the initiation of risdiplam, notable improvements in his motor function were observed. By 10 months, he regained the ability to hold his head upright but was still unable to lift his head while in a prone position. At 11 months, he could sit independently for 10 s; by 15 months, he managed to roll over with considerable difficulty. By 16 months, he demonstrated the ability to sit independently for several minutes. Despite consistent adherence to risdiplam therapy and a structured rehabilitation program, significant advancements in his motor function plateaued over the subsequent four months, particularly concerning lower limb weight-bearing capabilities. To enhance his motor function further, he received a dose of NKG001 at 1.2 × 10^{14} vg/kg via intravenous infusion at 19.5 months of age. Post-treatment, the patient experienced adverse effects including fever and upper respiratory infections; however, no immediate significant improvements in motor function were noted. Nevertheless, his parents reported an increase in his exercise endurance, as he was able to sustain longer durations during rehabilitation sessions and exhibited improved task completion and cooperation during exercises. By day 90 post-treatment, while wearing ankle-knee orthoses and with bilateral support, he could stand for 1–2 min. At the day 121 post-treatment assessment, he achieved standing with assistance (WHO Motor Milestones's definition). Specifically, the patient demonstrated the ability to maintain an upright posture while holding onto a stable support for at least 10 s. Follow-up evaluations on day 169 indicated further progress, as he was able to stand for 20 s with bilateral hand support, with trunk clearance from contact surfaces. During the follow-up on day 226, he attempted to crawl forward in a prone position and stand with unilateral hand support. Additionally, he attempted to take steps forward with assistance (Figure 2).

Improvements in motor function and electrophysiological outcomes in patients indicate NKG001's efficacy

After diagnosing two patients with SMA, we continuously monitored their motor function (Figure 3). Due to their high Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP INTEND) scores prior to receiving NKG001, we were

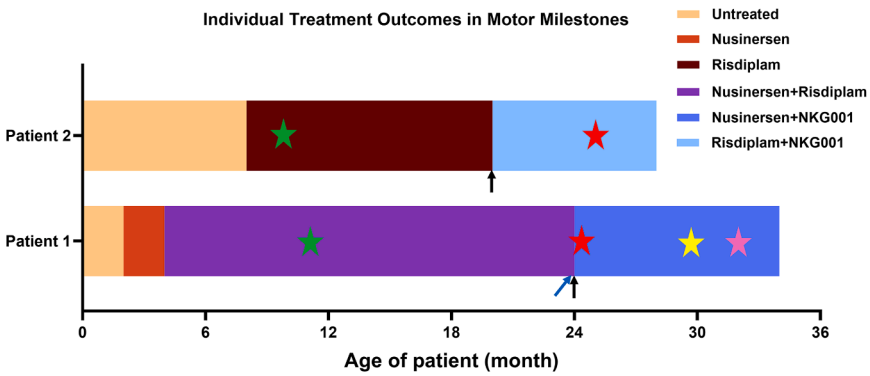

Figure 2. Treatment timeline and motor milestone achievement for two patients

The horizontal axis indicates patient age (months). Treatment regimens (drug dosage, administration intervals, and duration) are displayed for two cases. Green, red, yellow, and pink stars represent the ages at which independent sitting for 10 s or more, standing with bilateral support, walking with assistance, and standing alone for 10 s or more were achieved, respectively. Black arrows denote NKG001 formulation administration time points for two patients. In patient 1 (blue arrow), risdiplam was discontinued 0.5 months prior to NKG001 intervention, followed by nusinersen administration (≥1-month interval post-NKG001); patient 2 maintained continuous oral risdiplam throughout NKG001 treatment.

concerned about potential age-related issues or ceiling effects, so we incorporated the Hammersmith Functional Motor Scale for Children (HFMSE) scoring system post-treatment. Both patients had undergone at least one year of pharmacological treatment and rehabilitation prior to NKG001, resulting in significant improvements in their CHOP INTEND scores compared to baseline. However, patient 1 did not show significant improvement in either CHOP INTEND or HFMSE scores during the five months prior to NKG001 treatment, even exhibiting a downward trend. Following NKG001 treatment, her motor scores demonstrated a gradual increase. At the final follow-up on day 317, the CHOP INTEND score improved by 4 points, reaching the maximum score on the assessment scale. Additionally, there was an 8-point increase in HFMSE score and a 6-point increase in HINE-2 score. Similarly, patient 2 did not demonstrate improvements in CHOP INTEND scores during the four months prior to NKG001 treatment. However, by day 14 post-treatment, his CHOP INTEND score increased by 2 points and his HFMSE score by 4 points, with continued improvements noted in subsequent assessments. By the last follow-up on day 226, his CHOP INTEND score had increased by 7 points (the maximum score on the assessment scale), the HFMSE score had risen by 9 points, and the HINE-2 score had improved by 2 points (Figure 3).

Before receiving NKG001, both patients had only achieved the milestone of independent sitting. After treatment, they quickly attained new motor milestones. Specifically, patient 1 achieved the milestones of standing, assisted walking, and independent standing milestone, while patient 2 reached the milestone of supported standing. The rate of improvement in motor function was notably enhanced.

The neurophysiological findings align closely with the motor assessments conducted for both patients undergoing treatment with NKG001. We compared patient 1's compound muscle action potential (CMAP) results obtained at 22.5 months of age (during combined treatment) to those recorded the day before NKG001 administration at 23.9 months. During this time, there was no significant improvement in the CMAP values for either the median or ulnar nerves. Notably, after 14 days of NKG001 treatment, all nerve CMAP values exhibited increased; specifically, the CMAP amplitude of the right median nerve improved from 5.19 to 5.76 mV, while the left median nerve CMAP amplitude increased from 2.99 to 3.70 mV. By day 170 post-treatment (at 29.6 months of age), these values had further increased to 6.35 and 4.28 mV, respectively, representing an overall improvement of approximately 21.5%–43.1%. These findings indicate a substantial enhancement in neuromuscular function. Patient 2, assessed at 14.1 months of age, showed improvements in CMAP values for both the median and ulnar nerves after continuing risdiplam treatment for an additional 5.5 months (at 19.6 months of age). Following NKG001 treatment, the patient exhibited a sustained and rapid increase in CMAP amplitudes in bilateral upper extremity nerves. At day 169 (at 25.1 months of age), CMAP amplitudes across all nerves showed 15.3%–52.5% increase from baseline, with the left ulnar nerve demonstrating the greatest change (3.40 mV vs. 2.23 mV). These findings reflect ongoing improvement in neuromuscular transmission and function (Tables S1 and S2).

NKG001 was generally well tolerated in SMA patients

During our follow-up period, both patients experienced mild to moderate adverse events. Patient 1 did not exhibit fever or pulmonary infections post-treatment, and her liver transaminases remained below twice the upper limit of normal (ULN), allowing for the planned discontinuation of oral prednisone. On day 86 of post-treatment, she experienced transient elevations in serum creatine kinase, lactate dehydrogenase, and creatine kinase myocardial band isozyme, while troponin I levels were normal, and she had no significant clinical symptoms; these values quickly returned to normal without treatment. During the extended follow-up period from day 126 to day 317, comprehensive laboratory assessments revealed that the patient's hematological parameters, including liver function tests (e.g., transaminases), and coagulation studies, consistently fell within normal reference ranges. A slightly increase in cardiac enzymes was detected, which was similar in magnitude to pre-treatment baseline levels. This enzymatic elevation is likely attributable to the inherent pathophysiological characteristics of SMA rather than being a consequence of therapeutic intervention.

Patient 2 developed fever and pharyngitis on day 3 post-treatment, which resolved with appropriate treatment by day 7. However,

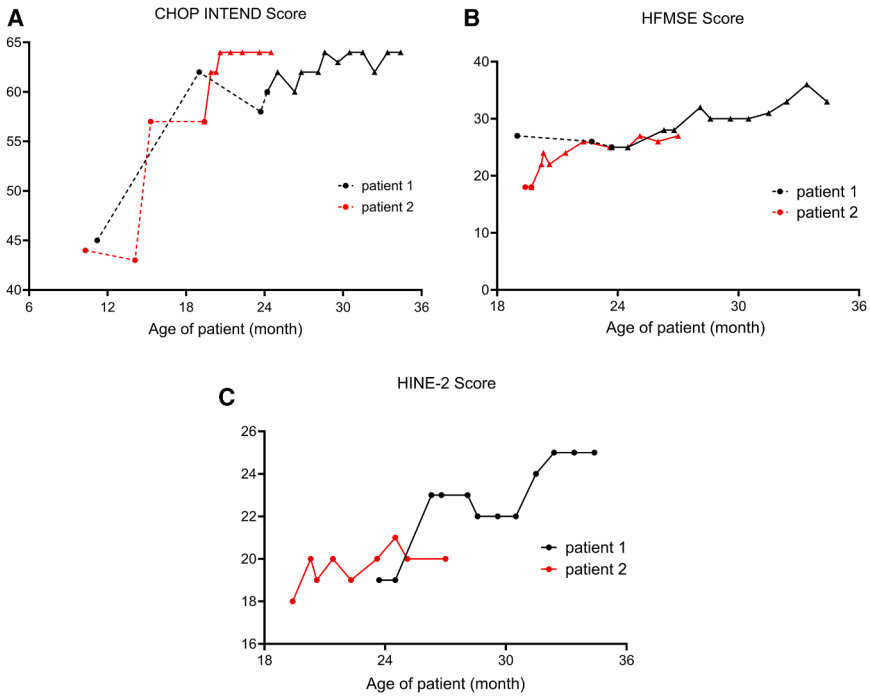

Figure 3. Motor function for two patients

The scoring results for two patients using the CHOP-INTEND (A), HFMSE (B), and HINE-2 (C) assessments are presented. The dashed lines indicate pre-treatment score trajectories, while the solid lines represent post-treatment score trajectories. HINE-2 assessments were not performed prior to NKG001 treatment. The purple arrows denote motor function scores at the baseline period.

during the follow-up period, an elevation in liver transaminases was observed on day 41 post-injection, with alanine aminotransferase (ALT) peaking at 161.8 U/L and aspartate aminotransferase (AST) at 95.7 U/L (both exceeding 2 times ULN). His prednisone dosage was continued for additional 4 weeks, and he received glutathione treatment. Subsequent evaluations showed a gradual decline in liver transaminases, ultimately allowing for the discontinuation of prednisone as planned at week 16. However, on day 120, an increase in liver transaminases was noted, prompting the continuation of glutathione therapy and close monitoring for any associated discomfort. By day 148, his transaminases levels had significantly improved. During the subsequent follow-up period spanning days 169–256, AST, ALT and gamma-glutamyl transferase (GGT) levels exhibited a progressive decline, and ultimately normalizing within the established reference range. Troponin I levels fluctuated between 0.016 and 0.049 ng/mL (normal value <0.012 ng/mL) from day 14 to day 120, but other cardiac enzyme markers remained within normal ranges with no clinical symptoms; therefore, no pharmacological intervention was provided. During the extended follow-up, troponin I levels remained within normal limits. Notably, mild elevations in lactate dehydrogenase and creatine kinase were detected, which were interpreted as being related to the intrinsic pathophysiology of SMA rather than reflecting acute cardiac injury. Liver transaminase changes, including AST, ALT, and GGT, can be observed in Figure 4.

Other tests conducted on the two patients, such as coagulation function assessments and complete blood counts for white blood cells, red blood cells, and platelets, remained within normal limits throughout monitoring. Echocardiograms indicated normal left ventricular ejection fraction and shortening fraction, with no evidence of intracardiac thrombi. To date, no adverse events such as vomiting, constipation, gastroesophageal reflux, diarrhea, or rash have been reported. All test values are detailed in Tables S3 and S4.

Immunogenicity of NKG001

Prior to and following the administration of NKG001, we conducted AAV9 antibody testing on the patients. Both patients exhibited anti-AAV9 binding antibody titers $\leq$1:50 and neutralizing antibody titers $\leq$1:20, meeting the eligibility criteria for receiving NKG001. After the injection, we monitored AAV9 neutralizing antibody titers weekly for the first month. The results indicated a significant increase in neutralizing antibody levels over time for both patients (Figure 5), reflecting a notable immune response. These fluctuations in antibody titers will be closely monitored, as they may impact the efficacy of subsequent treatments.

Pharmacokinetic characteristics of NKG001

We monitored the pharmacokinetics (PK) and viral shedding of NKG001 in patients. The PK assessment revealed a significant decline in viral DNA levels over time for both patients. Patient 1 showed a decrease from an initial viral load of approximately 2.69×10^9 vg/mL at day 0 to around 3316.34 vg/mL by day 180. Similarly, patient 2 started with a higher initial level of 1.99×10^{10} vg/mL on day 0, which decreased to approximately 1.37×10^7 vg/mL by day 90. We also assessed viral shedding in saliva, urine, and feces for both patients. Viral DNA in saliva and urine were reported as copies per microliter (copies/μL). To account for variations in fecal sampling, viral DNA levels were normalized to the total extracted gDNA concentration (vg/μg DNA). Patient 1 showed initial saliva levels of 983.2 copies/μL on day 1, decreasing to 0 by day 60. Urine levels started at 36,479.14 copies/μL on day 1 and dropped to negligible levels by day 30. Fecal shedding peaked at 606,235.27 vg/μg DNA on day 1, with subsequent fluctuations and a final measurement of 0 by day 180 (Figure 6). Patient 2 exhibited saliva levels starting at 263.71 copies/μL on day 1, peaking at 1,804.91 copies/μL on day 2 before reaching 0 by day 60. Urine levels began at 31,265.59 copies/μL on day 1 and also became undetectable by day 21. Fecal shedding for patient 2 was highest at 8.86×10^7 vg/μg DNA on

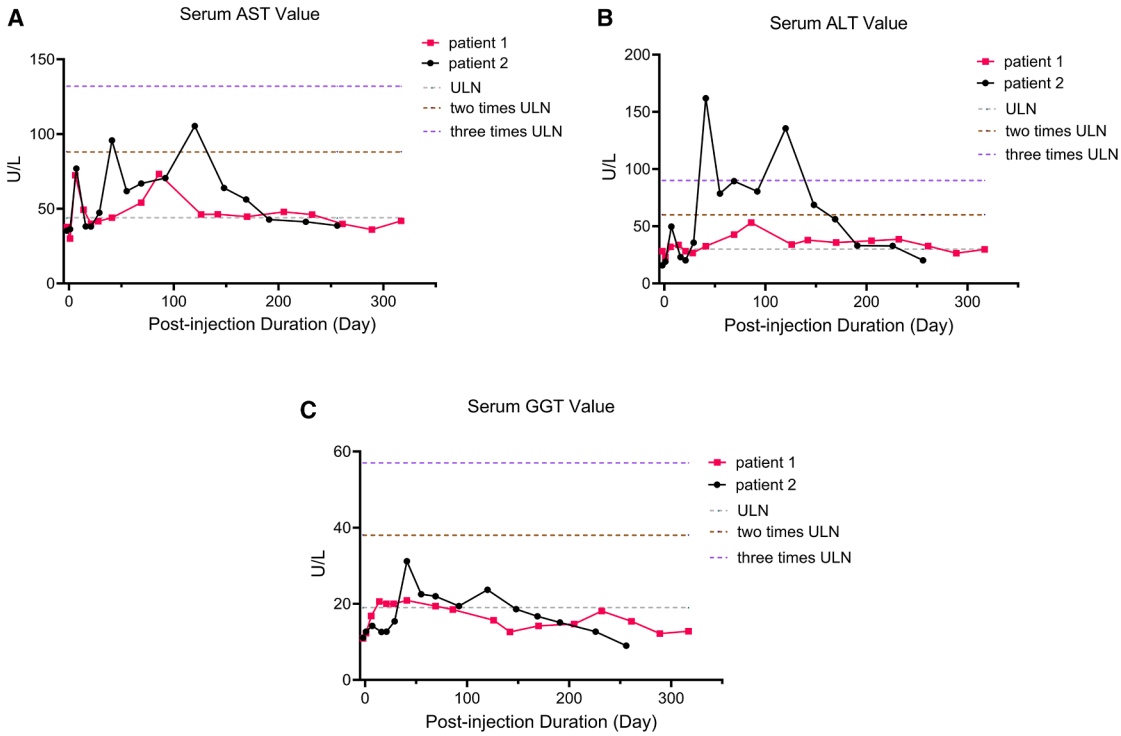

Figure 4. The transaminase changes in both patients

Patient 1: liver enzyme levels remained within normal ranges throughout the observation period. Patient 2: two transient transaminase elevations were observed, both resolving to baseline following glutathione treatment.

day 7, with a decline to 289.63 vg/μg DNA by day 120 and no detection thereafter (Figure 6). The PK of NKG001 in both patients indicates a consistent trend.

DISCUSSION

NKG-001 is a new AAV vector for the treatment of SMA, which is based on a unique self-complementary genome configuration, cceAAV.[23] The cceAAV was developed to produce scAAV genomes without the requirement of using a mITR. The inefficiencies in resolution of mITR in scAAV production were deemed as the most likely cause of vector genome heterogeneity in the scAAV vectors. *In vitro* experiments demonstrate that cceAAV vectors not only attenuate subgenomic particle formation but also significantly improve both transduction efficacy and transgene expression profiles compared to conventional AAV systems.[23] Current clinical trials and real-world data regarding the safe and effective administration of onasemnogene abeparvovec have primarily focused on monotherapy within homogeneous populations, particularly treatment-naive infants aged six months or younger who weigh less than 8.4 kg.[7–9,17,27,28] Consequently, the safety and efficacy of onasemnogene abeparvovec in older, heavier, and/or symptomatic children receiving combination or sequential therapies remain less well defined.[20,29]

Our study with cceAAV vectors provided valuable, albeit limited, real-world insights into the application of gene therapy in older patients with type 1 SMA, allowing for the concurrent use of other non-gene therapy SMA medications. The data collected from preclinical models to clinical evaluations robustly validate the effectiveness of NKG001 through intravenous injection, demonstrating that it is both safe and well tolerated when administered alongside risdiplam or nusinersen. However, due to the small sample size and lack of a direct comparative arm, further studies in a large pool of patients will be needed to remove the limitation in interpreting the results of this initial study.

Since the no-observed-adverse-effect level (NOAEL) for NKG001 was determined to be 1.5×10^{14} vg/kg, this is consistent with the NOAEL determined for scAAV vectors.[30] This dosage represents a critical threshold that balances therapeutic efficacy and safety. Clinical trial results further support these findings. Both patients treated with NKG001 exhibited substantial improvements in motor function compared to baseline assessments, with noticeable progress observed as early as 14 days post-treatment. Patient 1 achieved three key motor milestones during follow-up, while patient 2 also reached significant motor milestones despite having previously experienced a plateau in motor function prior to NKG001 treatment. These observations suggest that NKG001 has a rapid onset of action and could be a viable treatment option for patients who do not respond optimally to existing therapies. Additionally, CMAP assessments corroborated the effectiveness of NKG001 and its positive impact on motor neurons.

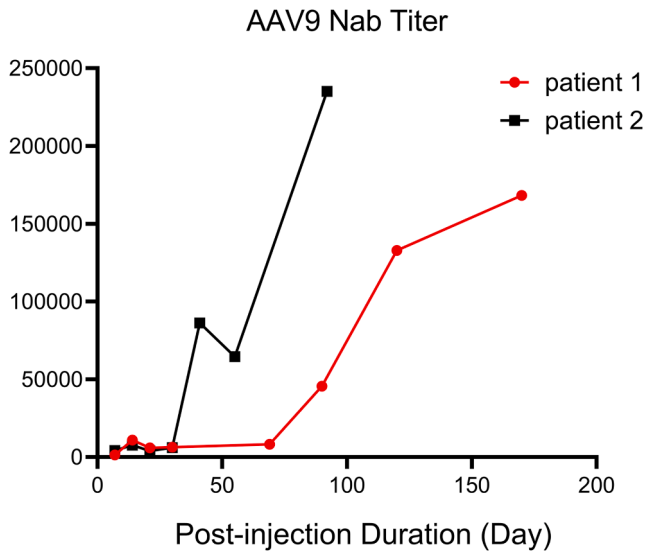

Figure 5. The AAV9 neutralizing antibody levels of two patients
AAV9 neutralizing antibody levels had significant increase in both patients.

The treatment was generally well tolerated, with no severe adverse events directly attributed to NKG001. While mild immune responses were observed, they were manageable and did not adversely affect overall treatment outcomes. The patterns of viral shedding observed in our study were consistent with those reported in clinical trials involving AAV-9 capsid-based vector.[17] Mild to moderate adverse events were noted in two patients, including fever and elevated serum levels of transaminases, myocardial enzymes, and cardiac troponin I; however, these adverse events did not manifest clinically. Similarly, fever, infections, and hepatotoxicity are among the most common adverse events reported in patients treated with AAV-9 capsid-based vectors.[6,7,19,20,27,28,30–34] Particularly, approximately one-third of patients experienced hepatotoxicity-related events,[32] with nearly 90% showing post-dosing elevations in liver function tests and some patients experienced elevated cardiac I levels.[33]

As reported for patients who received scAAV based on AAV9 vectors, elevations in AST and ALT levels are frequently observed within the first 14 days post-dosing[29,32] or between 4 and 6 weeks later.[29,32,35] Limited real-world data suggest that older SMA patients or those previously treated with nusinersen may also experience liver enzyme elevations around 13–14 weeks post-treatment.[19] Notably, patients receiving a combination of onasemnogene abeparvovec and nusinersen have reported instances of thrombocytopenia[18]; however, in patient 1 who received cceAAV, neither of these complications occurred, potentially due to the lower injection dosage. Conversely, in patient 2, significant elevations in liver transaminases were noted during the 4–6 weeks period post-injection, with an additional increase observed at 17–18 weeks. Initially, there were only slight elevations in liver transaminases during the first 30 days post-treatment, which quickly returned to normal levels. These findings underscore the importance of long-term monitoring even in patients who do not exhibit the more commonly observed initial peaks within the first two weeks. Despite not identifying any serious adverse events such as thrombocytopenia or thrombotic microangiopathy during our follow-up period, it is essential to acknowledge that the limited duration of our clinical trial may influence these findings. Monitoring for potential side effects is crucial as the landscape of SMA treatments evolves, especially considering that previous studies have highlighted safety concerns associated with gene therapies. While we are preparing to expand the study to phase 1/2 trials, the treated patients in this study will be monitored continuously for the next few years. Since enhanced capsids are believed to have a large impact on expression efficiency, an enhanced capsid should be combined to the cceAAV genome technology to maximize the therapeutic benefits in the patients.

As gene therapy becomes available, children who were initially treated with nusinersen or risdiplam have opted to switch to this new therapeutic option following diagnosis. As the therapeutic landscape evolves and more treatment options emerge, clinicians and families are increasingly considering factors beyond mere survival and motor function scores when making treatment decisions. These considerations include improvements in feeding and swallowing functions, fatigue management, respiratory support, and overall quality of life factors that significantly impact both patients and their families.

Thus, a therapy suitable for older children that enhances motor function while being safe for both monotherapy and combination treatments would expand therapeutic options for SMA significantly.

MATERIALS AND METHODS

Clinical trial study and participants

The study of NKG001 injection for the treatment of SMA 1/2 is a single-center, open-label, single-arm, non-randomized, dose-escalation trial initiated by researchers. The study was approved by the Ethics Committee of Xiangya Hospital of Central South University, with the ethical approval number TA2024-011. Written informed consent was obtained from all participants. All the patients had a genetically confirmed diagnosis of SMA1, homozygous *SMN1* exon 7 deletions, and two or three copies of *SMN2*. Details regarding the inclusion and exclusion criteria are provided in the supplemental text sections. NKG001 were provided by Niketherapeutics (Hangzhou) Company Limited. The primary objective is to assess the tolerability and safety of NKG001 injection for SMA 1/2 and to determine the recommended dose for further studies. Secondary objectives include evaluating the preliminary efficacy of NKG001 injection for SMA 1/2, as well as its immunogenicity, PK, and viral shedding characteristics.

In summary, eligible subjects were identified and provided consent in accordance with the protocols approved by the Institutional Review Board at Xiangya Hospital, of Central South University. Prior to injection, subjects underwent a series of evaluations, including assessments of AAV9 antibody titers, motor function, and upper limb electromyography, along with oral administration of prednisone acetate for prophylactic treatment.

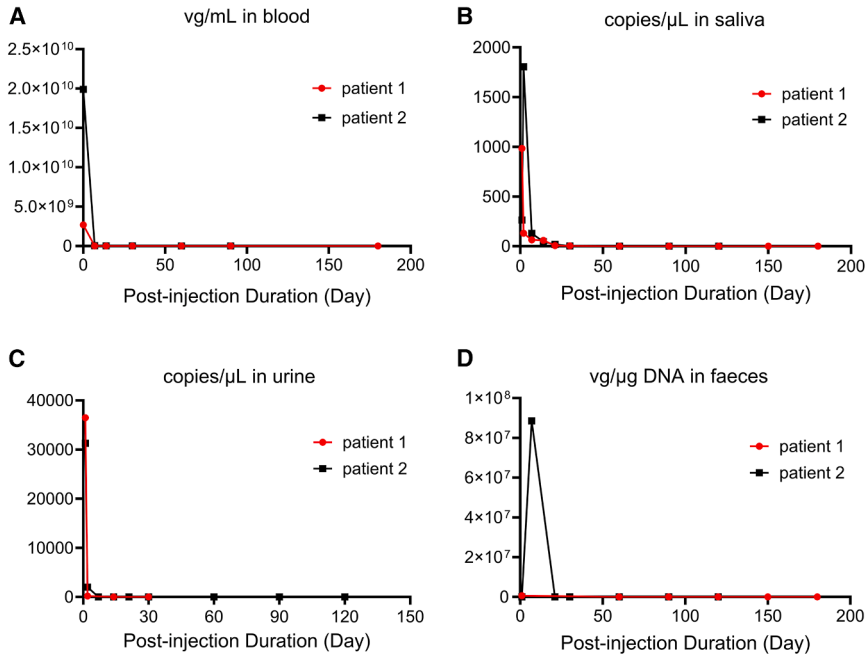

Figure 6. The PK and viral shedding of NKG001 for patient 1 and patient 2

The PK and viral shedding of NKG001 were measured in blood (A), saliva (B), urine (C), and feces (D) for patient 1 and patient 2.

Administration of gene therapy and follow up

On the first day of the treatment period, the patients received a single intravenous dose of NKG001, calculated based on body weight using a specified formula. Following dose calculation, the formulation was diluted with 10–20 mL/kg of normal saline and administered via slow intravenous infusion over 60 min. The administration of nusinersen was scheduled four weeks after NKG001, while risdiplam was given one week following the NKG001 treatment. The timeline between vaccination or recent illness and dosing was set at four weeks. Post-infusion, patients were monitored for 24 h and discharged if stable. They were then reviewed as inpatients within 72 h. The dosage calculation of NKG001 injection is as follows:

$$\text{S1 dose (mL)} = \frac{\text{weight(kg)} \times 6.0 \times 10^{13}\text{vg/kg}}{2.69 \times 10^{13}\text{vg/ml}}$$

$$\text{S2 dose (mL)} = \frac{\text{weight(kg)} \times 1.2 \times 10^{14}\text{vg/kg}}{2.69 \times 10^{13}\text{vg/ml}}$$

Specifically, all patients commenced oral prednisolone (2 mg/kg/day) the day prior to infusion. Prednisolone was continued for a minimum of 30 days then weaned, providing ALT and AST concentrations were less than two times ULN values and the AAV9 T cell response is <100 spot-forming cells/10^6 peripheral blood mononuclear cells.

Weekly clinical motor function assessments and laboratory tests for liver function (including ALT, AST, and GGT), along with a full blood count, urinalysis, coagulation function tests, and blood troponin I concentrations, were conducted during the first month. After this initial period, assessments continued monthly for 11 months, followed by quarterly evaluations for the final 12 months. Baseline electrocardiograms (ECGs) were recorded prior to dosing, with continuous 24-h ECG monitoring during the injection. One-month post-injection, an ECG and echocardiogram were performed, with subsequent evaluations every six months for a total of 24 months post-administration.

Motor function assessments

We utilized four established motor function assessments: the CHOP INTEND, the Expanded Hammersmith Functional Motor Scale (HFMSE), WHO Motor Milestones, and the HINE-2. Evaluators followed a standardized procedure manual and received training at annual in-person meetings.

The CHOP INTEND, designed for infants with SMA, consists of 16 items with a score range of 0–64. The HFMSE is a 33-item scale that evaluates gross motor abilities, with a total score range of 0–66. As the HFMSE was developed for SMA 2 and 3 patients who can sit independently, it was only used in patients who achieved this ability in the current study. It assessed attainment of gross motor development milestones (sitting without support, standing with assistance, hands-and-knees crawling, walking with assistance, standing alone and walking alone) by WHO Motor Milestones. The HINE-2, adapted from the general HINE, assesses motor developmental milestones in infants aged 2–24 months and includes eight motor items scored according to typical developmental progression. Higher scores on all three scales indicate better motor function.

Electromyography

We recorded the CMAP amplitude (mV) from both upper limbs using supramaximal stimulation of the ulnar, median, and radial nerves. The CMAP waveforms were measured by a single electromyographer.

Pharmacokinetic testing and viral shedding detection

We have engaged Saifu (Beijing) Testing Technology Service Co., Ltd. for pharmacokinetic testing, viral shedding detection, and immunological assessments. The details of the testing methods are as follows.

This study employs fluorescent quantitative PCR to measure the concentrations of target genes and vector DNA in human feces,

urine, saliva, and whole blood samples. Standard samples are diluted to various concentrations to create a standard curve. The QuantStudio Design and Analysis SE Software is utilized to plot the log values of standard sample concentrations on the *x* axis against the corresponding Ct values on the *y* axis, thereby generating a standard curve. The Ct value of DNA extracted from samples with unknown concentrations is then applied to this curve to determine the target gene concentration, which reflects the levels of the test substance in feces, urine, saliva, and whole blood.

Detection of anti-AAV9 antibodies in human plasma by the indirect ELISA

An indirect enzyme-linked immunosorbent assay (ELISA) was established to quantify anti-AAV9 antibodies in human plasma. The underlying principle is as follows. AAV9-Gluc is a pseudotyped AAV9 vector that carries a luciferase reporter gene for detection purposes. Upon infecting HeLa cells, the transduction by AAV9-Gluc generates a luminescent signal (RLU) in the presence of a luciferase substrate. In the presence of NAbs within the serum, AAV9-Gluc viral infection is inhibited, leading to a decrease in luciferase expression and a corresponding reduction in the RLU. The RLU intensity is inversely proportional to the concentration of neutralizing antibodies against AAV9 (NKG001). This constitutes the method used for quantifying neutralizing antibodies against NKG001.

Briefly, 96-well high-binding microplates were coated overnight at 2°C–8°C with NKG001 capsids at a final dilution of 1:300, prepared by sequential dilution (1:10 followed by 1:30) in coating buffer. Following three washes with 0.05% phosphate-buffered saline with Tween-20 (PBST), wells were blocked with 5% BSA in PBST for 1.5–2 h at room temperature.

The negative control (NC) was generated by pooling sera from at least 10 healthy human donors of both sexes. Positive control samples at high (HPC, 200 ng/mL) and low (LPC, 8 ng/mL) concentrations were prepared by serial dilution of anti-AAV9 monoclonal antibody (ADK9; stock concentration 50 μg/mL) into the NC matrix. A confirmation solution containing NKG001 at 2.5×10^{11} vg/mL was freshly prepared by diluting the NKG001 stock solution (2.69×10^{13} vg/mL).

Test plasma samples were diluted 1:20 with sample dilution buffer, while confirmation samples were diluted using the NKG001-containing confirmation buffer. A total of 100 μL of each diluted sample was added to wells in duplicate and incubated at 25°C for 2 h with shaking (450 rpm). After washing, wells were incubated sequentially with Biotin-labeled NKG001 (final dilution 1:1,000) for 1 h, followed by streptavidin-HRP conjugate (final dilution 1:3,000) for another 1 h, both at room temperature with shaking. Plates were washed and incubated with 100 μL of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate in the dark for 7–8 min at room temperature. The enzymatic reaction was terminated by the addition of 100 μL of stop solution, and optical density was measured at 450 nm within 30 min using a SpectraMax iD5 plate reader.

DATA AND CODE AVAILABILITY

Data can be made available upon request.

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AUTHOR CONTRIBUTIONS

H.D., C.Z., W.W., J.Z., W.X., and J.P. designed the research. Z.Z. and W.W. developed assays and managed the toxicology experiments. J.P. and W.X. oversaw logistics and project management. H.D., C.Z., J.P., and F.Y. designed the clinical protocol. C.Z., Li Yang, Lifeng Yang, F.H., Z.P., X.W., and L.M. managed patients. H.D., W.X., and J.P. analyzed the data and wrote the manuscript. H.D. generated the figures. R.H. and D. P. revised the manuscript. All authors reviewed and interpreted the data and revised the manuscript.

DECLARATION OF INTERESTS

Patent applications related to this work have been filed (W.W. and Z.Z.; "AAV vector, vector composition, and chimeric vector for treating or preventing SMA"). Z. Z. and W.W. are employees of Nikegen LLC.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.ymthe.2025.06.028.

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