Safety and Efficacy Evaluation of rAAV2tYF-PR1.7-hCNGA3 Vector Delivered by Subretinal Injection in CNGA3 Mutant Achromatopsia Sheep

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Applied Genetic Technologies Corporation (AGTC) is developing a recombinant adeno-associated virus (rAAV) vector expressing the human *CNGA3* gene designated AGTC-402 (rAAV2tYF-PR1.7-hCNGA3) for the treatment of achromatopsia, an inherited retinal disorder characterized by markedly reduced visual acuity, extreme light sensitivity, and absence of color discrimination. The results are herein reported of a study evaluating safety and efficacy of AGTC-402 in CNGA3-deficient sheep. Thirteen day–blind sheep divided into three groups of four or five animals each received a subretinal injection of an AAV vector expressing a *CNGA3* gene in a volume of 500 μL in the right eye. Two groups ($n = 9$) received either a lower or higher dose of the AGTC-402 vector, and one efficacy control group ($n = 4$) received a vector similar in design to one previously shown to rescue cone photoreceptor responses in the day-blind sheep model (rAAV5-PR2.1-hCNGA3). The left eye of each animal received a subretinal injection of 500 μL of vehicle ($n = 4$) or was untreated ($n = 9$). Subretinal injections were generally well tolerated and not associated with systemic toxicity. Most animals had mild to moderate conjunctival hyperemia, chemosis, and subconjunctival hemorrhage immediately after surgery that generally resolved by postoperative day 7. Two animals treated with the higher dose of AGTC-402 and three of the efficacy control group animals had microscopic findings of outer retinal atrophy with or without inflammatory cells in the retina and choroid that were procedural and/or test-article related. All vector-treated eyes showed improved cone-mediated electroretinography responses with no change in rod-mediated electroretinography responses. Behavioral maze testing under photopic conditions showed significantly improved navigation times and reduced numbers of obstacle collisions in all vector-treated eyes compared to their contralateral control eyes or predose results in the treated eyes. These results support the use of AGTC-402 in clinical studies in patients with achromatopsia caused by *CNGA3* mutations, with careful evaluation for possible inflammatory and/or toxic effects.

Keywords: achromatopsia, CNGA3, AAV, gene therapy

INTRODUCTION

Congenital achromatopsia (ACHM) is an inherited retinal disorder of cone photoreceptors characterized by markedly reduced visual acuity, extreme light sensitivity, and absence of color discrimination.[1] Current management of patients with ACHM consists of the use of heavily tinted lenses, which reduces the photophobia that occurs outdoors and in normally illuminated public spaces, but does not address the underlying defects in visual acuity, color discrimination, or daytime blindness. Low-vision aids such as high-powered

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magnifiers for reading are recommended and needed, even in low-light environments. There is currently no specific therapy for this disease.

In Western populations, approximately 25% and 50% of ACHM cases are caused by mutations in the cone photoreceptor-specific cyclic nucleotide gated channel alpha subunit (*CNGA3*) or beta subunit (*CNGB3*) gene, respecively.[2,3] In other populations, *CNGA3*-associated ACHM seems to be more prevalent.[4,5] *CNGA3* and *CNGB3* mutations result in a loss of cone photoreceptor function in humans and in animals with mutations in the homologous genes.[6–8]

Studies in mouse, sheep, and dog models of achromatopsia caused by mutations in the *CNGA3* and *CNGB3* genes indicate that gene augmentation therapy using a recombinant adeno-associated virus (AAV) vector expressing a normal *CNGA3* or *CNGB3* gene can produce the functional CNGA3 or CNGB3 proteins and restore cone photoreceptor function.[9–13] As part of the efforts to develop a product candidate for treatment of humans with ACHM caused by mutations in the *CNGA3* gene, an Investigational New Drug (IND)-enabling safety and efficacy study of AGTC-402, an AAV vector containing a cone-specific promotor (PR1.7),[14] a codon-optimized human *CNGA3* cDNA, and a SV40 polyadenylation sequence packaged in an AAV2 capsid containing three tyrosine to phenylalanine (YF) mutations, was conducted in CNGA3-deficient sheep.

RESULTS AND DISCUSSION

Relevance for clinical trials

Results of this study support a planned clinical trial in which individuals with achromatopsia caused by *CNGA3* mutations will receive AGTC-402 administered by subretinal injection. The clinical trial will be a dose escalation study in which successive groups of subjects will receive progressively increasing doses of study agent. Details are available at www.clinicaltrials.gov/ct2/show/NCT02935517.

Objectives and study design

The study was designed to evaluate the safety and efficacy of AGTC-402 administered by subretinal injection in day-blind Awassi sheep from the experimental flock of the Volcani Center, Israel, carrying a premature stop codon mutation in the *CNGA3* gene.[15] This large animal model has been used successfully in gene augmentation therapy studies in which treatment with an AAV5 vector carrying either a mouse or human *CNGA3* gene under the control of the 2.1 kb red/green opsin promoter (PR2.1) resulted in long-term recovery of visual function.[9] The current study design is summarized in Table 1.

A total of 13 animals were divided into three groups of four or five affected sheep. The age of the tested animals at the time of treatment ranged from 8 to 35 months, and animals were followed for 13 weeks post treatment. Each received a subretinal injection of 500 μL of an AAV vector expressing a human *CNGA3* cDNA in the right eye. Sheep in groups 2 and 3 received either a lower (1.8×10^{11} vector genomes [vg]/eye) or higher (1.5×10^{12} vg/eye) dose of AGTC-402, respectively. Sheep in group 1 that served as an efficacy positive control received an AAV5-PR2.1-hCNGA3 vector (6×10^{11} vg/eye) with the same design but from a different production batch of the one that was shown to be effective in a previous study.[9] The left eye of each animal received a subretinal injection of 500 μL of vehicle (four animals distributed across the three experimental groups) or was untreated (nine animals). The original protocol included 12 sheep but was amended to add one additional sheep (animal ID #7965) to group 3 to accommodate the possible loss of animal ID #7070 in this group, which was found to have considerable subconjunctival hemorrhage immediately after its surgical procedure and substantial inflammation in the posterior segment (retina and vitreous) at the 2-week evaluation.

Clinical evaluations were conducted before vector administration, 1 day postoperatively, and weekly thereafter. Ophthalmic examinations (slit lamp biomicroscopy and indirect ophthalmoscopy) were conducted before vector administration and 1 and 3 days and 1, 2, 4, 8, and 12 weeks after treatment. Electroretinography (ERG) and behavioral assessment by maze-navigation testing were performed before vector administration and 6 and 12 weeks after treatment.

Summary of data

Administration of AGTC-402 and the efficacy control vector by subretinal injections was generally well tolerated and not associated with any systemic toxicity. All animals reached the end of the study (week 13) with no major health problems and with good to very good body condition. One group 3 animal (#6404) had vitreous reflux of AAV vector during dose administration, resulting in a partial involuntary intravitreal injection ($\sim$100 μL) and an overall increase of vector dose volume from 500 to 600 μL. In all dosed eyes, visualization through the operating microscope showed that the site of retinal penetration by the injection cannula appeared to be sealed immediately after retraction of the cannula during surgery with a well-preserved

Table 1. *Design of a study to evaluate subretinal injection of AGTC-402 or AAV5-PR2.1-hCNGA3 in CNGA3-deficient sheep*

			Dose level		
Group	*Number of animals*	*Vector*	*(vg/mL)*	*Volume*	*(vg/eye)*
1	4	AAV5-PR2.1-hCNGA3	1.2×10^{12}	0.5 mL	6.0×10^{11}
2	4	AGTC-402	3.6×10^{11}	0.5 mL	1.8×10^{11}
3	5	AGTC-402	3.0×10^{12}	0.5 mL	1.5×10^{12}

Each animal received a subretinal injection of vector in the right eye on study day 1. Four animals (two in group 1 and one each in groups 2 and 3) received a subretinal injection of vehicle in the left eye. The left eye of the other nine animals was untreated.

subretinal bleb. Within 24 h, the subretinal bleb of fluid had mostly or completely resorbed. No bleb could be seen in any eye at study day 3.

Ophthalmic findings. Most animals had mild to moderate conjunctival hyperemia, chemosis, and subconjunctival hemorrhage immediately after surgery that generally resolved by postoperative day 7. The only significant postoperative complications were seen in the operated eye of one animal in group 3 (#7070) that developed severe posterior segment inflammation 1 week after surgery, with vitreal haze, hemorrhage, floaters, and a white plaque. Most of the inflammation subsided by the study week 8 examination, but a cataract and a chorioretinal scar, located within the area of the bleb that did not extend beyond its borders, remained as sequelae. Due to this inflammatory reaction, another animal (ID #7965) was added to the study. However, animal ID #7070 completed the study.

Ophthalmic examinations revealed no abnormal findings in the eyes of any of the animals before treatment and in untreated eyes throughout the study. No clear and consistent test article–related effects were noted on clinical examinations in any group.

Subretinal injection of the vehicle control article was associated with focal chorioretinal and injection site pigmentation, and vitreal and subretinal hemorrhage in one or two out of the four animals included in this group (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/humc). One animal developed an incipient posterior cataract on study day 1 after dosing, which persisted throughout the follow-up.

Ophthalmic findings in the four eyes given AGTC-402 at 1.8×10^{11} vg/eye (lower dose) are listed in Supplementary Table S2. One animal had subconjunctival hemorrhage (grade 1+ to 2+) on study days 1, 3, and 7 that resolved by study day 14. Two other animals had chemosis (1+ to 3+) on study days 1 and 2 that resolved by study day 7, and one of these animals developed a corneal scar on study day 3 that resolved by study week 12, and an incomplete posterior cortical cataract on study week 8 that persisted through the end of the study. A vitreal clot that resorbed was seen in animal ID #5600 treated with the low dose of AGTC-402 on study day 1 that resolved by study day 7.

Ophthalmic findings in the five eyes given AGTC-402 at 1.5×10^{12} vg/eye (higher dose) are listed in Supplementary Table S3. Three animals (ID #6404, #7070, and #7965) had conjunctival hyperemia (1+ to 2+) on study day 1, which resolved by study week 4. Two animals (#7965 and #7070) had subconjunctival hemorrhage (1+ to 3+) starting on study day 1 or 3 that resolved by study week 4. Four animals (animal ID #6404, #7070, #7299, and #7965) had chemosis (1+ to 3+) on study day 1 that resolved by study day 7. Three animals (animal ID #7088, #7299, and #7965) had focal chorioretinal pigmentation that resolved by study week 8. Animal #7070 had a chorioretinal scar at study week 4, a posterior cataract at study day 1 that persisted throughout the study, vitreal haze and hemorrhage at study day 7, and subretinal hemorrhage, white plaque, and vitreal floaters that dissipated by study week 8.

Ophthalmic findings in the four eyes given the rAAV5-PR2.1-hCNGA3 efficacy control vector at 6×10^{11} vg/eye are listed in Supplementary Table S4. Two animals (animal ID #7079 and #7090) had moderate conjunctival hyperemia (1+ to 2+), transient vitreous haze (1+), transient subconjunctival hemorrhage (1+), and chemosis (1+) on study days 1 and/or 3, which resolved by study day 7. One of these (animal ID #7090) also had focal vitreal hemorrhage at study day 1 through study day 7. One other animal (animal ID #6383) had focal vitreal hemorrhage on study day 3 that resolved by study day 7, and another animal (animal ID #7105) had pigment alterations at the injection site.

In summary, almost all of the clinical ophthalmic findings were postoperative and considered to be related to the surgical procedure, without significant clinical implications. They were distributed across all groups, and many of them were found in vehicle-treated eyes. One animal that

received the higher dose of AGTC-402 had a chorioretinal scar that could have been related to surgery, inflammation, or focal chorioretinitis.

ERG and visually guided behavior. ERG testing showed no treatment-related changes in rod-mediated responses (Fig. 1A). There was a significant increase of photopic ERGs results for both 30 Hz amplitude and critical flicker fusion frequency (CFFF)[16] for all treated groups at study weeks 6 and 12 compared to pre-dose ($p < 0.05$). There was also a significant increase of photopic ERGs (both 30 Hz and CFFF) at study weeks 6 and 12 when comparing treated and untreated eyes ($p < 0.05$). The one exception was that group 1 had a significant increase at study week 12 (both 30 Hz and CFFF) and study week 6 for 30 Hz ($p < 0.05$), but the increase was not significant at study week 6 for CFFF ($p > 0.05$; Fig. 1B and C).

Behavioral examinations by maze-navigation testing under photopic conditions showed that during post-treatment testing, navigation times and numbers of obstacle collisions were similar to the those recorded on the pre-dose test under the conditions where both eyes were patched or the right eye, the vector-treated eye, was patched. However, there was a significant reduction ($p < 0.05$) in navigation time and number of collisions in all groups when the left (untreated) eye was patched or while both eyes were unpatched (Fig. 2 and Supplementary Tables S5 and S6). Although there appears to be a decrease in transit time and number of collisions in all treatment groups between 6 and 12 weeks post treatment (Fig. 2), in all cases, this decrease was not significant ($p > 0.05$).

Taken together, both ERG and behavioral testing showed that a single subretinal injection of the AGTC-402 vector restored photopic vision in all treated eyes in a way similar to the effect of the AAV5-PR2.1-hCNGA3 vector tested previously.[9] A similar vector containing the *CNGB3* gene has been used successfully to restore vision in a canine model of CNGB3-related ACHM.[13]

Histopathological findings. Findings on gross pathology examination were observed in 6/13 animals distributed across all three experimental groups (Supplementary Table S7). None of the findings was considered to be related to the test article.

Findings on histopathological examination were observed in all eyes that received a subretinal injection of vehicle or vector (Table 2).

In animals treated with AGTC-402, microscopic findings of outer retinal atrophy with or without lymphocytic inflammation in the retina and histiocytic to lymphocytic inflammation in the choroid occurred with varying severity in the vector-treated right eyes of two animals in the higher-dose group (animal ID #6404 and #7070). Substantial photoreceptor loss in the treated area of the retina of animals ID #6404 and #7070 was observed, accompanied by RPE loss in animal ID #6404 (Fig. 3B). However, no retinal degeneration was observed in the area that was not treated (Fig. 3C), suggesting that the retinal atrophy was restricted to the treated area of the retina. No eyes that were untreated or treated with vehicle or the lower dose of AGTC-402 had such lesions. Ghost vessels were also seen on the right cornea of animal ID #6404. The vessels, which may have been missed in the preoperative examination, usually indicate a past history of keratitis that had resolved. As the retina in this eye showed signs of atrophy, it is considered possible that the two are linked, and that a historic inflammation caused the corneal and retinal inflammation. However, this is speculation, and the chances that the two are indeed linked are considered low.

It is noted that sheep ID #6404 and ID #7070 had surgery-related complications. Animal ID #7070 had a considerable subconjunctival hemorrhage immediately after operation and severe posterior segment inflammation recorded at study week 1 after surgery, and animal ID #6404 had vitreous reflux of AAV vector during dose administration. Therefore, the outer retinal atrophy and retinal and choroidal inflammation observed in these two animals could be procedure related and/or test-article related.

Photoreceptor displaced nuclei (PDNs) of varying severity were observed in the vector-treated eyes of 1/4 animals (ID #5600) and 1/5 animals (ID #7299) treated with the lower or higher dose of AGTC-402, respectively. Other findings included lipofuscin accumulation in the RPE cells in one eye each from the AGTC-402 high- (ID #7299) and low-dose (ID #5600) groups and choroidal and retinal congestions (in all eyes analyzed with the exception of the eyes that presented with retinal inflammation). These findings were considered to be biologically insignificant and/or not test-article related.

Microscopic findings of outer retinal atrophy with or without lymphocytic inflammation in the retina and histiocytic to lymphocytic inflammation in the choroid occurred with varying severity in the vector-treated eyes from 3/4 animals treated with rAAV5-PR2.1-hCNGA3 (ID #6383, #7090, and #7079). Similar findings had not been observed in the previous study in which different batches of

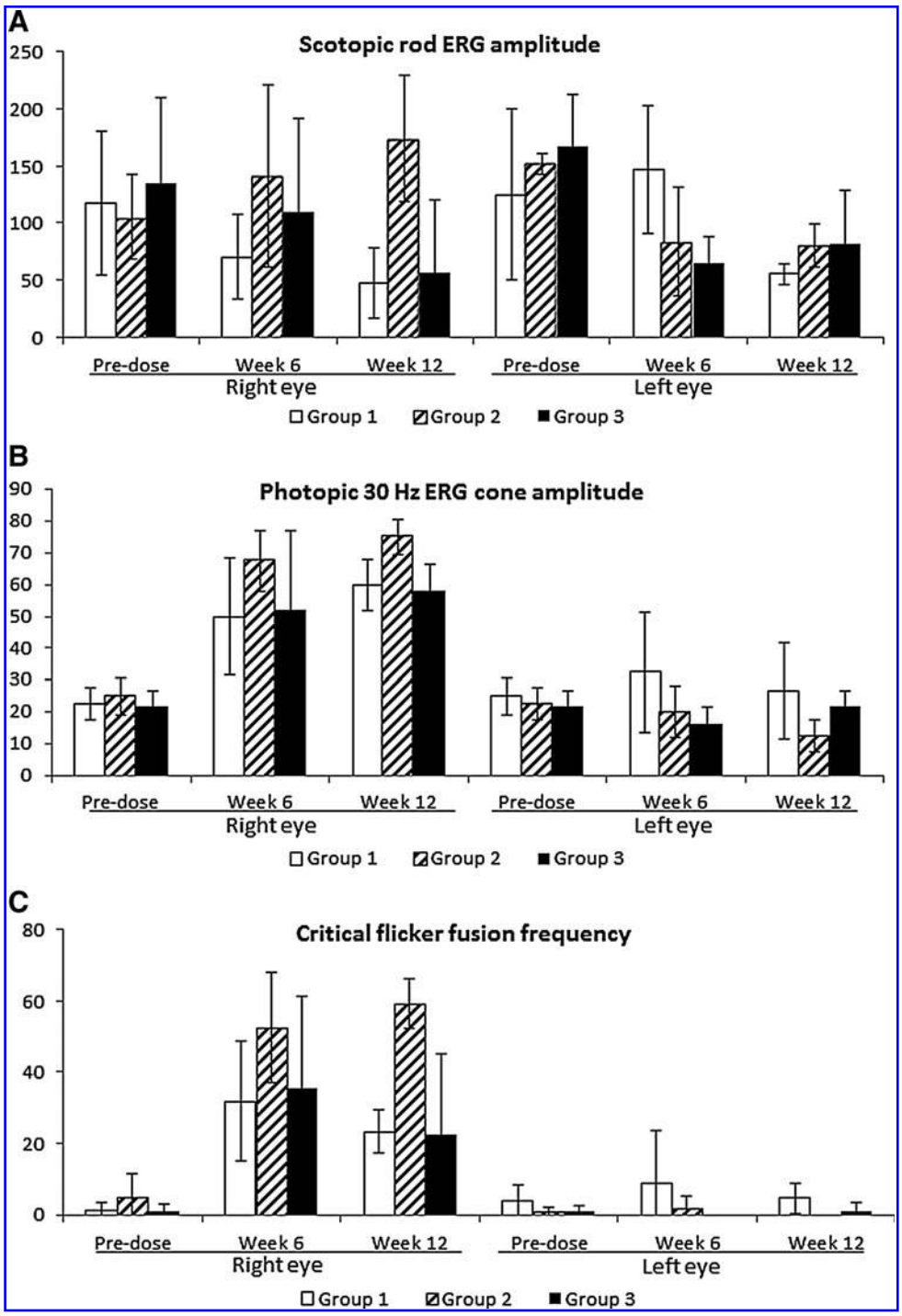

Figure 1. Electroretinography (ERG) responses in CNGA3-deficient sheep before and 6 and 12 weeks after treatment with an adeno-associated virus (AAV) vector expressing **CNGA3**. **(A)** Mean ± standard deviation (*SD*) dark-adapted ERG b-wave amplitude (μV). **(B)** Mean ± *SD* light-adapted 30 Hz cone ERG cone amplitude (μV). **(C)** Mean ± *SD* light-adapted critical flicker fusion frequency (Hz). Group 1 = AAV5-PR2.1-hCNGA3 at 6.0×10^{11} vector genomes (vg)/eye; group 2 = AGTC-402 at 1.8×10^{11} vg/eye; group 3 = AGTC-402 at 1.5×10^{12} vg/eye. The right eye was the vector-treated eye in each animal.

this vector had been administered by the same investigators.[9] Because determination of vector concentration can vary widely between different laboratories,[17,18] the vector concentration for the batch of AAV5-PR2.1-hCNGA3 vector used to treat the first four animals in the previous published study was tested using the quantitative polymerase chain reaction (qPCR) method used to determine the vector concentration in the present study. Based on the vector concentration determined by

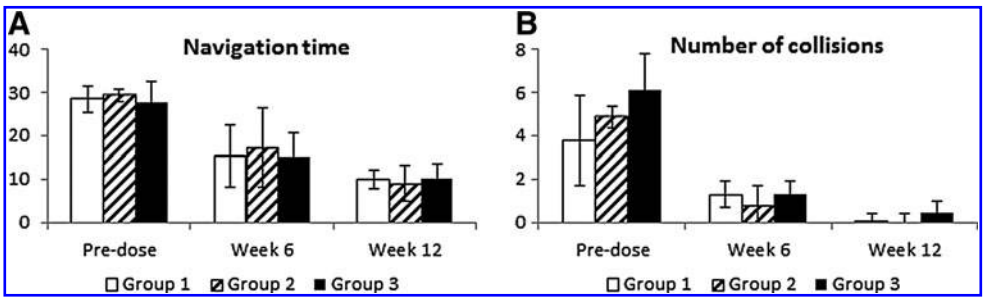

Figure 2. Maze navigation test results in CNGA3-deficient sheep before and 6 and 12 weeks after treatment with an AAV vector expressing *CNGA3*. **(A)** Mean ± *SD* time in seconds taken to move through a maze containing two obstacles. **(B)** Mean ± *SD* number of collisions during navigation through the maze. Each animal received a 0.5 mL subretinal injection. Animals in group 1 received rAAV5-PR2.1-hCNGA3 at a concentration of 1.2×10^{12} vg/mL. Animals in group 2 received AGTC-402 (rAAV2tYF-PR1.7-CNGA3co) at a concentration of 3.6×10^{11} vg/mL. Animals in group 3 animals received AGTC-402 at a concentration of 3.0×10^{12} vg/mL. All results were evaluated by an independent masked observer. Results with one or both eyes patched are provided in Supplementary Tables S5 and S6.

Table 2. *Histopathological findings after subretinal injection of AGTC-402 or AAV5-PR2.1-hCNGA3 in CNGA3-deficient sheep*

	Group 1		*Group 2*		*Group 3*	
	OS	*OD*	*OS*	*OD*	*OS*	*OD*
Retina						
Retinal fold, focal, 1+						1/5
Congestion						
1+	1/4	2/4	2/4	3/4	1/5	1/5
2+	2/4	1/4	2/4	1/4	4/5	3/5
3+	1/4	1/4				1/5
Atrophy/degeneration, outer retinal						
Multifocal						
1+		1/4				
2+		1/4				
Focally extensive, 4+		1/4				2/5
Inflammation						
Lymphocytic peri-vascular, 1+		2/4				
Photoreceptor displaced nuclei						
1+						
2+		1/4		1/4		
3+						1/5
Detachment, 3+						1/5
RPE						
Hypertrophy, 1+						1/5
Lipofuscin						
1+	1/4	1/4				
2+				1/4		1/5
Choroid						
Congestion						
1+	1/4	2/4	2/4	3/4	1/5	1/5
2+	2/4	1/4	2/4	1/4	4/5	3/5
3+	1/4	1/4				1/5
Inflammation						
Histiocytic/lymphocytic						
1+		1/4				2/5
3+		1/4				
Lymphocytic–multifocal						
1+		1/4				
2+		1/4				

The right eye was the vector-treated eye for each animal. Findings were graded as 1+ (minimal), 2+ (moderate), 3+ (marked), or 4+ (severe). Group 1 = AAV5-PR2.1-hCNGA3 at 6.0×10^{11} vector genomes (vg)/eye; group 2 = AGTC-402 at 1.8×10^{11} vg/eye; group 3 = AGTC-402 at 1.5×10^{12} vg/eye.
OS, left eye; OD, right eye.

the current method, the maximum total vector dose administered in the previous study was 1.66×10^{11} vg per eye, which is approximately fourfold lower than the dose used in the current study. Thus, it is possible that the toxicity observed in this group is dose related, although other factors (*i.e.*, different production facility or small differences in batch production or purification procedures) cannot be ruled out (Table 3).

Photoreceptor displaced nuclei, lipofuscin accumulation in the RPE cells, and choroidal and retinal congestions were also observed in this group.

Immunohistochemical findings. Immunohistochemical (IHC) staining showed the expected hCNGA3 staining of photoreceptors in all 13 vector-treated eyes, while control untreated or vehicle treated eyes remained unstained. Representative IHC sections showing hCNGA3-positive staining in one eye treated with AGTC-402 at the lower dose (animal ID #5600) is shown in Fig. 3E and F. Double staining for cone opsin and hCNGA3 protein showed that the hCNGA3 protein positively stained in cone photoreceptor inner segments was well aligned with the cone opsin positively stained in the outer segment.

Immunological findings. Results of anti-AAV neutralizing antibody testing are shown in Table 4. Two of nine animals (animal ID #6404 and #7070) treated with AGTC-402 had a four- to eightfold increase in NAb titer to AAV2 capsid, and 2/4 animals (animal ID #6363 and #7079) treated with rAAV5-PR2.1-hCNGA3 had a 16- and >128-fold increase in Nab titer to AAV5 capsid. The magnitude of the increase in NAb titer was greater in animals treated with rAAV5-PR2.1-hCNGA3 than in animals treated with AGTC-402. In a previous

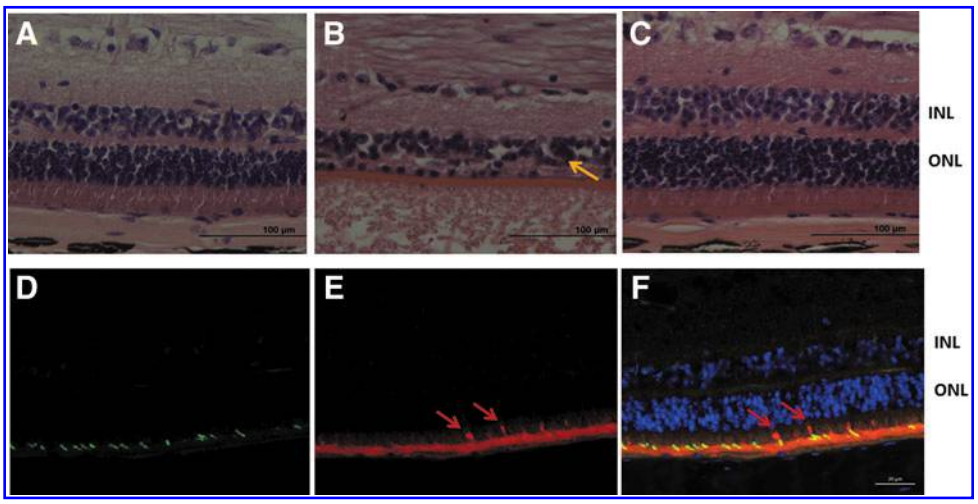

Figure 3. Representative images of histopathologic assessment and immunohistochemical (IHC) staining. Severe retinal atrophy is shown in the vector treated area of the right eye of animal ID #6404 **(B)** when compared to the control left eyes at the same location (mid-dorsal) **(A)**. No retinal atrophy was observed in the untreated retinal area (outside of bleb) of the vector treated right eye **(C)**. The arrow in **(B)** indicates disruption or thinning of inner and outer nuclear layers. L/M cone opsin (in green) and hCNGA3 protein expression (in red) was detected by IHC in eyes treated with AAV2tYF-PR1.7-hCNGA3 **(D, E, and F)**, (animal #5600, group 2). Arrows indicate examples of hCNGA3 protein positively stained (in red) in cone photoreceptor inner segments **(E)**, and alignment to the cone opsin positively stained (in green) in the outer segment when double stained for cone opsin and hCNGA3 protein **(F)**.

IND-enabling nonhuman primate (NHP) study evaluating the safety and biodistribution of rAAV2tYF-PR1.7-hCNGB3, a drug product analogous to AGTC-402 that has the same AAV2tYF capsid, serum NAb to AAV2tYF capsid was detected in 7/8 treated animals, with an 8- to 32-fold increase in titers.[19] In the current study, the two animals treated with AGTC-402 that had an increase in NAb titer had either vitreal reflux of the drug product or considerable subconjunctival hemorrhage that occurred during or immediately after surgery. In the other sheep, the NAb titer increase in the current study was significantly lower than that in the NHP study, suggesting a possible species-dependent effect on the development of NAb to AAV capsid.

Table 3. *Summary of ophthalmic and histopathological findings in the previous and current study*

Study	*Vector*[a]	*Ophthalmic exams*[b]	*Histopathology*[c]
1	AAV5-PR2.1-hCNGA3, $\leq 1.66 \times 10^{11}$ vg/eye ($n=8$)[d]	One animal had mild anterior uveitis that resolved within a few days	Normal retinal architecture with no signs of retinal damage or postoperative complications
2	AGTC-402, 1.8×10^{11} vg/eye ($n=4$)	Postsurgical findings	No major findings
	AAV5-PR2.1-hCNGA3, 6.0×10^{11} vg/eye ($n=4$)[e]	Postsurgical findings	Outer retinal atrophy with or without lymphocytic inflammation in the retina and histiocytic to lymphocytic inflammation in the choroid occurred in varying severity in the vector-treated eyes from 3/4 animals
	AGTC-402, 1.5×10^{12} vg/eye ($n=5$)	Postsurgical findings; one animal also had severe posterior segment inflammation 1 week after surgery, with vitreal haze, hemorrhage, floaters, and a white plaque	Outer retinal atrophy with or without lymphocytic inflammation in the retina and histiocytic to lymphocytic inflammation in the choroid occurred with varying severity in the vector treated eyes from 2/5 animals; substantial photoreceptor loss in the treated area was accompanied by RPE loss in one of these animals.

[a]Vector dose in the first study corrected for differences in vector concentration methods.
[b]Postsurgical findings were mild to moderate conjunctival hyperemia, chemosis, and subconjunctival hemorrhage immediately after surgery that generally resolved by postoperative day 7.
[c]In addition to the histopathological findings listed above, additional findings that were considered to be biologically insignificant and/or not test-article related were photoreceptor displaced nuclei (PDNs) of varying severity in the vector-treated eyes of 1/4 and 1/5 animals treated with the lower or higher dose of AGTC 402, respectively; lipofuscin accumulation in the RPE cells in one eye each from the AGTC-402 high- and low-dose groups; and choroidal and retinal congestions (in all eyes analyzed with the exception of the eyes that presented with retinal inflammation).
[d]Vector produced and purified in the laboratory of Dr. William Hauswirth, University of Florida.
[e]Vector produced and purified in the University of Florida Vector Core.

Table 4. *Serum AAV neutralizing antibody titers after subretinal injection of AGTC-402 or AAV5-PR2.1-hCNGA3 in CNGA3-deficient sheep*

Group	Animal #	Pre-dose	Week 6	Week 12
1	6383	<10	160	160
1	7105	160	160	80
1	7079	<10	>1,280	>1,280
1	7090	<10	10	10
2	6384	160	160	160
2	6956	40	10	20
2	7002	40	20	20
2	5600	20	20	20
3	7965	<10	20	<10
3	7070	40	160	80
3	6404	10	80	80
3	7088	80	80	80
3	7299	80	160	160

Results are expressed as the reciprocal of the highest serum dilution that inhibited expression of the luciferase transgene by ≥50%. Animals in group 1 were treated with AAV5-PR2.1-hCNGA3 at 6.0×10^{11} vg/eye. Animals in group 2 were treated with AGTC-402 at 1.8×10^{11} vg/eye. Animals in group 3 were treated with AGTC-402 at 1.5×10^{12} vg/eye.

No serum antibody to the transgene product hCNGA3 protein was detected in any vector treated sheep, which is consistent with the finding in the NHP study for the rAAV2tYF-PR1.7-hCNGB3 drug product.[19]

CONCLUSIONS

The objectives of this study were to evaluate the safety and efficacy of a viral vector targeted to treat ACHM patients by gene augmentation therapy in a large animal model. The viral vector used in this study, designated AGTC-402 or rAAV2tYF-PR1.7-hCNGA3, contains a codon optimized human *CNGA3* cDNA (hCNGA3) driven by a PR1.7 cone-specific promoter, packaged in an AAV2tYF capsid, and manufactured using a herpes simplex virus (HSV)-based AAV production system.

Subretinal injections of AGTC-402 were well tolerated and not associated with systemic toxicity. Most animals had mild to moderate conjunctival hyperemia, chemosis, and subconjunctival hemorrhage immediately after surgery that generally resolved by postoperative day 7. No clear or consistent test article–related adverse effects were noted in any group through the study. Two of five animals that received the high dose of AGTC-402 had outer retinal atrophy and retinal and choroidal inflammation that was considered procedure related and/or test-article related. There was an increase in cone-mediated ERG responses and resolution of day blindness in the treated eyes at both dose levels.

Results of this study support the use of AGTC-402 in clinical studies in patients with achromatopsia caused by *CNGA3* mutations. Treatment should be initiated at low doses and patients carefully evaluated for possible inflammatory and/or toxic effects, especially as the dose level is increased.

MATERIALS AND METHODS

Vector production

The AGTC-402 vector was designed based on the rAAV2tYF-PR1.7-hCNGB3 vector[19] in which the *CNGB3* cDNA was replaced by a codon-optimized *CNGA3* cDNA. The vector was produced by AGTC using a recombinant herpes simplex virus (rHSV) complementation system in suspension-cultured baby hamster kidney (sBHK) cells.[20] Two rHSV helper viruses, one containing the AAV2 *rep* and AAV2tYF *cap* genes and the other containing the *CNGA3* expression cassette, were used to co-infect sBHK cells grown in serum-free medium. One day later, the cells were lysed with Triton X-100 detergent, treated with Benzonase® (Merck), clarified by filtration, purified by AVB Sepharose® (GE Life Sciences) affinity chromatography followed by CIM $SO3^-$ (BIA Separations) cation-exchange chromatography, and eluted in 2.6× balanced salt solution containing 0.014% (v/v) Tween 20 (BSST). The purified bulk was concentrated and buffer exchanged to 1×BSST (drug substance) and sterile filtered (0.2 μm) to generate drug product and stored at ≤-65°C.

The AAV5-PR2.1-hCNGA3 vector, which was used in the efficacy control group, was produced by the University of Florida vector core using a plasmid transient transfection method and purified by iodixanol gradient centrifugation followed by HiTrap Q HP column chromatography.[21] Briefly, HEK 293 cells (1×10^9 cells) seeded in a cell factory (Nunc) were co-transfected with plasmids pTR-PR2.1-hCNGA3 and pXYZ5, a helper plasmid encoding the AAV, and adenovirus genes required to pseudotype AAV2 ITR-containing expression cassettes into AAV5 capsids.[21] Transfected cells were harvested 60 h post transfection and resuspended in lysis solution (150 mM of NaCl, 50 mM of Tris, pH 8.4). The crude lysate was clarified by centrifugation and the vector-containing supernatant was subjected to a discontinuous iodixanol step gradient centrifugation followed by HiTrap Q HP column chromatography (GE, Life Sciences). The vector eluted from the Q HP column was concentrated and buffer exchange to Alcon balanced salt solution containing 0.014% Tween 20 (1×BSST), sterile filtered through a 0.22 μM filter, and dispensed in small aliquots that were stored at ≤-65°C.

Vector characterization

Vector concentrations (vg/mL), purity (silver-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis), and concentrations of endotoxin and HSV protein were measured, as previously described.[22] Concentrations of BHK protein, bovine serum albumin, Benzonase, and AVB ligand were measured by enzyme-linked immunosorbent assay (ELISA) using commercially available kits. HSV and BHK DNA were measured by qPCR. Testing for mycoplasma, bacteria, and fungi was performed using standard microbiological methods.

The AGTC-402 drug product had a vector concentration of 4.5×10^{12} vg/mL, purity >90%, endotoxin 1.83 EU/mL, and was negative for bacteria and mycoplasma. Concentrations of process residuals in the drug substance used to generate this drug product were 351 ng/mL for HSV DNA, 117 ng/mL for BHK DNA, 6.43 ng/mL for bovine serum albumin, 12.97 ng/mL for BHK protein, less than the lower limit of quantification (LLOQ) for HSV protein and Benzonase, and negative for microbial enumeration.

The efficacy control vector AAV5-PR2.1-hCNGA3 made by plasmid transfection method had a vector concentration of 3.3×10^{13} vg/mL, purity >90%, <LLOQ for endotoxin, and negative for bioburden testing.

Subretinal injections

Animals were premedicated with acepromazine and pethidine, induced with propofol, and anesthetized and ventilated with isoflurane. Eyes were prepped with 1% povidone iodine solution and rinsed with sterile saline. An adhesive sterile drape was applied on the eye and around it. Then a single 23G pars-plana port was created on the ventral aspect of the limbus, 2 mm from it. A 41G retractable subretinal injection needle (DORC 1270.ext, Dutch Ophthalmic Research Center), attached to a short extension tube and a 1 mL syringe containing the viral vector, was introduced through this port and slowly advanced into the subretinal space under direct visualization using a Zeiss ophthalmic microscope. Approximately 500 μL of viral vector formulated in BSS containing 0.014% Tween 20 was injected into the subretinal space, forming a bleb. The injection needle was withdrawn, and the 23G port removed. If leakage was observed, a single 7-0 Vicryl suture was placed. Following surgery, animals were treated with a topical chloramphenicol, polymyxin B, and dexamethasone solution three times daily for 1 week.

To minimize potential study bias, animals were dosed in a predetermined systematic manner. One animal from each group was dosed, followed by a second animal from each group, and so on until all animals had been dosed according to a prespecified randomization scheme. Dosing occurred on three separate days.

Residual dose formulations from each day of dosing were frozen at ≤-65°C for assessment of vector concentration in the formulated material.

Safety evaluations

Assessment of tolerability was based on mortality, clinical observations, ophthalmic examinations, ERG (scotopic and photopic tests), and anatomic pathology. A veterinarian conducted clinical examinations 1 day prior to dosing, after dosing on study day 1, and weekly thereafter. Abnormal findings, including any observed eye abnormality, were recorded on a data-collection form. A veterinary ophthalmologist or veterinary ophthalmology resident performed in-life ophthalmic examinations, including slit lamp biomicroscopy and indirect ophthalmoscopy prior to vector administration and 1 and 3 days and 1, 2, 4, 8, and 12 weeks after vector administration. Abnormalities were recorded on an ophthalmic exam data-collection form.

ERG testing

Recordings were conducted, as previously described.[16] Pupils were dilated with 0.5% tropicamide and 10% phenylephrine hydrochloride solutions ≥20 min before recording. Animals were premedicated with an intramuscular injection of acepromazine (0.1 mg/kg) and pethidine (3 mg/kg), and anesthesia was induced with an intravenous injection of propofol (4 mg/kg) and diazepam (0.15 mg/kg). Animals were intubated and anesthesia maintained with 3% isoflurane in 100% oxygen (5–6 L/min). During anesthesia, animals were ventilated (2% isoflurane and 1.5–2 L/min oxygen), hydrated with intravenous 0.9% saline infusion, and constantly monitored by a veterinary surgeon.

Animals were positioned in sternal recumbency, eyelids retracted with Barraquer eyelid retractor, and globes center-positioned using one or two subconjunctival stay sutures. Signals were recorded using a Jet contact lens electrode. Subcutaneous needles placed at the ipsilateral lateral canthus and the pinna of the ear served as reference and ground electrodes, respectively. The recorded eyes were kept moist with a drop of 1.4% hydroxymethylcellulose, and impedance was maintained <5 kΩ. To avoid confounding effects of adaptation and anesthesia, both eyes were recorded simultaneously using a dual stimulating and recording system (HMsERG 2000; Ocuscience) with a bandpass of 0.3–300 Hz. Background adaptation light and stimuli were delivered

with two handheld mini Ganzfelds (one per eye). Rod function was evaluated using the scotopic component of the "dog diagnostic" protocol.[23] Under dark-adapted conditions, responses to 10 flashes, presented at 0.5 Hz at a light intensity of 0.01 $cd \cdot s/m^2$, were recorded and averaged to generate a single scotopic flash response. This was repeated every 4 min for a total of five scotopic flash responses. Following scotopic evaluation, mixed rod-cone function was recorded; responses to four flashes presented at 0.1 and 0.05 Hz at two light intensities (3 and 10 $cd \cdot s/m^2$) were recorded and averaged. Animals were then light adapted for 10 min (30 cd/m^2), and cone function was recorded. Responses to 32 flashes, presented at 1 Hz (1 $cd \cdot s/m^2$), were recorded and averaged to generate the single photopic flash response. This was followed by eight cone flicker responses (flashes presented at 10–80 Hz, with 128 responses averaged at each frequency). The paradigm was then repeated at intensities of 2.5, 5, and 10 $cd \cdot s/m^2$. Recordings were conducted in a fluorescent-lit, indoor experimental facility with an ambient light intensity of 0.06 cd/m^2. Results were recorded as the amplitude of scotopic b-wave amplitudes, cone flicker amplitudes, and the critical flicker fusion frequency (the highest frequency of flickering light stimulus that an animal can resolve).[24]

Visually guided behavior testing

Photopic maze-navigation testing was conducted prior to surgery and at 6 and 12 weeks post treatment, as described previously.[25] Briefly, animals were directed to pass through a 9 m maze with two barrier obstacles. A group of sheep positioned at the end of the maze attracted the test animal to pass through the maze as quickly as possible. For each animal, a test consisted of two successive trials, with the barriers randomly rearranged in their right-left orientation between trials to avoid a learning effect. Passage time and number of collisions with the obstacles were recorded for each trial. A trial lasted up to 30 s, and a passage time of 30 s was ascribed to animals that failed to transit the maze. As only the right eye was treated with a CNGA3 vector, maze testing of treated animals was conducted with alternate patching of the treated and untreated eyes. All trials were videotaped for later review by an experienced independent masked observer (Dr. Andras Komaromy, Michigan State University).

Detection of immune responses to AAV and CNGA3

Serum was obtained before treatment and at 6 and 12 weeks after treatment and stored at <-60°C. Each sample was tested by ELISA at Covance Laboratories for antibodies to CNGA3 and for antibodies to AAV2 or AAV5 using a previously described neutralization assay.[26]

Necropsy and gross pathology observations

During study week 13, all animals were euthanized with 20% embutramide, 5% mebezonium iodide, and 0.5% tetracaine hydrochloride (0.1 mL/kg; T-61, Intervet Canada Corp.). Comprehensive gross pathology examinations were performed and tissue collected for histopathology from all eyes and any gross lesions. Lesions identified during gross pathology examination were fixed in 10% paraformaldehyde and stained with hematoxylin and eosin (H&E).

Eye globes were collected within 10–15 min after euthanasia and fixed in Davidson solution. After 48 h fixation, the eye globes were grossly sectioned at Hadassah Medical Center into three sections, dehydrated, and embedded in paraffin wax (Paraplast; Leica). From each section of the treated right eye and from the middle section of the left eye, 15 slides of 5 μm sections were cut. One slide was stained with H&E, and the other 14 slides were prepared for IHC.

IHC

Deparaffinized sections of the eyes were incubated in a decloaking chamber (Biocare Medical) with 10 mM of citrate buffer (pH 6.0) at 125°C, blocked with phosphate-buffered saline solution containing 1% (w/v) bovine serum albumin, 0.1% (w/v) Triton X-100, and 10% (w/v) normal donkey serum, and subsequently incubated at a temperature of 4°C with anti-hCNGA3 antibody (goat polyclonal, 1:50; Santa Cruz Biotechnology, Inc.), with or without anti-red/green cone opsin antibody (rabbit polyclonal, 1:100; Chemicon International). After washing, the following secondary antibodies were applied for 1 h at room temperature: CY2-conjugated donkey anti-rabbit IgG and rhodamine red-X-conjugated donkey anti-goat IgG (1:200; both from Jackson ImmunoResearch Laboratories). In some cases, secondary staining with Cy5 donkey anti-goat IgG (1:250; Jackson ImmunoResearch Laboratories) was used. Nuclei were counterstained with 4,6-diamidino-2-phenylindole-containing mounting medium (Vectashield, Vector Laboratories). To determine the specificity of the antigen–antibody reaction, corresponding negative controls were exposed to secondary antibody alone.

Data evaluation and statistical analyses

For ERG responses, statistical analysis was performed using SAS v9.4 (SAS Institute, Inc.).

Descriptive statistics (number of observations, mean, standard deviation [*SD*], minimum, median, and maximum) by dose group, eye, and visit (pre and post dose) were presented for each three ERG response parameters: b-wave amplitude of the fifth scotopic recording, 30 Hz, $10\,cd\cdot s/m^2$ flicker amplitude and the critical flicker fusion frequency (CFFF) at $10\,cd\cdot s/m^2$. Differences between eyes (experimental vs. untreated or vehicle-treated control) at different visits (pre and post dose) within each dose group, and differences between pre- and post-dose visits were tested for the three types of ERG response parameters. The analysis was performed using a linear model with repeated measures. An UN@UN covariance structure was used to model within-subject (animal) error in SAS Proc Mixed. The UN@UN structure accommodates the correlation between right and left eye at each visit, correlation over time between repeated measures within a specific eye, cross-correlation between a measurement at one visit in the right eye and measurement at another visit in the left eye of the same subject, and different variances between eyes as well as over time. The least squares means and corresponding standard errors and *p*-values for comparisons within treatment and between treatments were presented.

For the maze -navigation results, navigation time and number of collisions were tested using analysis of variance. The statistical model included the following main effects: treatment (time × patching type); time (pre dose vs. week 6 vs. week 12); patching type (both eyes unpatched, both eyes patched, left eye patched, right eye patched); rAAV vector (AAV5-PR2.1-hCNGA3, 6.0×10^{11} vg/eye; AAV2tYF-PR1.7-hCNGA3, 1.8×10^{11} vg/eye; AAV2tYF-PR1.7-hCNGA3, 1.5×10^{12} vg/eye), and animal nested within vector and treatment × vector interaction.

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AUTHOR DISCLOSURE

G.Y., P.M.R., J.D.C., and M.S.S. are employees and shareholders of AGTC and have a conflict of interest to the extent that this work potentially increases their financial interests.

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