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A mutation in gene *CNGA3* is associated with day blindness in sheep ☆

Shay Reicher [a,b], Eyal Seroussi [a], Elisha Gootwine [a,*]

[a] Institute of Animal Science, Agricultural Research Organization, The Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel
[b] The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Israel

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ABSTRACT

Lambs with congenital day blindness show diminished cone function, which is characteristic of achromatopsia, a congenital disorder described in humans and dogs. To identify gene(s) associated with sheep day blindness, we investigated mutations in the *CNGA3*, *CNGB3*, and *GNAT2* genes which have been associated with achromatopsia. Sequencing the coding regions of those genes from four affected and eight non-affected lambs showed that all affected lambs were homozygous for a mutation in the *CNGA3* gene that changes amino acid R236 to a stop codon. By PCR-RFLP-based testing, homozygosity for the stop codon mutation was detected in another 19 affected lambs. Non-affected individuals ($n = 386$) were non-carriers or heterozygous for the mutation. While a selection program has been launched to eradicate the day blindness mutation from Improved Awassi flocks, a breeding nucleus of day-blind sheep has been established to serve as animal models for studying human achromatopsia.

Introduction

A novel congenital day blindness in sheep that is inherited in an autosomal recessive mode has been recently reported in the Improved Awassi breed [1]. Sporadic birth of visually impaired lambs was first recorded in 2003 at the Ein Harod flock, Israel. With time, the proportion of the affected lambs born in the flock gradually increased, reaching by 2008 the value of 3.5% (unpublished results). This newly discovered illness is associated with diminished function of cones but not of rods, resembling achromatopsia described in humans in its incomplete mode [2,3]. This form of blindness differs in its etiology from other known cases of inherited sheep blindness such as ceroid lipofuscinosis [4] or blindness related to microphthalmia [5].

Complete or typical achromatopsia is an autosomal recessive disease, in which patients manifest reduced visual acuity, lack of color vision, photophobia and nystagmus essentially from birth, while maintaining a normal- or near-normal-looking fundus. Cone electroretinogram (ERG) responses and color vision are absent [6]. The presentation of incomplete or atypical achromatopsia is similar, except that some color vision and improved acuity are present [7].

Most cases of human achromatopsia have been associated with mutations in three genes that play a major role in the cone phototransduction cascade. Two of these genes code for the cone photoreceptor cyclic nucleotide-gated (CNG) channel subunits α and β—*CNGA3* and *CNGB3*, respectively [8,9]. Cone CNG channels are tetramers of two α and two β subunits and are non-selective cation channels located in the membrane of cone photoreceptors. CNG channels are open in the dark. Light stimulation leads to CNG channel closure and consequently to the absence of cation influx and membrane hyperpolarization, which diminishes glutamate release at the photoreceptor synapse [10].

CNGB3 has been estimated to account for 40–60% of achromatopsia in humans [11,12]. About 28 pathogenic mutations have been found within the human *CNGB3* gene, most of them nonsense or frameshift mutations. Mutations in or deletion of the canine *CNGB3* gene have been also associated with day blindness in German Shorthaired Pointer and Alaskan Malamute dogs [13,14]. *CNGA3* mutations account for about 20–30% of the cases of human achromatopsia. About 69 mutations have been detected within the *CNGA3* gene in human patients with achromatopsia, most of them of the missense type [2,12,15–20]. A minor fraction, about 2%, of achromatopsia cases in humans have been associated with mutations in a third gene, *GNAT2*, encoding the α-subunit of the cone photoreceptor transducin G-protein gene [21–23].

Applying a "candidate gene approach," we searched for the mutation that underlies day blindness in sheep by comparing the sequences of exons of ovine *CNGA3*, *CNGB3* and *GNAT2* genes between affected and non-affected individuals. Our results show that a stop codon mutation in the ovine *CNGA3* gene is associated with day blindness in Improved Awassi sheep.

☆ Sequence data from this article have been deposited in the DDBJ/EMBLGenBank Data Library under GenBank Accession nos. FN377574, FN377575 and FM946176. (*CNGA3*); FM946177 (*CNGB3*) and FM946178 (*GNAT2*).

* Corresponding author. Fax: +972 3 9603678.
E-mail address: gootwine@agri.gov.il (E. Gootwine).

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Results and discussion

Cloning of major ovine cone phototransduction genes

RNA extracted from retinas of non-affected lambs belonging to the Improved Awassi ($n=4$) and local Awassi ($n=2$) breeds, as well as to an American Suffolk cross ($n=2$) was reverse-transcribed using PCR primers designed based on the orthologous bovine genes (Supplementary Table 1). We were able to PCR amplify the complete *CNGA3* and *CNGB3* coding regions and partial *GNAT2* coding region of the putative candidate genes.

CNGA3 sequencing results: sequencing of the PCR-amplified products showed that the entire ovine *CNGA3* coding region comprises 2103 nucleotides (GenBank accession FN377574 and FN377575), coding for 700 amino acids. The polypeptides encoded by these sequences showed 95% identity and 97% similarity to the orthologous bovine *CNGA3* protein (GenBank accession no. NP_776704, Supplementary Fig. 1).

Alignment of ovine *CNGA3* cDNA sequences of the eight non-affected individuals revealed a total of 18 synonymous and nine non-synonymous polymorphic sites (Table 1). The two Suffolk crossbred lambs were homozygous for the same *CNGA3* haplotype. At least seven additional haplotypes were evident in the other six normal Improved Awassi and local Awassi individuals.

Two of the nine non-synonymous polymorphic sites, namely L217F and K265R, were located in conserved transmembrane domains S2 and S3, respectively (Supplementary Fig. 1). Other non-synonymous polymorphic sites were in the less conserved sequence regions.

CNGB3 sequencing results: sequence analysis showed that the entire coding region of the ovine *CNGB3* comprises 2304 nucleotides (GenBank accession no. FM946177), coding for 767 amino acids, with 92% identity and 93% similarity to the orthologous bovine protein (GenBank accession no. XP_597439, Supplementary Fig. 2).

Sequence alignment of eight non-affected individuals revealed seven synonymous and 10 non-synonymous (Supplementary Table 2) polymorphic sites within the ovine *CNGB3* coding region. One of the Improved Awassi lambs was found to be homozygous for the same *CNGB3* haplotype and at least six additional haplotypes were evident in the other seven non-affected individuals.

Three of the 10 non-synonymous polymorphic sites found in non-affected individuals, namely E188K, R336Q and T564M, were located in conserved regions of the *CNGB3* gene (Supplementary Fig. 2). Other non-synonymous polymorphic sites were in the less conserved sequence regions.

GNAT2 sequencing results: partial sequencing (exons 4-8) of ovine *GNAT2* cDNA (GenBank accession no. FM946178) showed a single synonymous polymorphism (T439C). The polypeptide encoded by this sequence was identical to the orthologous bovine, equine and canine proteins (GenBank accession nos. NP_776751, XP_001495066 and XP_547240, respectively).

Sequencing candidate genes in day-blind sheep

CNGA3

All four day-blind lambs were homozygous for a *CNGA3* haplotype that was not observed in non-affected individuals, and which differed from the Suffolk-cross haplotype by four non-synonymous mutations (GenBank accession no. FM946176.) (Table 1). One of the mutations (C706T) truncated the encoded polypeptide at position 236 (Fig. 1) at the end of transmembrane domain S2.

Such early truncation is likely to render a non-functional cone CNG channel, leading to day blindness. Interestingly, a similar C→T substitution leading to the appearance of a stop codon was detected in the human *CNGA3* gene at residue 221 [17], which is orthologous to ovine residue 236. In contrast to the day-blind sheep in this study, which manifested impaired but not abolished cone function [1], the patient that carried the mutation in a homozygous situation had complete achromatopsia with photophobia, nystagmus, decreased visual acuity (OD-OS 6/60-6/36), and absence of photopic (cone) ERG.

To further investigate the association between the C706T mutation in *CNGA3* and day blindness in sheep, we examined this position by PCR-RFLP in 19 affected and 386 non-affected Improved Awassi rams and ewes from flocks where day blindness has been segregating (Fig. 2). All 19 affected lambs were homozygous T/T, while 289 and 97 of the healthy lambs were either homozygous C/C for the wild-type allele, or heterozygous C/T, respectively.

CNGB3

While all four affected lambs were homozygous for the same *CNGA3* haplotype, a different result was obtained upon sequencing of their *CNGB3* gene—one affected lamb was homozygous for a haplotype found in normal lambs, and the three others were heterozygous for ovine *CNGB3* haplotypes (Supplementary Table 2). These results make the ovine *CNGB3* locus an unlikely candidate for the Improved Awassi day blindness, as homozygosity is expected near the locus carrying the deleterious recessive alleles in the genomes of all affected individuals.

Table 1
Non-synonymous polymorphism detected in the ovine *CNGA3* cDNA in normal and day-blind sheep.

	Exon no.	2	2	4	8	8	9	9	9	9
	Nucleotide no.	50	61	269	649	706	794	1064	1898	2037
Animal no.	Status	Genotype at nucleotide position								
Suffolk cross 1	normal[a]	CC	TT	GG	CC	CC	AA	TT	GG	GG
Suffolk cross 2	normal									
Improved Awassi 1	normal	CT	TA	GA			AG		AG	CG
Improved Awassi 2	normal				CT		AG			
Improved Awassi 3	normal[b]						GG	CC		
Improved Awassi 4	normal									
Local Awassi 1	normal	CT	TA	GA				CC		
Local Awassi 2	normal									
Improved Awassi 5	affected[c]					TT		CC	AA	CC
Improved Awassi 6	affected[c]					TT		CC	AA	CC
Improved Awassi 7	affected[c]					TT		CC	AA	CC
Improved Awassi 8	affected[c]					TT		CC	AA	CC
Amino acid. substitution		S17L	S21T	R90Q	L217F	**R236STOP**	K265R	T355I	K633R	H679Q

Suffolk cross 1 sequence marked in gray, is used as a reference sequence. Only deviations from the reference sequence are indicated.
[a] GenBank accession no. FN377574.
[b] GenBank accession no. FN377575.
[c] GenBank accession no. FM946176.

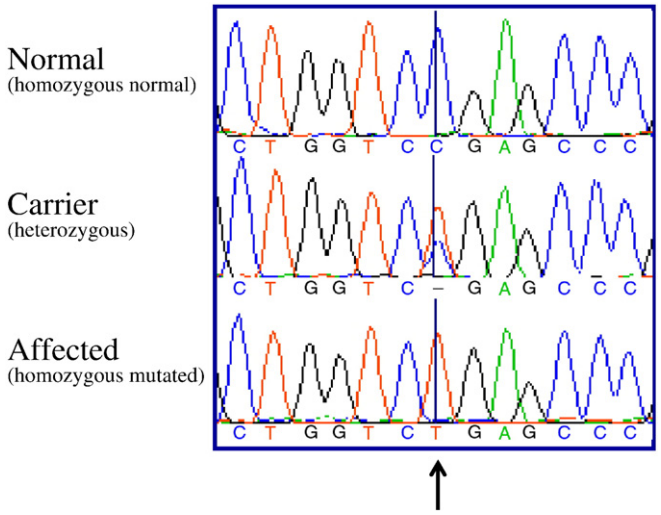

Fig. 1. Mutation analysis of the ovine *CNGA3* gene. Sequence analysis of cDNA from a non-carrier, heterozygous carrier, and homozygous day-blind mutant. An arrow denotes the position of the mutation.

GNAT2

All four affected lambs were heterozygous for the T439C polymorphism, excluding *GNAT2* homozygocity in the affected lambs. Although the gene was partially sequenced, this observation, coupled with that of identical proteins being encoded by *GNAT2* sequences obtained from both unaffected and affected lambs, support the notion that ovine *GNAT2* is an unlikely candidate locus for the Improved Awassi day blindness.

Use of the "candidate gene approach" in the present study effectively identified *CNGA3* as the gene responsible for day blindness in Improved Awassi sheep. This approach was also effective in associating human achromatopsia with a mutation in the *GNAT2* gene [21]. However, a similar approach failed to identify candidate genes associated with microphthalmia in sheep [5], or candidate genes associated with day blindness in the Standard Wire-Haired Dachshund dog [24]. In the latter case, a whole-genome association study of discordant sib-pairs, genotyped with the canine single nucleotide polymorphism (SNP) array, was applied to identify the gene that causes the illness [25].

The origin of the *CNGA3* mutation described in the present study is the Ein Harod Improved Awassi flock [1]. This has been a closed flock, with no entry of foreign genetic material, for several decades. The *CNGA3* mutation event occurred before 2003, the year in which the birth of blind lambs was first recorded. From 2004 to 2008, the frequency of affected lambs in that flock rose from 0.5% to 3.5% (unpublished data). Interestingly, the appearance and spread of the ovine *CNCA3* mutation in a genetically closed population resembles

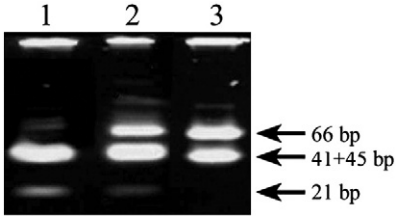

Fig. 2. PCR-RFLP analysis after digestion of the PCR product with *Eco*471 (*Ava*II). Restriction enzyme: Lane 1, homozygous normal-vision lamb; A major band of 41 + 45 bp and a minor band of 21 bp are detected. Lane 2, heterozygous carrier lamb; Major bands of 66 bp and 41 + 45 bp are detected, along with a faint minor band of 21 bp. Lane 3, homozygous day-blind lamb; Two major bands are detected: one of 66 bp and the other of 41 + 45 bp.

that of the *CNGB3* mutation causing achromatopsia among the closed population of the Pingelapese islanders of Micronesia [26].

Following the identification of *CNGA3* as the gene responsible for day blindness, selection against carrying the day-blindness mutation was launched in Improved Awassi flocks carrying the mutation. All males are PCR-RFLP-genotyped (Fig. 2) and rams and ram–lambs that carry the mutation are culled (unpublished results). On the other hand, the finding that similar stop codon mutations cause day blindness in both humans [17] and sheep promotes use of the day-blind sheep as a large animal model for studying and possibly treating human achromatopsia. Indeed, gene therapy has been reported to restore cone function in a mouse model for human achromatopsia with a *CNGA3* mutation [27]. However, it should be noted that some differences may exist between human and ovine photoreceptor CNG ion channel function, as patients homozygous for the stop codon mutation at residue 221 of the human *CNGA3* gene show the absence of photopic ERG [17], while in the day-blind affected sheep, it is impaired, but not completely absent [1].

Relatively extensive non-synonymous polymorphism was found in the present study in the *CNGA3* and the *CNGB3* genes, in both conserved and non-conserved amino acid residues (Table 1, Supplementary Table 2). In contrast, no non-synonymous polymorphism was detected in the *GNAT2* gene. Whether this difference is due to the relative tissue specificity of the *GNAT2* expression pattern as compared to expression of the *CNGA3* gene, where transcripts have been detected in various non-retinal tissues in humans [28] and other species [29], requires further investigation.

In conclusion, our results show that a stop codon mutation in the ovine *CNGA3* gene is associated with day blindness in Improved Awassi sheep. This finding has paved the way for molecular-based selection against carrying the mutation in commercial sheep flocks. As gene therapy is becoming a real possibility for treating retinal genetic illnesses [30–32], sheep homozygous for the *CNGA3* mutation may serve as an animal model for studying and treating this illness in humans.

Materials and methods

Experimental protocols were approved by the Volcani Center's Animal Care Committee (authorization no. 16208). Four normal Improved Awassi lambs and four impaired-vision Improved Awassi lambs were from the Ein Harod Improved Awassi dairy flock, Israel [33]. Based on the breeding records of that flock, each Improved Awassi lambs (affected and non-affected) was sired by a different ram. Day blind lambs were assigned based on their inability to orientate during day time and their fail to manifest menace response [1]. Non-affected American Suffolk crossbred lambs ($n=2$) were progeny of American Suffolk rams kept at the Insemination Center at Illania, Israel. Non-affected Local Awassi lambs ($n=2$) were from an unknown Bedouin flock from the south of Israel.

Retinas from the four affected and the eight non-affected lambs were dissected from eyes collected at the abattoir a few minutes after slaughter, immediately immersed in liquid nitrogen and kept at −80 °C until RNA extraction. Total RNA extraction was carried out utilizing Tri-reagent (Sigma-Aldrich, Israel), according to manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed in a final volume of 20 μl containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 5 mM MgCl2, 10 mM dithiothreitol, 0.5 mM of each deoxynucleotide, 0.5 μg oligo-dT/hexamer primers (Promega, Israel), and 1 U avian myeloblastosis virus reverse transcriptase (Promega, Israel). The reverse-transcription reaction temperatures were 42 °C for 1 h and 95 °C for 10 min.

To search for possible causative mutations within the ovine *CNGA3*, *CNGB3* and *GNAT2* genes, fragments covering the complete (*CNGA3*, *CNGB3*) or partial (*GNAT2*) coding regions of those genes were amplified from the cDNA samples obtained from the four affected and the

eight non-affected lambs. Primers (Supplementary Table 1) were designed using Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), based on the orthologous bovine genes (Supplementary Table 1). PCRs were carried out on a Thermal Cycler 2720 (Applied Biosystems, Foster City, CA, USA) in a total volume of 40 μl, containing cDNA template (0.5–1.0 μg/reaction), 8 pmol of each primer, 2 units of Taq DNA polymerase (Fermentas, Burlington, Canada), 1×Taq Buffer with $(NH_4)_2SO_4$ (Fermentas, Burlington, Canada), final concentration of 0.2 mM each dNTP, 2 mM $MgCl_2$ and 200 ng BSA. Amplification parameters for each PCR are presented in Supplementary Table 1.

PCR products were size-separated by electrophoresis on 1.2% agarose gels stained with ethidium bromide and were extracted from the gel using QIAquick gel extraction kit (Qiagen, Hilden, Germany) according to manufacturer's instructions, and sequenced from both directions with the ABI BigDye Terminator Sequencing Kit (Applied Biosystems) on an ABI 3730. Sequences were assembled and compared using the GAP4 program [34]. Protein sequences were aligned with ClustalW software (http://clustalw.genome.jp), using default settings and the Gonnet weight matrix. The graphical image of the multiple alignments was created with BoxShade (http://www.ch.embnet.org/software/BOX_form.html).

Population screening for the day-blindness mutation in *CNGA3* was carried out using the PCR-RFLP method. Genomic DNA was extracted from blood samples using standard DNA extraction methods. PCR products of 107 bp were amplified using primers designed according to the ovine gene sequence (GenBank accession no. FN377574): sense primer 5′-GTTTCGATGAAGCTCCAGTCC-3′; antisense primer 5′-CTCACCTGGTCCGGGCTC-3′. PCR products were digested with *Eco*471 (*Ava*II) restriction enzyme, and size-separated and visualized by 3.5% Metaphor agarose gel electrophoresis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2009.10.003.

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