Genomics 97 (2011) 121–129

Contents lists available at ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

Mapping of equine cerebellar abiotrophy to ECA2 and identification of a potential causative mutation affecting expression of *MUTYH*☆

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ARTICLE INFO

Article history:
Received 7 August 2010
Accepted 20 November 2010
Available online 30 November 2010

Keywords:
Abiotrophy
Neurodegenerative
Ataxia
Cerebellum
Purkinje
Horse

ABSTRACT

Equine Cerebellar Abiotrophy (CA) is a neurological disease found in Arabian horses. CA is characterized by post-natal degeneration of the Purkinje cells of the cerebellum. Signs of CA include ataxia, head tremors, and a lack of balance equilibrium. We have discovered a linkage of the CA phenotype to a microsatellite marker on ECA2 and identified a region of conserved homozygosity spanning approximately 142 kb. Complete sequencing of the four genes in this region identified one SNP found only in Arabian horses, located in exon 4 of TOE1 and approximately 1200 base pairs upstream of MUTYH, adjacent to a possible binding site for the transcription factor GATA2. qPCR analysis of cDNA from the cerebella of affected and unaffected horses suggested that MUTYH expression is down-regulated in affected horses. This SNP may therefore have a function effect on TOE1, or a regulatory effect on MUTYH by negatively affecting the binding affinity of GATA2.

1. Introduction

Cerebellar Abiotrophy (CA) is a neurological condition, characterized by a post-natal degeneration of Purkinje cells, which affects a number of animal species. Cerebellar cortical degeneration involving extensive loss of Purkinje cells has been reported in dogs, cattle, cats and chickens. CA has been identified in several breeds of dogs, including Papillons [1], Border Collies [2], English Bulldogs [3], Brittany Spaniels [4], and Staffordshire Terriers [5]. Both Angus [6] and Holstein Friesian cattle [7] have been affected by CA. Cats show variability in the onset of CA; while CA can manifest itself in kittens before a year of age, published case reports indicate that symptoms of CA can appear as late as seven years of age [8,9]. In chickens, CA is known as congenital quiver, although the symptoms and pathological features are the same as in other species [10]. The etiologies of CA in these species are unknown and may differ.

In horses, CA is found almost exclusively in the Arabian breed [11–13]. Symptoms of CA in horses generally appear between six weeks and four months of age and include intention head tremors, ataxia, exaggerated or paddling action of the forelegs, a wide-based stance and a lack of menace response [12,14,15]. Affected horses may startle easily and fall, and are often unable to rise from a reclining position. Because the symptoms of CA are similar to other neurological conditions, correct diagnosis of the disease can be a challenge and is often reached only after other possible conditions have been eliminated [13].

Conclusive diagnosis of the disease can only be made post-mortem by a histopathological exam of the cerebellum. The Purkinje cells are the most prominently and consistently affected component, but the degeneration of these cells leads to a concurrent degeneration of the granular neurons of the cerebellum [11,12,14] and to a disorganization of the molecular and granular layers, with remaining Purkinje cells being small and shrunken [16]. Overall size and thickness of the cerebellum, however, is largely unaffected [14].

We have previously shown that CA in horses is inherited as a recessive trait [17]. Therefore, the mating of two carriers will result in an affected foal in 25% of breedings. While this information is of some use to Arabian breeders in choosing breeding stock, it is only relevant if the animals in question are known to have produced affected foals. Otherwise, the risk of producing an affected foal from a particular mating is still unknown.

Despite the number of species affected by CA, the genes responsible for the disease have yet to be identified. An apoptosis mechanism has been implicated in the loss of Purkinje cells in CA-affected horses [13] but no specific genes, or even genomic regions, have been identified. While CA is not fatal, most owners elect to euthanize affected horses as their lack of coordination makes handling them dangerous for both the horses and handlers. There is thus a considerable need to identify the genetic mutation responsible for CA, so that a molecular diagnostic test can be developed to help breeders avoid producing affected foals.

☆ SNP data from this article were deposited in the GenBank SNP Library dbSNP. Submitted SNP reference numbers are noted in tables detailing these polymorphisms.

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doi:10.1016/j.ygeno.2010.11.006

Table 1
SNPs identified by gene sequencing and used for CA mapping. Locations of SNPs on ECA2, surrounding sequence, and type of SNP with associated residues for exonic SNPs are shown.±

Gene	Location	dbSNP ss #	SNP sequence	Type	Residues
AKR1A1	12866110	252444779	GAGGCTGGGGCTGC [G/A] GCTGGAGGGATC		
DMAP	14144436	252444780	GGGACCGAGAATAT [G/A] TGTCACCTTGAGC		
DMAP	14137370	252444781	CAGGACCTGCAGAA [G/A] CTGATAACAGCG	Synonymous	Lys → Lys
EIF2B3	13452583	252444782	TGTTGTTATTAAATC [T/A] AGAAACAGAACA		
FAAH	12120045	252444783	CTGGACTCAGAGGC [G/A] CTGCTGGCCCTGC	Synonymous	Ala → Ala
IPO13	14370561	252444784	TTCATCCTAGTCAGC [G/C] ATCTGATCCAGGG		
PIK3R3	12442822	252444785	TTGCACCTCTTTTGA [A/G] TTGAATTGCCTGA		
PRNPIP	14048685	252444786	TGATTAGAATAACA [T/G] TTGCTGTCACATCA		
PRNPIP	14112315	252444787	ACTCAGACAGACAC [A/G] TATCCATACTGAT		
SLC6A9	14333337	252444788	ACCAGGGACAGCAT [C/T] ATCATCAGCATCA	Synonymous	Ile → Ile
ZSWIM5	13189648	252444789	CTCTCTGCACCAGCC [C/G] TGGGCCGAGACC		
ZSWIM5	13417395	252444790	ACACGTATTCACCAT [C/T] GCCCGCTACATGG	Synonymous	Ile → Ile

In this study, we mapped CA to the p-arm of ECA2 by performing a whole genome scan on four paternal families of Arabian horses segregating for CA. Further marker development in the identified region, as well as homozygosity analysis in affected foals, refined the CA region to approximately 142 kb. The CA region contains four annotated genes, including *MUTYH* and *TOE1*, both of which are potential candidate genes. Complete sequencing of the genes in this region, including introns and intergenic spaces, identified 22 mutations segregating with CA. One SNP identified was found to be exclusive to the Arabian breed and to be completely concordant with the CA trait, making it an excellent candidate for the CA mutation. This SNP is located in exon 4 of *TOE1* but results in a relatively benign amino acid substitution [18]. However, it is also located approximately 1200 base pairs 5— of the start site of *MUTYH*, which is transcribed from the opposite strand, and is immediately adjacent to a possible binding site for the transcription factor GATA2 [19]. The results of a qPCR analysis on RNA extracted from the cerebella of affected and unaffected horses suggest that *MUTYH* expression is reduced in affected horses. The putative CA SNP may therefore have a regulatory effect on *MUTYH* by affecting the binding affinity of GATA2.

2. Results

2.1. Mapping of CA

Genome scan analyses using CRIPMAP and data from 335 markers showed significant linkage between CA and microsatellite marker TKY615 (GenBank: AB103833.1) on ECA2 (RF = 0, LOD = 5.12). SIBPAL analysis also supported association of TKY615 to CA ($p = 0.01075$). There was no evidence of significant linkage to any other genomic regions. To further refine the map position, genotypes of the CA families were obtained for an additional 25 microsatellites spanning 6 Mb around TKY615. CA showed significant linkage (LOD ≥ 3.0) to 13 of these additional microsatellites, with 11 of these having recombination fractions of zero with CA.

Sequencing of genes considered candidates for CA based on function and/or expression in the cerebellum—*DMAP1*, *PRNPIP*, *IPO13*, *AKR1A1*, *PIK3R3*, *SLC6A9*, *EIF2B3*, *ZSWIM5* and *FAAH*—yielded no causative mutations but led to the discovery of 12 additional SNPs that were also genotyped on the CA families (Table 1). Eight of these SNPs showed significant linkage (LOD ≥ 3.0) to CA, and all but two had recombination fractions of zero with CA. Four markers had LOD scores of 5.12: *TG(15)*, *AC(16)2*, *TG(17)2*, and a SNP in *PRNPIP* (intron 5–6). SNPs segregating completely with the CA phenotype were excluded as being causative by their location within non-coding regions of the gene or by virtue of being synonymous substitutions.

2.2. Allele frequencies and haplotype construction using markers associated with CA

Allele frequencies were calculated for eight selected microsatellite markers in the vicinity of the CA region, including the four microsatellites showing the highest LOD scores. Allele frequencies were obtained for the group of affected horses (n = 23), known carriers (n = 45) and a control group of Arabian horses drawn from the general population (n = 41). All of the 8 markers tested exhibited allele frequencies in the affected population that were significantly different ($p < .001$) from the control population (Table 2).

Haplotypes were constructed for the 83 horses segregating for CA using all 39 genetic markers across 6 Mb of ECA2. A common haplotype was identified in foals affected with CA and known carriers that spanned approximately 3.36 Mb and that corresponded with those microsatellites having the highest LOD scores in the linkage analysis. This further confirmed the linkage analysis results and, with the additional 51 horses genotyped, refined the location of CA. Homozygosity was observed for 75% of affected foals in this region. However, in 87.5% of the affected foals, homozygosity was further restricted to a 2.24 Mb region, 95.8% of affected foals displayed homozygosity in a 550 kb region, and 100% of affected foals displayed homozygosity in a 142 kb region that spanned from intron 3–4 of *TESK2* to a SNP approximately 95 kb distal to *HPDL* (Table 3). Of the 15

Table 2
Allele frequencies for eight microsatellites in the CA region having the highest association with CA (based on LOD scores) in random Arabian horses, known carriers, and horses affected with CA. X represents any allele other than that associated with CA.

	TKY615		AC(16)2		TG(17)2		TG(15)		TC(32)3i		AC(13)		TG(15)2		TG(13)	
	274	X	296	X	123	X	206	X	401	X	234	X	156	X	245	X
Random (n = 41)	.037	.963	.037	.963	.037	.963	.049	.951	.061	.939	.244	.756	.220	.780	.134	.866
Carriers (n = 45)	.522	.478	.522	.478	.522	.478	.522	.478	.522	.478	.533	.467	.511	.489	.511	.489
Affected (n = 24)	.958	.042	.958	.042	.958	.042	.979	.021	.979	.021	.958	.042	.958	.042	.958	.042
χ^2 (df = 1)	105.64		105.64		105.64		106.06		102.57		59		63.2		80.22	
p value	$p < 0.001$		$p < 0.001$		$p < 0.001$		$p < 0.001$		$p < 0.001$		$p < 0.001$		$p < 0.001$		$p < 0.001$	

Table 3
Regions of conserved homozygosity across 18 genetic markers spanning 3.36 Mb on ECA2 in 23 affected Arabian horses. Regions of homozygosity are shown in blue; regions of heterozygosity are shown in pink.

Horse/marker	AT(18)	AC(38)	IP013	481101	DMAP	PRNPIP	TKY615	AC(16)2	TG(17)2	TG(15)	ZSWIM5	CA SNP	TESK2	TC(32)3i	AC(13)	TG(15)2	TG(13)	TG(19)
Genomic location on ECA2	15264935	14559094	14364252	14294111	14150809	1403739	14067934	13691925	13577416	13293497	13189648	13074277	13047413	12307781	12057997	12049739	12029626	11898349
AFF1	172	228	C	C	A	G	274	296	123	206	C	A	A	401	234	156	245	200
AFF2	172	228	C	C	A	G	274	296	123	206	C	A	A	401	234	156	245	200
AFF3	172	228	C	C	A	G	274	296	123	206	C	A	A	401	234	156	245	200
AFF4	172	228	C	C	A	G	274	296	123	206	C	A	A	401	234/236	156/160	243/245	200/204
AFF5	172	228	C	C	A	G	274	296	123	206	C	A	A	401	234	156	245	200
AFF6	172	228	C	C	A	G	274	296	123	206	C	A	A	401	234	156	245	200
AFF7	172	228	C	C	A	G	274	296	123	206	C	A	A	401	234	156	245	200
AFF8	172	228	C	C	A	G	274	296	123	206	C	A	A	401	234	156	245	200
AFF9	172	228	C	C	A	G	274	296	123	206	C	A	A	401	234	156	245	200
AFF10	172	228	C	C	A	G	274	296	123	206	C	A	A	401	234	156	245	200
AFF11	172	228	C	C	A	G	274	296	123	206	C	A	A	401	234	156	245	200
AFF12	172/172	228/232	C/G	C/T	A	G	274	296	123	206	C	A	A	401	234	156	245	200
AFF13	172	228	C	C	A	G	274	296	123	206	C	A	A	401	234	156	245	200
AFF14	172	228	C	C	A	G	274	296	123	206	C	A	A	401	234	156	245	200
AFF15	172	228	C	C	A	G	274	296	123	206	C	A	A	401	234	156	245	200
AFF16	172	228/234	C/G	C/T	G/A	G/A	274/280	288/296	117/123	206	C	A	A	401	234	156	245	200
AFF17	172/190	228/286	C/G	C	A	G	274	296	123	206	C	A	A	401	234	156	245	200
AFF18	172	228	C	C	A	G	274	296	123	206	C	A	A	401	234	156	245	200
AFF19	172	228	C	C	A	G	274	296	123	206	C	A	A	401	234	156	245	200
AFF20	172	228	C	C	A	G	274	296	123	206	C	A	A	401	234	156	245	200
AFF21	172	228	C	C	A	G	274	296	123	206	C	A	A	401	234	156	245	200
AFF22	172	228	C	C	A	G	274/280	296	123	206	C	A	A/G	401/429	234/236	156/162	243/245	170/200
AFF23	172	228	C	C	A	G	274	296	123	206	C	A	A	401	234	156	245	200
AFF24	172	228/286	C/G	C/T	G/A	G/A	274/280	288/296	117/123	198/206	C/G	A	A	401	234	156	245	200

unaffected horses in the CA sample set and the 41 horses from the general Arabian population, only three, or 5.3%, were heterozygous for the extended haplotype spanning the 142 kb region.

2.3. Sequencing of genes in the CA region

The 142 kb region contains four annotated genes in the horse: *TESK2*, *TOE1*, *MUTYH*, and *HPDL*. Together, these genes occupy approximately 46.7 kb of the 142 kb region; the other 95 kb contain no known genes in the horse. (Fig. 1) Complete sequencing was performed on the approximately 49.7 kb, from the SNP in intron 3–4 of *TESK2* that formed the upper boundary of the region to approximately 3000 base pairs beyond the 5′-end of *HPDL*. The sequencing included exons, introns, and intergenic spaces between all four genes. At least one affected Arabian horse from the CA families and one unaffected Arabian horse were sequenced in order to identify polymorphisms between the samples. Mutations were selected for further genotyping if they were not present in the unaffected sample and were homozygous in the affected sample. A total of 22 mutations were identified: 19 SNPs, 1 double nucleotide polymorphism (DNP), one indel, and one insertion (Table 4). Two SNPs were located in exons, 14 SNPs, the DNP, the insertion and the indel were located in introns, and one SNP was found in intergenic space.

The 22 polymorphisms were genotyped on the CA families, and all showed segregation with the CA phenotype. However, when genotyped on the mixed breed samples, 21 polymorphisms were

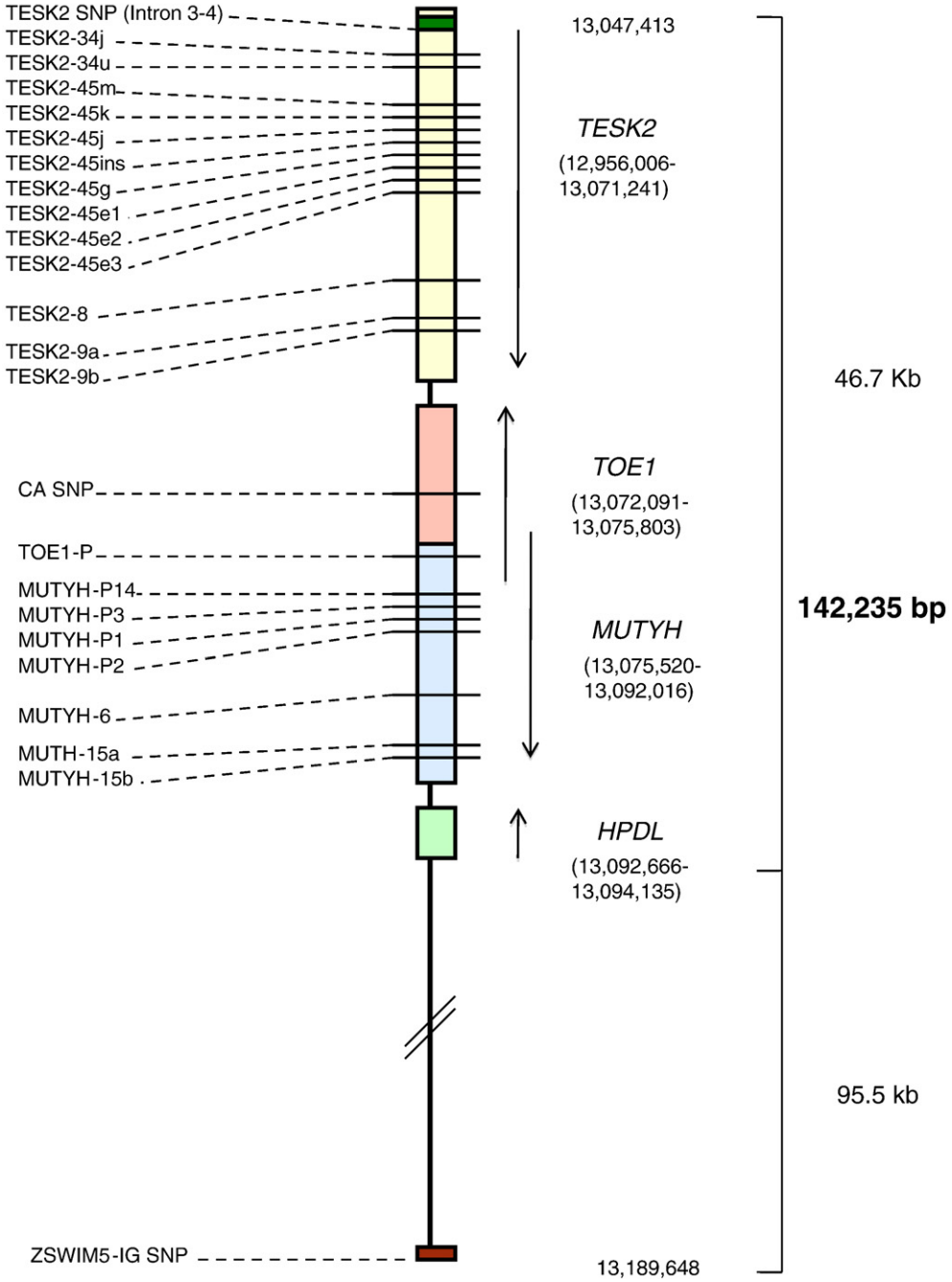

Fig. 1. Region of ECA2 identified as containing the causative CA mutation by haplotype analysis. Boxes indicate annotated genes; arrows indicate direction of transcription. Physical location (by base pair count) is indicated to the right. Relative location of 22 identified mutations in the region is noted on the left.

Table 4
Twenty-two polymorphisms identified in a 142 kb region containing the causative mutation for CA. The type of mutation identified, the nucleic acid change, location (exonic, intronic, or intergenic), and physical base pair position on ECA2 (EquCab2 draft assembly) are noted. For exonic mutations, the amino acid change (position and amino acid substitution) are indicated.

Polymorphism	Type	Base change	Source	AA change?	Location	dbSNP ss #
TESK2-34j	SNP	C –> T	Intron		13050853	252444759
TESK2-34u	SNP	C –> T	Intron		13058558	252444760
TESK2-45m	SNP	T –> C	Intron		13059524	252444761
TESK2-45k	DNP	GC –> TA	Intron		13060692	252444762
TESK2-45j	SNP	T –> C	Intron		13061799	252444763
TESK2-45ins	Insertion	T	Intron		13063606	252444791
TESK2-45g	SNP	T –> C	Intron		13063718	252444764
TESK2-45e1	Indel	delGAGGinsT	Intron		13065301	252444792
TESK2-45e2	SNP	A –> G	Intron		13065644	252444765
TESK2-45e3	SNP	T –> G	Intron		13065756	252444766
TESK2-8	SNP	G –> A	Intron		13069693	252444767
TESK2-9a	SNP	A –> G	Intron		13070359	252444768
TESK2-9b	SNP	C –> T	Intron		13070428	252444769
CA SNP	SNP	G –> A	Exon	95:Arg –> His	13074277	252444770
TOE1-P	SNP	T –> G	Intergenic		13075982	252444771
MUTYH-P14	SNP	C –> T	Intron		13078958	252444772
MUTYH-P3	SNP	T –> G	Intron		13085553	252444773
MUTYH-P1	SNP	C –> A	Intron		13087492	252444774
MUTYH-P2	SNP	C –> T	Intron		13087753	252444775
MUTYH-6	SNP	C –> T	Exon	129:Pro –> Leu	13089117	252444776
MUTYH-15a	SNP	T –> C	Intron		13091070	252444777
MUTYH-15b	SNP	G –> A	Intron		13091320	252444778

found in at least three breeds other than Arabians and only one, identified as ECA2:13074277, was absent in all samples. (Table S1) This SNP (G –>A) is located in exon 4 of *TOE1*, and results in a non-synonymous substitution of histidine for arginine at this position (R95H). As arginine and histidine are both small, polar, positively-charged amino acids [18], it is unclear if this substitution would lead to a significant alteration in protein structure or function. However, analysis of this amino acid change using the program Polyphen-2 [20] results in a prediction that this mutation is "probably damaging" (score: 1.000), due in part to a high degree of evolutionary conservation at this site. Interestingly, the 5′-end of *TOE1* overlaps with the 5′-end of *MUTYH*, leading to transcription of these two genes from opposite strands of partially overlapping DNA sequences [21]. The sequence preceding the ECA2:13074277 SNP (5′-GAGGATAGAA-3′) is an 88.1% match for the GATA-2 site (http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html), thus presenting the alternative possibility that the SNP could have a regulatory function for *MUTYH*.

2.4. Quantification of MUTYH expression

To investigate the possibility that regulation of *MUTYH* expression is affected by the ECA2:13074277 SNP, we performed a qPCR on cDNA generated from cerebellar RNA from affected and unaffected (control) horses. The expression of *MUTYH* showed considerable variability between affected and unaffected horses and also between horses of different ages (Table 5). *MUTYH* expression increased with the age of the animal in control and CA-affected horses but affected horses had comparatively lower levels of expression than the controls. Affected horses showed a 2.2-fold increase in *MUTYH* expression between the 5-month-old and the 9-month-old horses, and another 17-fold increase in expression between the 9-month-old and the 6-year-old horses. In contrast, the control horses showed 67.25-fold increase between the 2-3-month-old and the 23.5-month-old horses and another 1.16-fold increase between the 23.5-month-old and the 4-year-old horse.

3. Discussion

Cerebellar abiotrophy is a disease of increasing concern to Arabian horse owners. Once thought to be a problem of particular families or specific lines of breeding, affected CA foals have been identified that are of Polish, Egyptian, and Spanish types. While some interbreeding between these lines is common, analysis of the pedigrees of affected foals often shows common ancestry more than five generations removed. Therefore, it has been unclear whether the CA mutation arose prior to the geographic segregation and selection of these various strains, or if perhaps separate CA mutations occurred in different lineages.

Linkage mapping placed CA on the p-arm of ECA2. Further marker development and homozygosity analysis in this region has refined the map location of CA to ECA2: 13,047,413–13,189,648, which spans approximately 142 kb. We identified a haplotype in this region that segregates closely with the CA phenotype. The conservation of this haplotype among all affected horses strongly suggests that CA arose as a single mutation prior to the segregation of the Arabian breed into more closed lineages. This conclusion is further supported by the specificity of the CA haplotype; allele frequencies between affected and control populations are significantly different for each of the alleles associated with CA, and only three of the unaffected horses (5.3%) that we tested were heterozygous for this haplotype. Given the number of Arabian lineages producing affected foals, it is entirely possible that these horses are indeed carriers for CA.

The region of ECA2 identified in this study has four annotated genes in the horse: *HPDL*, *MUTYH*, *TOE1*, and *TESK2*, which span about

Table 5
Levels of *MUTYH* and reference gene *SYP* observed in cDNA samples isolated from the cerebella of three control and three affected horses. Quantities shown are averaged from all samples for which qPCR analysis was performed. The ratio of *MUTYH* expression to *SYP* expression is shown.

Sample	Age	Quantity mean (*MUTYH*)	Quantity mean (*SYP*)	*MUTYH:SYP*	Sample	Age	Quantity mean (*MUTYH*)	Quantity mean (*SYP*)	*MUTYH:SYP*
CONT-1	2–3 mo.	3.798 ± 2.23	522.629 ± 24.77	0.00727	AFF-1	5 mo.	4.659 ± 1.02	114.485 ± 32.47	0.0407
CONT-1	23.5 mo.	255.423 ± 15.68	1256.958 ± 46.85	0.20321	AFF-2	9 mo.	10.224 ± 3.2	1263.639 ± 13.51	0.00809
CONT-3	4 yr.	284.822 ± 20.3	1985.516 ± 37.65	0.14345	AFF-3	6 yr.	173.138 ± 13.74	1860.81 ± 54.24	0.09304

46.7 kb of the 142 kb region. Complete sequencing of the approximately 49.7 kb that contain these four genes revealed 22 mutations that segregated with the CA trait. Of these, only one SNP, ECA2:13074277, was shown to be present only in the Arabian breed; all other mutations were found in at least four non-Arabian breeds, and non-Arabian horses homozygous for each of these mutations were identified. As CA has only been identified in horses with Arabian ancestry, the occurrence of homozygotes for each of these SNPs effectively excludes them as candidate mutations for CA. The ECA2:13074277 SNP is therefore the best and only candidate mutation identified in our study.

Two of the four genes in the CA region, *MUTYH* and *TOE1*, are expressed in the brain, although *TOE1* is not expressed at high levels in the cerebellum (BioGPS database, http://biogps.gnf.org/#goto=genereport&id=114034). The ECA2:13074277 SNP is located in exon 4 of *TOE1*, but the amino acid change produced (Arg→His) is not generally considered to be significant [18]. It is therefore unclear if this SNP affects the functionality of *TOE1*, although prediction software suggests that this mutation has a high probability of negatively affecting functionality of the protein. *TOE1*, or *Target of EGR1*, is a gene target of *Early Growth Response 1 (EGR1)*, which functions as a transcriptional regulator. *TOE1* is a 510-amino acid protein that mediates the inhibitory growth effect of *EGR1*, which directly binds to the promoter region of *TOE1* and activates its expression [22]. *TOE1* plays a role in cell cycle regulation by inducing the expression of TGFβ and p21, and may also modify the activity of p53 [22,23].

The second possibility is that the ECA2:13074277 SNP affects the regulation of the *MUTYH* gene. This SNP lies immediately adjacent to the 3′-end of a possible GATA-2 binding site. *MUTYH*, or *MutY Homolog*, is a DNA glycosylase that removes adenine residues that are incorrectly added opposite of 8-oxo guanine, a chemical decay product of oxidative damage [24]. *MUTYH* expression is found in the hippocampal, cortical and cerebellar regions of the brain, and is particularly robust in the cerebellum [25]. There is abundant *MUTYH* expression in Purkinje neurons, with moderate expression in the cytoplasm of granular cells [26].

Four isoforms of MUTYH exist in the brain [26]. Among these, only one is expressed during embryonic development in the rat brain [27]. This isoform is present at a high level at E14, but gradually declines in the embryo and neonate, while the other three isoforms appear and gradually increase throughout post-natal development, reaching their highest levels in the adult brain [27]. At the E14 stage, MUTYH is present primarily in the nuclear fraction of Purkinje cells [27], while it is present primarily in the mitochondrial fraction of adult Purkinje cells [26,27]. The appearance of the three additional MUTYH isoforms in the adult brain thus coincides with the end of proliferative neuron growth and the maturation of mitochondrial function [27]. This evidence suggests that *MUTYH* is involved in post-replicative DNA repair in the nuclei of rapidly proliferating Purkinje cells, while in post-mitotic Purkinje cells it is involved in the repair of oxidative damage to the mitochondrial genomes.

An analysis of the sequence immediately surrounding the ECA2:13074277 SNP revealed that this SNP lies immediately 3′ adjacent to a possible binding site for the transcription factor GATA-2. GATA-2 is a transcription factor required for hematopoiesis and is dynamically expressed in the central nervous system, giving rise to hematopoietic progenitors, enveloping layer (EVL) cells and neurons [28]. Each member of the GATA family is characterized by its ability to bind to cis-acting DNA elements with the consensus core sequence WGATAR. To data, six members of the GATA family have been identified in vertebrates [29].

In the early phases of neurogenesis, a spot-like pattern of GATA-2 expression is observed in the ventral parts of the hindbrain [30]. Expression moves slowly into the hindbrain and ventral midbrain regions, and GATA-2 expressing cells are found in a layer inserted between the ventricular zone and the layer of maturing neurons. Later in development, the proliferative zone narrows markedly as neurogenic cell proliferation comes to an end. GATA-2 expressing cells accumulate in the border of the proliferative zone, suggesting a direct link between neuron formation and GATA-2 expression. This also supports the view that GATA-2 is expressed by mid- and hind-brain precursors in the immediate post-mitotic stage.

qPCR analyses in CA affected and unaffected horses, showed that expression of *MUTYH* increased with the age of the horse. This is not surprising, as it has been shown that *MUTYH* is one of a number of genes that is upregulated in aged (24 months) versus young (8 months) rats in the basal forebrain, which is selectively vulnerable in human brain diseases [31]. However, we observed that the level of upregulation was much greater in the control horses when compared with the affected horses. While in the control horses there was a 75-fold increase from the 2–3-month-old sample to the 4-year-old sample, affected horses showed only a 37.16-fold increase overall from the 5-month-old sample to the 6-year-old sample. This suggests that *MUTYH* expression is negatively altered in horses affected with CA. Given the proximity of the candidate SNP to the possible GATA-2 binding site, we hypothesize that it may impair the enhancer, thus leading to a lower than required level of expression of *MUTYH*. Lower levels of MUTYH could result in reduced protection of either nuclear or mitochondrial DNA from oxidative damage in Purkinje cells and apoptosis. The level of MUTYH reduction could be variable enough to account for variation in age of onset of the disease and the level of debilitation. Additional control and affected samples of various ages will be needed in order to fully investigate this hypothesis. Further studies should also include evaluating the binding capacity of the sequence encompassing ECA2:13074277 for the GATA-2 protein in affected and unaffected horses.

The identification of a conserved haplotype, including the putative CA mutation, has allowed testing of suspected affected foals and potential breeding animals for the presence of the disease. The haplotype has been used to identify carrier animals and to diagnose several foals for which CA was later confirmed by histopathology post-euthanasia. To date, approximately 4200 horses have been tested for the presence of CA. A carrier rate of 19.7% has been observed, although because of the preferential selection by owners for testing animals from particular lineages, this may overestimate the carrier rate for the entire population. Three of the 41 horses from the general Arabian population tested were heterozygous for the extended haplotype spanning the 142 kb region, and given the carrier rate among Arabians, it is very possible that these three animals are carriers of CA. Among tested animals, 1.4% were determined to be affected by CA (two copies of the CA haplotype and the putative CA mutation). Most of these animals display the characteristic signs of CA. However, to date nine horses have been identified as genotypically affected with CA that do not show outward signs of the disease based on owners' observation.

The occurrence of homozygosity of the CA haplotype and the putative CA mutation in phenotypically unaffected horses raises the question of variable expression of the disease. Age of onset and severity of signs is variable among horses affected with CA; some present signs of the disease almost immediately after birth, while others are several months of age when signs are first observed. Additionally, some affected horses show severe expression of the disease, with almost constant head tremors and a high degree of ataxia, making them extremely difficult to handle. Other affected horses are very high functioning, with only occasional missteps in gait and a very mild head tremor during intentional movement. In some instances, “mildly affected” horses will show increasing severity of their signs into adulthood, but this is not always the case. The range of expression therefore raises the possibility that, in some cases, horses could have the affected genotype and show no outward signs. If lowered *MUTYH* expression is the cause of CA in horses, the variable

expression of the disease could be explained by differing amounts of oxidative damage to nuclear or mitochondrial DNA suffered by individual horses. It is also possible that a small subset of the Arabian population carries a suppressor mutation that can compensate for the effects of the disease.

The cost of an affected foal to an Arabian breeder in terms of lost revenue can be significant, as many of these foals come from valuable breeding stock. As well, the birth of a CA foal causes emotional distress to the owners. Affected foals are often euthanized, as they are never able to perform in hand or be ridden safely. Development of a genetic test to screen for the specific CA mutation will allow diagnosis of CA carriers prior to breeding and it will provide data to estimate the carrier frequency in the Arabian breed. Pedigree analyses of the horses in the CA families indicate that the mutation was present in related horses that were breeding in the 1930s and that are common ancestors in many Arabian bloodlines. Therefore, the frequency of carriers in the breed may not be negligible. Genetic screening of breeding stock will allow breeders to avoid mating two carriers together, thus eliminating the chances of producing an affected foal.

4. Materials and methods

4.1. Mapping resource

DNA samples were prepared from blood or hair root samples collected from 32 horses in four paternal families that comprised 16 known carriers, 11 affected and five unaffected horses. Familial structure of these four families is shown in Fig. S1. Based on pedigree records, three of the four stallions shared common ancestors within four generations; the fourth had a common ancestor nine generations removed. The cerebellum from one affected foal in each family was harvested post-mortem, fixed in formalin and submitted to the Anatomic Pathology Service, Veterinary Medicine Teaching Hospital, University of California, Davis for confirmation of CA diagnosis. Genomic DNA was extracted from whole blood using a salt extraction method [32]. Extraction of DNA from hair roots was performed according to standard protocols at the Veterinary Genetics Laboratory (VGL) [33].

4.2. PCR amplification and fragment size analysis

Amplification of microsatellites and SNP markers by PCR was performed in 12.5 μL total volume reactions containing 2 μL of DNA extract, 1× PCR buffer (Applied Biosystems, Foster City, CA, USA), 2.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 U Taq DNA Polymerase, and 3.5 μL of primer mix at optimized concentrations. Annealing temperatures were also optimized for each pair of primers, and varied from 56 °C to 60 °C. PCR cycling conditions were as follows: A hot start of 90 °C for 5 min was performed, followed by four cycles of 94 °C for 1 min, optimized annealing temperature for 30 s and 72 °C for 30 s, 25 cycles of 94 °C for 45 s, optimized annealing temperature for 30 s and 72 °C for 30 s, and a final extension of 72 °C for 30 min. PCR products were labeled using fluorescent M13 tails and separated by capillary electrophoresis on ABI 3730 DNA Sequencers (Applied Biosystems, Foster City, CA, USA). STRand software (http://www.vgl.ucdavis.edu/informatics/strand.php) was used to determine allele fragment lengths.

4.3. Linkage analysis

A whole genome scan was conducted on the four paternal families segregating for CA using 335 microsatellites that spanned all 31 horse autosomes. These microsatellites were obtained from published reports. Co-segregation analyses were performed with the TWOPOINT option of CRI-MAP [34] between each informative marker and CA status to identify linked markers. Genotype data for ECA2 markers and CA were also analyzed with linkage software SIBPAL from the S.A.G.E. package. (SAGE, Department of Epidemiology and Biostatistics, Case Western Reserve University, Cleveland, OH, USA) SIBPAL calculates the excess allele sharing by comparison with the null hypothesis under no linkage by *t* statistics.

4.4. Marker development

BLAST comparisons of TKY615 with ECA2 genomic sequence (http://genome.ucsc.edu, version 2) were used to identify an additional 25 microsatellites spanning approximately 6 Mb around TKY615. Twelve SNPs were identified by direct sequencing of nine potential candidate genes using horse-specific primers developed from genomic sequence. One additional SNP in the region of interest was identified from SNPs available on the Illumina Equine SNP50 Bead Chip. Allele-specific primers that differed in size by 2–3 base pairs as well as fluorescence color were designed using Netprimer (http://www.premierbiosoft.com/netprimer/) to genotype the mapping families for identified SNP markers. Direct sequencing was performed using the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. Total reaction volume for cycle sequencing was 10 μL, including 2 μL of PCR product and 0.5 μL each of 20 μM forward or reverse primers.

4.5. Haplotype analysis

Association of marker alleles with the CA phenotype was performed on 83 Arabian horses segregating for CA as well as a control group of 41 Arabian horses drawn from the general population. The 83 horses segregating for CA consisted of 23 affected horses, 45 carriers, and 15 unaffected horses. Haplotypes and allele frequencies were obtained from genotypes collected for all horses at each of the 39 loci. Allele frequencies were calculated by direct count and haplotyping was done by hand. Chi-squares and associated p values for the probability of allele frequencies in the CA population were calculated using a 2×2 contingency table and one degree of freedom. A *p* value was assigned based on the null hypothesis that the alleles of each marker were randomly distributed throughout both populations.

4.6. Primer development and PCR of genes in the CA region

At least one affected and one unaffected horse were sequenced for the 49.7 kb region identified as containing the CA mutation. Horse-specific primers designed to cover the exons and introns of genes within the genomic region of interest were developed from the equine reference genome (available at http://www.genome.ucsc.edu/). Amplification of genomic regions by PCR was performed in 25 μL total volume reactions containing 2–4 μL of DNA extract, 1× PCR buffer (Applied Biosystems, Foster City, CA, USA), 2.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 U Taq DNA Polymerase, and 7.0 μL of primer mix at optimized concentrations. Annealing temperatures were optimized for all primer pairs. The cycling program used for PCR was identical to that described in subsection 4.2. PCR products were electrophoresed on 1.5% agarose gels to identify correct band sizes, and optimized PCR products were purified for sequencing using either Sephadex gel filtration (Edge Biosystems, Gaithersburg, MD, USA) or gel extraction with resin filtration (Invitrogen, Carlsbad, CA, USA).

4.7. Sequencing of PCR products

Sequencing was performed using the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Total reaction volume was 10 μL, including 2 μL of PCR product and 0.5 μL of 20 μM forward or reverse primers. Separation of sequencing products

was performed by capillary electrophoresis on ABI 3730 DNA Sequencers (Applied Biosystems, Foster City, CA, USA). Analysis of sequences was performed using Lasergene 6 SeqMan software (DNASTAR, Madison, WI, USA). The sequence of affected horses was compared with that of the sequenced unaffected horses and the equine reference genome (available at http://www.genome.ucsc.edu/). Mutations that displayed homozygosity in the affected horses but were absent in unaffected horses were selected for further study.

4.8. Genotyping of SNPs

A total of 22 mutations were identified across all genes in the CA region. Allele-specific primers that differed in size by 2–3 base pairs as well as fluorescence color were designed to genotype the mapping families for each SNP. SNPs were genotyped on the CA families to validate segregation with the trait and on 140 horses representing 10 breeds: Standardbreds, Andalusians, Thoroughbreds, Miniature Horses, Morgans, Hackneys, Hanoverians, Norwegian Fjords, Percherons, Quarter Horses, and Dutch Warmbloods.

4.9. Isolation of total RNA and generation of complete cDNA

Three unaffected and three affected animals were used for quantification of gene expression. The control (unaffected) horses included a 2–3-month-old Paso Fino foal, a 23.5-month-old Thoroughbred horse, and a 4-year-old Thoroughbred horse. The absence of CA was confirmed histopathologically by the Pathology service at the UC Davis School of Veterinary Medicine. The affected horses included a 5-month-old Arabian foal, a 9-month-old Arabian foal, and a 6-year-old Arabian gelding. Confirmatory CA diagnosis for the three affected horses was done by histopathological examination, the first at the School of Veterinary Medicine, University of Pennsylvania and the latter two at the UC Davis School of Veterinary Medicine. Cerebellar tissues were collected immediately post-mortem. Briefly, the cerebellum of each horse was removed and cut sagittally through the vermis. Tissue samples were then taken from the midline of the cerebellum at the junction of the molecular and granular layers to ensure recovery of Purkinje cells. Total RNA was isolated from the cerebellar samples of each animal using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) per manufacturer's instructions. RNA was quantified by spectrophotometry on a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and quality was determined by loading 1 μg of RNA on a 1% MOPS/formaldehyde agarose gel. Samples were first DNase-treated to remove any residual DNA by heating for 10 min at 65 °C. For complete cDNA synthesis, 1 μg of RNA was combined with 1 μL of an $OligoDT_{(18)}$ primer, 1 μL 10 mM dNTPs, DNaseI, and DEPC dH_2O to 13 μL. Samples were heated for 5 min at 65 °C. Following this step, a mastermix of 4 μL 5× RT Buffer (Invitrogen, Carlsbad, CA, USA), 1 μL 0.1 M DTT, 1 μL RNasin, and 1 μL SuperScriptII reverse transcriptase (Invitrogen, Carlsbad, CA, USA) was then added. cDNA synthesis was achieved by heating at 25 °C for 5 min, followed by 40 °C for 1 h and then heating to 70 °C for 15 min to destroy any remaining enzyme. All resulting cDNA samples were stored at −70 °C until used. Total RNA was successfully retrieved from 2 samples of the 2–3 month-old control horse, 1 sample of the 23.5-month-old control horse, 2 samples of the 4-year-old control horse, 1 sample of the 5-month-old affected horse, 3 samples of the 9-month-old affected horse, and 2 samples of the 6-year-old affected horse, for a total of 8 RNA samples.

4.10. qPCR

All qPCR reactions were run in 96-well Microamp Fast Optical well plates (Applied Biosystems, Foster City, CA, USA) on an ABI 7500 fast thermocycler (Applied Biosystems, Foster City, CA, USA). *Synaptophysin* (*SYP*) was used as a reference gene, because of its stability of expression in brain tissue and its specificity of expression in neurons [35]. All 9 samples were run in triplicate on both *MUTYH* and *SYP* except for the 5-month-old affected horse, for which four reactions were performed, and the 23.5-month-old control horse, for which six reactions were performed. Because only one cDNA sample was retrieved from each of these horses, additional reactions were performed to increase the number of data points. Each reaction contained 10 μl of Fast SYBR Green PCR mastermix, *SYP*- or *MUTYH*-specific forward and reverse primers at optimized concentrations, 50 ng of cDNA and dH_2O to a total sample volume of 20 μl. Standard curves for each gene were constructed using cDNA template consisting of pooled cDNA from different samples at concentrations of 100, 50, 25, 12.5, 6.25, 3.125, and 0.0 ng/reaction. Both genes were amplified on the same plate with primers *MUTYH*-forward: 5′-TACGACAGAGAGAAGCGGGA-3′ and *MUTYH*-reverse: 5′-AGAACACAGGTGGCAGAGCA-3′, *SYP*-forward: 5′-GGCACTACCAAAGTCTTCCTG-3′ and *SYP*-reverse: 5′-CCGATGAACTAACAAGCCACA-3′.

Each 96-well plate was run using a 3-stage thermocycling program. Stage 1 consisted of a 1 min hot start at 95 °C. Amplification was carried out in stage 2 and consisted of 3 steps: denaturation for 15 s at 94 °C, annealing for 30 s at 60 °C, and product extension for 30 s at 72 °C. Fluorescence data was collected on the annealing step. Stage 2 was repeated for a total of 40 cycles. In stage 3, dissociation was measured in 3 steps: 95 °C for 15 s, 60 °C for 1 min, and 95 °C for fifteen seconds to create a melt curve. Data from the qPCR reactions were analyzed using ABI 7500 Software v2.0.1 (Applied Biosystems, Foster City, CA). The identified quantities of gene expression, measured in ng, were averaged among all samples for each horse.

Supplementary materials related to this article can be found online at doi:10.1016/j.ygeno.2010.11.006.

Acknowledgments

The authors wish to gratefully acknowledge Dr. Jim Mickelson at the University of Minnesota for his assistance in acquiring the 2-month-old control cerebellum sample, Dr. James MacLeod at the University of Kentucky for providing the 23.5-month-old control cerebellum sample, Mr. Lee Millon at the Veterinary Genetics Laboratory, University of California, Davis for his assistance in genotyping genetic markers, and Ms. Christina Lindquist at the Veterinary Genetics Laboratory for her assistance with qPCR data analysis. We also express deep appreciation to the many Arabian owners and breeders who generously provided DNA samples for this research.

Role of the funding source: This research was supported in part by a generous gift from the Arabian Horse Foundation. The funding source had no involvement in study design, the collection, analysis or interpretation of data, writing the report, or the decision to submit the paper for publication.

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