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Genetic risk factors for insidious equine recurrent uveitis in Appaloosa horses

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Summary

Appaloosa horses are predisposed to equine recurrent uveitis (ERU), an immune-mediated disease characterized by recurring inflammation of the uveal tract in the eye, which is the leading cause of blindness in horses. Nine genetic markers from the ECA1 region responsible for the spotted coat color of Appaloosa horses, and 13 microsatellites spanning the equine major histocompatibility complex (ELA) on ECA20, were evaluated for association with ERU in a group of 53 Appaloosa ERU cases and 43 healthy Appaloosa controls. Three markers were significantly associated (corrected *P*-value <0.05): a SNP within intron 11 of the *TRPM1* gene on ECA1, an ELA class I microsatellite located near the boundary of the ELA class III and class II regions and an ELA class II microsatellite located in intron 1 of the *DRA* gene. Association between these three genetic markers and the ERU phenotype was confirmed in a second population of 24 insidious ERU Appaloosa cases and 16 Appaloosa controls. The relative odds of being an ERU case for each allele of these three markers were estimated by fitting a logistic mixed model with each of the associated markers independently and with all three markers simultaneously. The risk model using these markers classified ~80% of ERU cases and 75% of controls in the second population as moderate or high risk, and low risk respectively. Future studies to refine the associations at ECA1 and ELA loci and identify functional variants could uncover alleles conferring susceptibility to ERU in Appaloosa horses.

Keywords association analysis, ERU, eye disorder, major histocompatibility complex, MHC

Introduction

Equine recurrent uveitis (ERU), the leading cause of blindness in horses, is characterized by recurring inflammation of the uveal tract in the eye (Dwyer *et al.* 1995) and infiltration of $CD4^+$ T cells (Gilger *et al.* 1999; Deeg *et al.* 2001). These helper T cells have been shown to be responsive to multiple retinal autoantigens (e.g., Deeg *et al.* 2006a,b), suggesting that ERU is the result of an autoimmune reaction against retinal proteins (Deeg *et al.* 2007a).

It is unclear whether the pathological autoimmune response in ERU is triggered by a cross-reactive microbial antigen or an autoantigen derived from intraocular tissue itself (Deeg *et al.* 2001). The argument for the former is supported by a strong correlation between *Leptospira* infection and ERU (Wollanke *et al.* 2001) as well as evidence that *Leptospira* organisms trigger cross-reactive immunity against equine ocular antigens (Dwyer *et al.* 1995; Lucchesi & Parma 1999; Pearce *et al.* 2007). The cross-reactive immunity between *Leptospira* and equine ocular antigens (Dwyer *et al.* 1995; Lucchesi & Parma 1999; Pearce *et al.* 2007) is consistent with *Leptospira* infection leading to autoimmunity

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when autoantigens are presented by class II molecules to $CD4^+$ T cells in the uveal tract of the eye. In one study, more than 90% of vitreal samples from ERU-affected eyes were positive for *Leptospira* DNA and anti-*Leptospira* antibodies (Brandes *et al.* 2007); however, other studies have failed to identify *Leptospira* DNA and anti-*Leptospira* antibodies in horses with uveitis (Gilger *et al.* 2008).

Alternate immune-mediated mechanisms in ERU also have been suggested. A study in German Warmblood horses demonstrated an association between ERU and equine MHC (ELA) class I serological haplotypes; at least one copy of the ELA-A9 haplotype appeared in 13 of 32 ERU cases and in none of the 37 controls, implicating self-/non-self-recognition mediated by class I antigen-presenting cells in ERU (Deeg *et al.* 2004). Another study also implicated the innate immune response in ERU, where complement component C3 was found to be up-regulated in the ERU retina and an activated complement system was identified in the sera of ERU cases (Zipplies *et al.* 2009). Thus, although the pathophysiology of ERU is not completely understood, it is widely considered to be a complex, immune-mediated disease, with development likely influenced by the initial cause of uveitis, the environment and the genetic makeup of the horse (Gilger & Deeg 2011).

Insidious ERU is a clinical subclassification of ERU that is commonly seen in Appaloosa horses and is distinguished by a persistent low-grade intraocular inflammation with a gradual and cumulative destructive effect, rather than outwardly painful episodes of uveitis (Gilger & Deeg 2011). Examination of an eye affected by insidious ERU often reveals changes that are diagnostic of chronic inflammation, and more often than not, cases of insidious ERU are bilateral, although the degree of severity may differ between the two eyes (Gilger & Deeg 2011). Although uveitis occurs in all horse breeds, Appaloosa horses are eight times more likely to develop uveitis, frequently have insidious ERU and are nearly four times more likely to develop blindness compared to other breeds (Dwyer *et al.* 1995). Being an Appaloosa is a risk factor for ERU (Angelos *et al.* 1988), thus suggesting a shared underlying genetic basis for susceptibility to ERU in this breed.

Appaloosa horses are a breed that has been selected for an allele at the Leopard complex spotting locus (*LP*). Most horses within the breed are homozygous or heterozygous for the incompletely dominant *LP* white-spotting allele, as opposed to the *lp* allele for the absence of spotting. The effect of a single copy of the *LP* allele is to produce a white pattern centered over the hips that can extend over the majority of the body and can have oval pigmented spots (Sponenberg 2009). *LP* has been mapped to a 6-cM region on equine chromosome 1 (ECA1) (Terry *et al.* 2004), and *transient receptor potential cation channel, subfamily M, member 1* (*TRPM1*) has been identified as a positional candidate gene for *LP* (Bellone *et al.* 2008, 2010a,b). *TRPM1* expression is decreased in the retina and skin of Appaloosa horses, and the mechanism for this reduced expression is thought to be caused by premature polyadenylation resulting from an long terminal repeat insertion in intron 1 of LP horses (Bellone *et al.* 2008, 2013). Forms of uveitis in humans have been linked to autoimmune reaction against melanocyte-expressed proteins in the pigmented epithelium of the uveal tract (Yamada *et al.* 2001; Otani *et al.* 2006), and autoantibodies against *TRPM1* have been detected in the sera of patients with paraneoplastic retinopathy (Kondo *et al.* 2011), leading to the hypothesis that altered TRPM1 in uveal tract melanocytes may contribute to an autoimmune response and insidious uveitis in Appaloosa horses.

In this study, we investigated the hypothesis that the increased risk of ERU in the Appaloosa breed is associated with the *LP* gene and/or MHC genes among the breed. To do so, we used a candidate region approach to evaluate the association between insidious ERU in Appaloosa horses and genetic markers within the *LP* region on ECA1 and the MHC gene complex on ECA20. Further, we used statistical modeling to demonstrate the relative predictive contribution of each marker allele to developing insidious ERU, and we tested this marker-based risk model in a second population of Appaloosa horses.

Materials and methods

Sample collection and phenotyping

Whole-blood samples were collected from 136 Appaloosa horses. Genomic DNA was prepared using either the Qiagen QIAamp Blood minikit or the Gentra Systems Puregene Blood DNA Kit according to the manufacturer's protocols. All horses were phenotyped for ERU by a board-certified ophthalmologist (HJK) with slit-lamp biomicroscopy and direct or indirect ophthalmoscopy. The severities of ocular lesions were scored from 1 to 5 as follows: grade 1 = no observed pathology; grade 2 = persistent ocular discharge that was not associated with nasal lacrimal disease; grade 3 = subtle changes in iris pigmentation suggestive of past inflammation; grade 4 = ocular discharge, miosis, marked depigmentation or hyperpigmentation of the iris and/or glaucoma; and grade 5 = active uveitis as evidenced by aqueous flare and miosis and/or fibrin in the anterior chamber. Grade 5 also was assigned to horses with signs of persistent uveitis including anterior or posterior synechia, cataract, glaucoma, degenerative/discolored vitreous and/or peripapillary chorioretinal scarring. Sample collection and phenotyping protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee (Animal Subjects Code-0201A15441).

Selection of cases and controls

Seventy-seven of the phenotyped horses were graded between 2 and 5 and classified as ERU cases. Fifty-nine

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of the phenotyped horses were classified as controls (grade 1). Cases and controls were divided into two populations. An initial population for association analysis (Population 1) included 53 of the most severe ERU cases (mean grade 4.8; consisting of 36 grade 5, 11 grade 4.5 and six grade 4; mean age: 18 years). The 43 controls in Population 1 were the oldest controls (≥5 years of age, mean age: 12 years), as younger controls may not yet have developed clinical disease. A follow-up population (Population 2) included 24 less severe cases that had a grade <4 (13 horses of grade 3; seven horses of grade 2) or were cases that had some uncertainty about ophthalmologic grade (three horses for which grade was not recorded and one horse of grade 5 that had possible signs of ocular disease unrelated to ERU). The mean grade for cases in Population 2 was 2.8. The 16 controls in Population 2 (mean age: 3 years) were significantly younger than were the controls in Population 1.

Genotyping and quality control

Thirty-three microsatellite markers and two SNPs were genotyped in ERU cases and controls (Table S1). Seven microsatellites and two SNPs were located within the *LP* region associated with Appaloosa coat color spotting on ECA1 (Terry *et al.* 2004), 13 microsatellites were located within the regions encompassing the ELA on ECA20 (Table S1) (Brinkmeyer-Langford *et al.* 2013), and 13 unlinked microsatellite markers in other genomic regions were genotyped for the estimation of relatedness between individual horses (see below).

Microsatellite markers were obtained from previously published reports or were identified for use in this study (Tables S1 and S2). Standard PCR for microsatellite locus amplification used a forward PCR primer, which contained an additional 5′ sequence that bound to a colored fluorescent primer (Mickelson *et al.* 2004). Microsatellites 168–482, 174–495 and 172–492 were evaluated on an ABI-3730 Genetic Analyzer (Applied Biosystems), and the ABI GENEMAPPER 3.5 software (Applied Biosystems) was used to score alleles. All other microsatellites were evaluated on a Beckman Coulter Ceq 8000, the genotypes were analyzed with Beckman Coulter Ceq 8000 software and the alleles were scored manually. Microsatellite minor alleles (frequency of <5% across the entire study population) were summed for all statistical analyses. SNPs in *TRPM1* and *OCA2* (Table S1) were genotyped using restriction fragment length polymorphism assays, as previously reported (Bellone *et al.* 2008; Bellone *et al.* 2010a). Individuals and markers with genotyping rates under 90% were excluded from statistical analysis. Mean genotyping rates across remaining markers were 95.6% (range 90.1–100%) for markers on ECA1, 96.3% (range 90.0–100%) for markers on ECA20 and 99.4% (range 96.9–100%) for markers distributed across the genome.

Tests for association

Basic allelic association was performed using a Pearson's chi-squared test of independence to identify significant differences in the allele distribution between the case and control populations. *P*-values for chi-square tests were computed by Monte Carlo simulation with 10×10^6 replications each, as many of the tables had <5% expected observations in at least one cell.

To control for potential confounding due to relatedness, an additive logistic mixed model was fit on the case–control status for each marker separately, with random effects for individual and relationships between individuals. A relationship matrix was constructed using pairwise relationship values estimated from genotype data from 13 microsatellites distributed throughout the genome (Vazquez *et al.* 2010; see Supporting information), as complete pedigree data were not available for all individuals. An additive effect of the marker was then tested for using the likelihood ratio test. Because this association method cannot handle missing data, genotypes from ECA1 or ECA20 were phased *in silico* with PHASEV2.1 (Stephens & Scheet 2005), and the missing alleles were imputed from the data. Imputed genotypes were retained in the analysis when imputations had >90% confidence score. After imputation, individuals with any missing alleles at that marker were removed from the analysis and alleles with <5% overall frequencies were combined into a single category (as above). Mean genotyping rates after imputation for markers on ECA1 and ECA20 were 99.4% and 99.7% respectively. A correction for the false discovery rate (FDR) was subsequently applied using the method of Benjamini and Hochberg (1995) as implemented in R. After FDR correction, $P < 0.05$ was considered significantly associated with ERU. Relative odds and associated 95% confidence intervals for each allele compared with the average of all other alleles were then computed for each significant marker.

Risk model

To simultaneously consider the effect of all three significantly associated markers, a risk model was formed by fitting an additive logistic mixed model including alleles from all associated markers as predictors. Relative odds and associated 95% confidence intervals for each allele compared with the average of all other alleles were then computed.

Follow up in Population 2

Basic allelic association was performed as described above independently in Population 2 and in Populations 1 and 2 combined. Additionally, probabilities of developing ERU for horses in Population 2 were predicted using the risk model developed using Population 1. These predicted probabilities were then compared with the actual case–control status.

All calculations were made in the R software package (R Development Core Team 2011).

Results

The ECA1 markers spanned a total of 14.14 Mb, and the average distance between the ECA1 markers was 1.77 Mb, whereas the ECA20 MHC microsatellites spanned a total of 4.60 Mb with an average intermarker distance of 0.38 Mb. Simple chi-square tests for association identified markers on both ECA1 and at the MHC locus putatively associated with ERU in Population 1 (Table 1). After controlling for relatedness using a mixed model with an additive logistic regression and correction for multiple testing using FDR, the SNP within intron 11 of the *TRPM1* gene ($P = 0.045$), two MHC microsatellites, *472-260* ($P = 0.029$); *EqMHC1* ($P = 0.015$) were identified as being significantly associated with insidious ERU in Appaloosa horses (Table 1). An additional marker in the MHC class I region, *UMN-JH34-2*, had an uncorrected mixed model $P < 0.05$, but was not significant after correction for multiple testing ($P = 0.074$). Similarly, two ECA1 microsatellites, *UMN-L29* (uncorrected $P = 0.020$) and *UMN-L31* (uncorrected $P = 0.011$), did not reach significance after correction for multiple testing.

The A allele at the *TRPM1* locus, previously associated with *LP*, was over-represented in ERU cases compared to controls (Table 2). Two alleles of marker *472-260* (224, 227) and two alleles of *EqMHC1* (205, 207) were over-represented in ERU cases (Table 2). To determine whether *472-260* and EqMHC1 were tagging the same risk locus on ECA20, *P*-values for all the MHC markers were calculated after controlling for the *EqMHC1* genotype (Table S3). After this calculation, *472-260* was still significantly associated with ERU ($P = 0.011$), suggesting that the marker genotypes at both *472-260* and *EqMHC1* were adding information about ERU risk.

Intermarker correlations and haplotype analysis were performed on both ECA1 and ECA20. The *TRPM1* SNP was highly correlated with the four ECA1 markers positioned between 105.37 Mb and 113.54 Mb (Fig. S1). *472-260* and *EqMHC1* were highly correlated with each other as were three additional markers (Fig. S2). Interestingly, there was also a strong correlation between *472-260* and three markers within the class II region of the MHC (Fig. S2). Haplotypic analysis of Populations 1 and 2 combined yielded 63 unique haplotypes across the four markers on ECA1 and 107 unique haplotypes across the five markers on ECA20. No haplotypes or pairwise marker combinations increased the significance of the association beyond that of the individual markers alone.

Relative odds and associated 95% confidence intervals for each allele compared with the average of all other alleles were first computed in Population 1 for each significant marker individually (Table 3). The alleles for each marker with the highest relative odds of being a case and 95%

Table 1 Marker associations with ERU in 53 cases and 43 controls in Population 1.

Marker name	Chromosome position (bp)[1]	MHC class	*P*-value χ^2	Raw *P*-value mixed model	Adjusted *P*-value[2] mixed model
UMN-L29	chr1.103622116	–	0.339	0.020	0.074
UMN-L31	chr1.104251379	–	0.031	0.011	0.063
UMN-L1	chr1.105372996	–	0.123	0.214	0.336
UMN-L2	chr1.106204477	–	0.056	0.062	0.171
TRPM1 RFLP	chr1. 108249293	–	0.019	0.006	0.045*
1CA43	chr1.110280065	–	0.065	0.099	0.218
TKY002	chr1.111173101	–	0.205	0.281	0.412
OCA2 RFLP	chr1. 113537217	–	0.700	0.716	0.772
1CA025	chr1.117758895	–	0.116	0.112	0.224
UMN-JH38	chr20.28905580	I	0.653	0.729	0.772
UMN-JH34-2	chr20.29232086	I	0.005	0.017	0.074
UMNe56	chr20.29288934	I	0.405	0.487	0.630
168-482	chr20.31384834	III	0.785	0.703	0.772
174-495	chr20.31419317	III	0.451	0.986	0.986
UMNe65	chr20.31474974	III	0.191	0.160	0.271
172-492	chr20.31498487	III	0.019	0.335	0.461
472-260	chr20.32544516	I	0.008	0.003	0.029*
EqMHC4	chr20.32624556	II	0.005	0.057	0.171
UMN-JH36	chr20.32629040	II	0.077	0.079	0.193
EqMHC1	chr20.32689801	II	0.0003	0.001	0.015*
464-243	chr20.33119703	II	0.771	0.737	0.772
UM011	chr20.33510120	II	0.464	0.133	0.243

*Marker significantly associated with ERU (adjusted *P*-value < 0.05).
[1]Chromosomal start position of the microsatellite.
[2]*P*-value after correction for false discovery rate.

Table 2 Allele frequencies of the three significantly associated markers in Population 1 ($n = 53$ cases and $n = 43$ controls).

		Allele frequencies	
Marker name	Allele	Cases	Controls
TRPM1 RFLP	A	0.651	0.477
TRPM1 RFLP	G	0.349	0.523
472-260	219	0.066	0.070
472-260	221	0.076	0.023
472-260	222	0.047	0.128
472-260	224	0.132	0.023
472-260	227	0.283	0.174
472-260	228	0.170	0.302
472-260	231	0.113	0.105
472-260	Minor[1]	0.112	0.175
EqMHC1	197	0.057	0.058
EqMHC1	203	0.245	0.547
EqMHC1	205	0.415	0.256
EqMHC1	207	0.208	0.128
EqMHC1	Minor[1]	0.075	0.012

[1]Minor alleles have <5% overall allele frequency.

Table 3 The relative odds of being a case and 95% confidence intervals for developing ERU in Population 1 with an additional allele compared with average of other alleles, for each significant marker separately.

Marker name	Allele	Relative odds	95% confidence interval
TRPM1 RFLP	A	2.97	1.33–6.65
TRPM1 RFLP	G	0.34	0.15–0.75
472-260	219	1.35	0.32–5.74
472-260	221	3.25	0.61–17.26
472-260	222	0.32	0.09–1.20
472-260	224	12.16	1.79–82.48
472-260	227	2.58	0.99–6.72
472-260	228	0.31	0.11–0.88
472-260	231	0.90	0.31–2.63
472-260	Minor[1]	0.33	0.11–0.99
EqMHC1	197	0.74	0.17–3.17
EqMHC1	203	0.25	0.12–0.51
EqMHC1	205	1.84	0.91–3.72
EqMHC1	207	2.33	0.91–5.99
EqMHC1	Minor[1]	6.82	0.66–70.54

[1]Minor alleles have <5% overall allele frequency.

confidence intervals were 2.97 (1.33, 6.65) for the A allele of *TRPM1*; 12.16 (1.79, 82.5) for the 224 allele of *472-260*; and 2.33 (0.91, 5.99) for the 207 allele of *EqMHC1*. To account for the additive effect of risk at each locus, a risk model was then built using all alleles from these three markers together and the relative odds and associated 95% confidence intervals for each allele compared with the average of all other alleles were computed (Table 4). Relative odds for each allele in the combined analysis were 2.36 (0.83, 6.69), 12.38 (2.24, 124.4) and 4.02 (0.86, 18.91) for the A allele of *TRPM1*, the 224 allele of *472-260* and the 207 allele of *EqMHC1* respectively.

Table 4 The relative odds of being a case and 95% confidence intervals for developing ERU in Population 1 with an additional allele compared with average of other alleles, for the three significant markers together in the risk model.

Marker name	Allele	Relative odds	95% confidence interval
TRPM1 RFLP	A	2.36	0.83–6.69
TRPM1 RFLP	G	0.42	0.15–1.20
472-260	219	0.39	0.05–2.97
472-260	221	3.48	0.63–19.31
472-260	222	0.90	0.20–4.08
472-260	224	12.38	1.24–124.2
472-260	227	2.82	0.81–9.81
472-260	228	0.38	0.10–1.48
472-260	231	0.78	0.23–2.68
472-260	Minor[1]	0.23	0.06–0.89
EqMHC1	197	0.39	0.06–2.74
EqMHC1	203	0.48	0.15–1.56
EqMHC1	205	0.56	0.17–1.90
EqMHC1	207	4.02	0.86–18.91
EqMHC1	Minor[1]	30.61	1.51–622.0

[1]Minor alleles have <5% overall allele frequency.

Association between these three markers and ERU was tested in a second, less severely affected population of ERU cases and younger controls. In Population 2, the *TRPM1* locus was significant both before ($P = 0.0032$) and after correction for multiple testing ($P = 0.044$). Similarly, *472-260* (uncorrected $P = 0.002$ and corrected $P = 0.035$) and *EqMHC1* (uncorrected $P = 0.041$ and corrected $P = 0.050$) were significantly associated with ERU. Combined *P*-values for chi-squared tests of association across both populations after correction for false discovery rate were $P = 0.0027$, 0.0001 and 0.0001 for TRPM1, *472-260* and *EqMHC1* respectively.

Population 2 also was used to survey how well the risk model could predict disease in a separate population of Appaloosa horses (Fig. 1). The median predicted probability of insidious ERU among cases in Population 2 was 0.73, and the median predicted probability of insidious ERU among controls was 0.20. When the predicted probability of being a case was classified as low, moderate or high, 62.5% of cases (15 of 24) were classified as high probability, 16.7% of cases (4 of 24) were classified as moderate probability and 20.8% of cases (5 of 24) were classified as low probability. In contrast, only 6.25% of controls (1 of 16) were classified as high probability, whereas 75% of controls (12 of 16) were classified as low probability.

Discussion

Nine genetic markers encompassing the *LP* region on ECA1 responsible for the spotted coat color pattern in Appaloosa horses and 13 microsatellites spanning the entire ELA on ECA20 were evaluated for significant associations with insidious ERU in a group of 53 Appa-

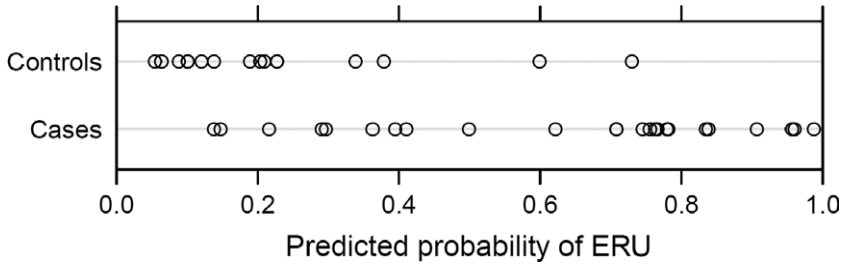

Figure 1 Results of a risk model developed from data in Population 1 ($n = 96$) used to predict the probabilities of developing ERU for horses in Population 2 ($n = 40$). The *x*-axis represents the probability of each horse being an ERU case, as predicted by the risk model. Each circle represents a horse Population 2 assigned to its true disease status of 'control' ($n = 16$) or 'case' ($n = 24$) on the *y*-axis.

loosa horses with ERU and 43 Appaloosa controls. Three markers, namely a SNP strongly correlated with *LP* and located within intron 11 of *TRPM1*, the MHC class I microsatellite *472-260* positioned near the boundary of the MHC class III and class II regions and the MHC class II microsatellite *EqMHC1* located in the first intron of the *DRA* gene, were significantly associated (corrected *P*-value <0.05) with insidious ERU and thus support our hypothesis that both the *LP* locus and other genetic factors contribute to ERU risk in the Appaloosa breed. After accounting for the MHC class II microsatellite *EqMHC1*, which was the most significantly associated marker, the MHC class I microsatellite *472-260* was still significantly associated with insidious ERU (Table S3). Other markers came close to reaching significance, including ECA1 microsatellite *UMN-L29*, ECA1 microsatellite *UMN-L31* and MHC class I microsatellite *UMN-JH34-2*; however, inclusion of these markers in a haplotype analysis did not add information beyond the single marker associations.

The *TRPM1* SNP is also highly correlated with the LP coat color pattern in Appaloosa horses (Bellone *et al.* 2010a). Autoimmune reaction against uveal tract melanocytes has been described in some forms of human and canine uveitis, in which an autoimmune reaction against melanocyte-associated protein tyrosinase incites inflammation (Yamaki *et al.* 2000; Uamaki *et al.* 2005). It has recently been demonstrated that *TRPM1* cation channels of retinal ON bipolar cells are the primary target of autoantibodies in melanoma-associated retinopathy (Dhingra *et al.* 2011; Kondo *et al.* 2011). It may be that an autoimmune reaction to *TRPM1* cation channels in melanocytes of the pigmented epithelium of the uveal tract is the link between the Appaloosa coat color and an increased risk of ERU. However, work to date has only demonstrated that *TRPM1* expression is altered in the skin and retinal bipolar cells in Appaloosa horses (Bellone *et al.* 2008); thus, it is unknown whether abnormal *TRPM1* cation channels exist in uveal melanocytes in ERU-affected horses, or whether these channels can elicit an autoimmune response.

Markers positioned greater than approximately 3 Mb away from the *TRPM1* SNP were not significantly associated with insidious ERU in Appaloosa horses; however, the *TRPM1* SNP was significantly correlated with ECA1 markers positioned between 105.37 Mb and 113.54 Mb (Fig. S1). Thus, another plausible hypothesis is that an allele of one of the other 29 genes within this 8-Mb region (Table S4) is harboring a genetic risk allele for insidious ERU, and this true risk allele is hitchhiking with selection for the *LP* mutation in the Appaloosa breed. In the future, genotyping a more dense set of ECA1 markers and genotyping for the *LP* causal mutation would help refine the associated region on ECA1, help substantiate or refute the association between ERU and the *LP* mutation and help aid in the precise identification of a susceptibility allele for insidious ERU in Appaloosas.

Two microsatellites in the ELA also were independently associated with ERU in this population of horses. Microsatellite *EqMHC1* is located in the first intron of the class II *DRA* gene. The *Equus* genus appears to have the highest *DRA* allelic diversity of any vertebrate studied to date (Kamath & Getz 2011), and it is possible that the high allelic diversity could play a very meaningful role in the equine immune response. Microsatellite *472-260* is located within a stretch of class I sequence near the boundary of the MHC class III and class II regions, which was predicted to have two class I pseudogenes, 3.8 and 3.9 (Tallmadge *et al.* 2005). It is possible that one or both of these loci are not pseudogenes in the Appaloosa horses tested. It is important to note that a single horse was used initially to identify the ELA loci by BAC contig sequencing (Gustafson *et al.* 2003), and several loci were characterized as pseudogenes because mRNAs were not detected in two tissues or because the sequenced allele contained a premature stop codon (Tallmadge *et al.* 2005). Our sequencing of the antigen-binding pocket domains of the *ELA3.1* and *ELA3.6* MHC class I loci in Appaloosa horses has identified a large number of alleles at both these loci, including a large number of *ELA3.6* null alleles containing a stop codon in the $\alpha 2$ domain (Fig. S3), and over 50% of horses sequenced had two null alleles at the *ELA3.6* locus (data not shown). Thus, ELA haplotypes in Appaloosa horses contain variable numbers of expressed MHC class I genes that could present autoantigens and

contribute to insidious ERU, and more work is needed to determine whether Appaloosas (or other breeds) have functional alleles at *ELA3.8* or other class I loci previously designated as pseudogenes. Finally, there was a strong correlation between MHC class I and II microsatellites, suggesting that certain combinations of alleles of MHC class I and II genes may be under selective pressure to segregate together. A list of known ELA genes and their current positions in the EquCab2 genome assembly can be found in Table S5.

Although many diseases have been associated with the MHC, it has been difficult to identify the specific causal genetic elements that contribute to disease phenotypes (Stewart *et al.* 2004). Characteristic features of the MHC, including high levels of polymorphism, extended linkage disequilibrium, clustering of genes with similar function and epistatic interactions between different combinations of alleles at multiple loci, can convolute the association of diseases with specific genetic elements. With recent advances in sequencing technologies, sequencing and annotating the entire MHC region in a cohort of cases and controls is feasible and would allow for refinement of associations between insidious ERU and the MHC and potentially the identification of functional risk alleles.

Within this cohort of Appaloosa horses, the relative risk of ERU is two to three times greater with each allele for the *TRPM1* SNP, and horses also are more likely to develop insidious ERU with certain alleles at ELA microsatellites *472-260* and *EqMHC1* (Table 3, Table 4). Despite relatively large confidence intervals associated with predicted odds of being an ERU case, the risk model built in the Population 1 was useful in predicting the case–control status of horses in a second population of 24 insidious ERU Appaloosa cases and 16 Appaloosa controls, even though ERU severity was, on average, lower in Population 2 (Table 5). In conclusion, our data suggest that markers at both the *LP* locus on ECA1 and within the MHC on ECA20 are associated with insidious ERU risk and suggest that further investigation of these genomic regions is warranted to identify putative functional alleles underlying ERU susceptibility.

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Supporting information

Additional supporting information may be found in the online version of this article.

Figure S1 Correlations between markers on ECA1.

Figure S2 Correlations between markers on ECA20.

Figure S3 Predicted amino acid alignment for ELA3.6 locus alleles.

Table S1 Markers included in the study.

Table S2 Newly discovered microsatellite markers used in this study.

Table S3 MHC marker associations in Population 1 after accounting for EqMHC1.

Table S4 Genotypic frequencies for each of the three associated markers.

Appendix S1 Materials.