Investigation of genetic risk factors for squamous cell carcinoma of the equine eye

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Background

- Squamous cell carcinoma (SCC), the most common cancer of the equine eye, has a high prevalence in Haflinger horses indicating a genetic predisposition.
- Potential contributors to ocular SCC include UV radiation, viral exposure, hormonal regulation, coat color and breed predispositions.[1]
- A recently identified recessive missense variant *(c.103 C>T p.Thr338Met)* in *damage specific DNA binding protein 2 (DDB2)* explains 76% of limbal and third eye lid SCC-affected Haflingers.[1,2]
- **Hypothesis:** Genetic heterogeneity explains risk for ocular SCC in horses.
- **Aim:** The specific aim of this study is to identify additional genetic risk variants for ocular SCC in Haflinger horses.

Materials & Methods

Sample Collection

- Horses were phenotyped by complete ocular examination and included as cases with histopathologic confirmation of SCC. Controls had no evidence of ocular disease per examination by a board-certified veterinary ophthalmologist and were at least 13 years of age.[2]
- DNA was extracted from blood or hair by routine extraction using the Puregene whole-blood extraction kit (Qiagen Inc., Valencia, CA) or from formalin-fixed, paraffin-embedded (FFPE) tissue using the Quick-DNA FFPE kit (Zymo Research, Irvine, CA).[1]

Variant Identification

- Whole genome sequencing data available from four Haflingers (**Table 1**)[2] were mapped to Equcab2.0[3] and variants called using Freebayes.[4]
- Variant caller files were filtered to prioritize polymorphism based on (1) location in an associated genomic locus from previous GWAS (published[1] and unpublished data) (2) allele distribution between cases and controls for different genetic models (**Table 2)** (3) predicted functional effect[5,6], and (4) the biologic relevance of the gene in cancer.

Genotyping Methods

- Twenty nine variants from the ranked variant list were used to design a multiplex SNP genotyping assays for genotyping 76 horses (14 cases and 62 controls) on the Agena MassArray platform.
- *KRT6A (T>G p.Lys42Gln*) and *NOTCH1 (A>G p.Ile157Val)* were genotyped via a PCR restriction fragment length polymorphism testing using PVUII and HpyCH4IV enzymes, respectively. Products were separated and visualized on the ABI3730 (ThermoFisher Scientific, Inc).
- Association testing was performed by Chi-squared test under several different models with predetermined significance level of α=0.05.

Table 1: Haflinger samples selected for high-throughput sequencing based on phenotype and *DDB2* c.1013 *C>T* genotype.

Horse	Ocular SCC Status	*DDB2* genotype c.1013 *C>T**
HF-14-62	Affected	*C/C*
HF-14-55	Affected	*C/C*
HF-13-17	Unaffected	*C/C*
HF-13-33	Unaffected	*T/T*

* The *T* Allele was previously associated with ocular SCC phenotype[1,2].

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Abstract

Squamous cell carcinoma (SCC) has a high prevalence in Haflinger horses indicating a genetic predisposition. A missense variant on equine chromosome 12 (ECA12) (*c.103 C>T p.Thr338Met*) in *damage specific DNA binding protein 2* (*DDB2*) explains 76% of ocular SCC-affected Haflingers.[1] This variant was not perfectly concordant with SCC status, as 24% of the affected horses were not homozygous for the risk allele. Further investigation of the 1.5 Mb region on ECA12 via serval methodologies did not identify another variant that was more strongly associated with the disease phenotype.[2] It is hypothesized that genetic heterogeneity contributes to cancer risk. To investigate this hypothesis, whole genome Illumina sequencing data from four Haflingers (two ocular SCC affected horses without the *DDB2* risk variant and two clinically unaffected horses) were evaluated to identify variants segregating with disease status. Thirty one variants were selected for further investigation based on genomic location, estimated allelic frequency differences between cases and controls, gene function, and SNPeff predicted effects.[3] Assessment of these variants within a sample set of 76 horses (14 ocular SCC-affected horses not homozygous for the *DDB2* risk variant and 62 unaffected controls) identified two risk alleles that warrant further evaluation.

Table 2. Filtering parameters used to prioritize variants for further investigation.

Filter 1: Greatest estimated difference in allele frequency
Cases: homozygous alternate. Controls: homozygous reference.
Filter 2: Identifying variants that fit a recessive model
Cases: homozygous alternate. Controls: homozygous reference or heterozygous.
Filter 3: Identifying variants that fit a dominant model
Cases: homozygous alternate or heterozygous. Controls: homozygous reference.
Filter 4: Dominant model allowing one control to carry variant
Cases: homozygous alternate or heterozygous. One control may have an alternate allele.

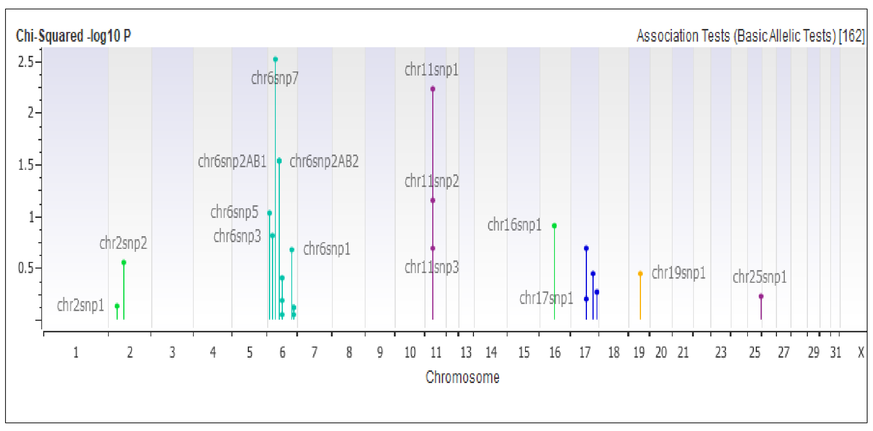

Figure 1: Basic Allelic Association Testing of Prioritized Variants for Ocular SCC in Haflinger Horses. Chromosomes are differentiated by color.

Results

- 31 variants from 7 chromosomes were selected from the 603,480 variants identified in filters 1 through 4 (Table 3).
- 24 variants were successfully genotyped either by RFLP or multiplex SNP genotyping assays.
- Basic allelic association testing identified a SNP on ECA6 ($p=3.0X10^{-3}$) and a SNP on ECA 11 ($p=1.02x10^{-6}$) warranting closer examination as genetic risk factors for ocular SCC (**Figure 1**).
- The associated missense variant on ECA6 *(COPS8 G>C p.Val174Leu)* is predicted to alter protein function. Allelic frequencies significantly differed between cases and controls (**Table 4**).
- All of the cases were homozygous for the KRT40 alternate allele *(*ECA11 SNP 1, *KRT40, A>G p.Ser57Gly),* suggesting that homozygosity for this variant may contribute to cancer risk. (**Table 5**).

Table 3: Number of variants identified and prioritized from Haflinger whole genome sequencing data for each filter applied along with the predicted effect of variant.

Filter	# of Variants	Low	Moderate	High	Prioritized
1	26,784	90	65	0	11
2	191,610	737	510	22	5
3	383,186	1,656	1,153	68	14
4	1900*	43	31	1	1

*From analysis of Chr6: 66476920 - 74079162

Table 4: Allelic association testing for chr6 SNP7 *COPS8 (G>C p.Val174Leu).*

Allelic Model	C	G	Total
Affected	16	12	28
Unaffected	34	88	122
Total	50	100	150
X^2 P-value = $3.0X10^{-3}$			

Table 5: Association testing under a recessive model for chr11 SNP1 *KRT40 (A>G p.Ser57Gly)*

Recessive Model	AA or AG	GG	Total
Affected	0	14	14
Unaffected	22	39	61
Total	22	53	75
X^2 P-value = 1.02×10^{-6}			

Conclusion and Future Directions

- In evaluating a small percentage of the variants identified by the whole genome sequencing analysis, we identified two SNPs that warrant further evaluation.
- COPS8 plays a role in regulating ubiquitin E3 ligase complexes and *COPS8 p.Val174Leu* may explain increased risk for ocular SCC by playing a role in the UV damage DDB2 DNA repair process.[8]
- KRT40 is a type 1 keratin with known roles in proper hair fiber formation. However, several keratin genes have been implicated in human SCC. The role of different keratins have not been well studied in equine cancer biology.[9]
- Further evaluation of more complex risk models are needed. However, 29% of our cases and only 1.5% of our controls are homozygous alternate at both loci.
- Whole genome sequencing data from 4 additional horses (2 cases and 2 controls) are currently being evaluated to prioritize additional variants for further investigation.
- We will continue to collect cases and controls for increased power to detect loci with small effects.
- Associated variants will be evaluated to establish the most robust risk model to inform clinical management and breeding decisions to benefit the health of this equine population.

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