EQUINE OCULAR SQUAMOUS CELL CARCINOMA: RETROSPECTIVE AND DIAGONOSTIC STUDIES

by

CORY BAIRD MOSUNIC

(Under the Direction of Karen Paige Carmichael)

ABSTRACT

Squamous cell carcinoma is the most prevalent tumor of the eye and adnexa in the horse. The first part of this study revealed retrospectively that ocular squamous cell carcinoma is most prevalent in chestnut, middle aged, Quarter Horse geldings at the University of Georgia. Post treatment recurrence versus non-recurrence was evaluated and radiation treatment at all tumor locations yielded a significantly lower recurrence rate when compared to non-radiation therapies. The second part of the study evaluated histopathological tools to assist in the diagnosis of borderline cases of squamous epithelial neoplasms that range from the benign squamous plaque through squamous cell carcinoma in situ and finally invasive squamous cell carcinoma. Our study revealed that AgNOR score is a proliferation assay that is able to distinguish between types of all ocular epithelial neoplasms in the horse. p53 immunoreactivity and Ki-67 proliferation assay are more limited in their ability to distinguish between equine epithelial neoplasms.

INDEX WORDS: Squamous cell carcinoma, equine, ocular, radiation therapy, AgNOR, p53, Ki-67
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DEDICATION

I dedicate this work to my loving husband, who without his patience and support, this project could not have been completed.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ACKNOWLEDGEMENTS</th>
<th>vi</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
</tbody>
</table>

## CHAPTER

1. **INTRODUCTION**
   - Background ......................................................... 1
   - Purpose of the study ............................................ 4
   - Expected Results .................................................. 5

2. **A RETROSPECTIVE ANALYSIS OF EQUINE OCULAR SQUAMOUS CELL CARCINOMA: 91 CASES**
   - Introduction ..................................................... 6
   - Criteria for Selection of Cases ............................. 8
   - Procedures ......................................................... 8
   - Results ............................................................ 8
   - Discussion ....................................................... 11
DETECTION OF ARGYROPHILIC NUCLEOLAR ORGANIZER REGIONS (AgNORs), AND p53 AND KI-67 IMMUNOREACTIVITY IN OCULAR SQUAMOUS EPITHELIAL TUMORS IN THE HORSE

Introduction

Experimental Methods and Design

Results

Discussion

CONCLUSION

REFERENCES
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Most Prevalent Breeds with Equine Ocular Squamous Cell Carcinoma</td>
<td>18</td>
</tr>
<tr>
<td>2.2</td>
<td>Most Prevalent Coat Colors of Horses with Ocular Squamous Cell Carcinoma</td>
<td>19</td>
</tr>
<tr>
<td>2.3</td>
<td>Percent Recurrence of Ocular Squamous Cell Carcinoma after Radiation versus Non-Radiation Treatments Regardless of Location</td>
<td>20</td>
</tr>
<tr>
<td>2.4</td>
<td>Outcome of Treatment of Equine Squamous Cell of the Eyelid/Canthus</td>
<td>21</td>
</tr>
<tr>
<td>2.5</td>
<td>Outcome of Treatment of Equine Squamous Cell Carcinoma of the Cornea</td>
<td>22</td>
</tr>
<tr>
<td>2.6</td>
<td>Outcome of Treatment of Equine Squamous Cell Carcinoma of the Limbus/Bulbar Conjunctiva</td>
<td>22</td>
</tr>
<tr>
<td>2.7</td>
<td>Outcome of Treatment of Equine Squamous Cell Carcinoma of the Third Eyelid</td>
<td>23</td>
</tr>
<tr>
<td>2.8</td>
<td>Outcome of Treatment of Equine Squamous Cell Carcinoma of the Palpebral Conjunctiva</td>
<td>24</td>
</tr>
<tr>
<td>3.1</td>
<td>Significant Differences Between Squamous Epithelial Neoplasms for AgNOR Score, and p53 and Ki-67 Immunoreactivity</td>
<td>50</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Photograph of equine ocular squamous cell carcinoma of the third eyelid</td>
<td>17</td>
</tr>
<tr>
<td>3.1</td>
<td>Photomicrograph of a squamous cell carcinoma with mean AgNOR score of 3.37</td>
<td>41</td>
</tr>
<tr>
<td>3.2</td>
<td>Photomicrograph of a squamous cell carcinoma <em>in situ</em> with mean AgNOR score of 1.93</td>
<td>42</td>
</tr>
<tr>
<td>3.3</td>
<td>Photomicrograph of a squamous plaque with the mean AgNOR score of 1.56</td>
<td>43</td>
</tr>
<tr>
<td>3.4</td>
<td>Photomicrograph of a squamous cell carcinoma with 84.7% p53 immunoreactivity</td>
<td>44</td>
</tr>
<tr>
<td>3.5</td>
<td>Photomicrograph of a squamous cell carcinoma <em>in situ</em> with 21.7% p53 immunoreactivity</td>
<td>45</td>
</tr>
<tr>
<td>3.6</td>
<td>Photomicrograph of a squamous plaque with 1% p53 immunoreactivity</td>
<td>46</td>
</tr>
<tr>
<td>3.7</td>
<td>Photomicrograph of a squamous cell carcinoma 87.7% Ki-67 immunoreactivity</td>
<td>47</td>
</tr>
<tr>
<td>3.8</td>
<td>Photomicrograph of a squamous cell carcinoma <em>in situ</em> with 15.7% Ki-67 immunoreactivity</td>
<td>48</td>
</tr>
<tr>
<td>3.9</td>
<td>Photomicrograph of a squamous plaque with the 3.3% Ki-67 immunoreactivity</td>
<td>49</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

Background

Squamous epithelial neoplasms are both prevalent in and devastating to many animal species. Squamous epithelial neoplasms can be either benign or malignant. Squamous cell carcinoma (SCC), a malignant squamous epithelial neoplasm, is prevalent in humans, dogs, cats, horses, and cows.\textsuperscript{1,2,3} It is a locally aggressive tumor originating from squamous epithelium throughout the body including the eye and adnexa.

SCC is reportedly the most common neoplasm of the equine, feline, and bovine globe and associated adnexa.\textsuperscript{4,5} Ocular squamous cell carcinoma is locally aggressive with a high recurrence rate, averaging between 40-45\%, and metastasis rates reportedly between 6-15\%. Metastasis is typically to the surrounding bony orbit, intra-cranial, regional lymph nodes, salivary glands, and thorax.\textsuperscript{6,7} Many reports have shown that there is a strong association between local invasion and metastasis of the tumor with short survival times and loss of vision of the affected globe either functionally or secondary to enucleation.\textsuperscript{8} As a result, ocular squamous cell carcinoma has a widespread impact financially, emotionally, and functionally with the potential for blindness or death of the animal.

SCC is the most common tumor of the eye and adnexa in horses,\textsuperscript{3,9} and the second most common tumor of horses.\textsuperscript{2,10} An increased prevalence for squamous cell carcinoma is reported in heavy drafts breeds,\textsuperscript{7} Appaloosas,\textsuperscript{4} Paint horses, Thoroughbreds, and Quarter Horses.\textsuperscript{9,6,11,12} A
sex predilection for the development of squamous cell carcinoma is reported for geldings.\textsuperscript{4,6,9} Increased exposure to UV light is thought to be a risk factor for the development of squamous cell carcinoma in light colored horses and those with non-pigmented skin surfaces, and in horses with exposure to a higher intensity and duration of solar radiation regardless of coat color. This suggests an actinic solar response in the pathogenesis of the tumor.\textsuperscript{4,13}

Non-radiation and radiation therapies are reported for the treatment of SCC. Non-radiation treatments included surgical excision,\textsuperscript{14,15} cryotherapy,\textsuperscript{16,17,18,19} radiofrequency hyperthermia (RFHT),\textsuperscript{7,20,21} immunotherapy,\textsuperscript{21,22} chemotherapy (cisplatin),\textsuperscript{12} and Co2 laser ablation.\textsuperscript{23} Radiation therapies include strontium-90,\textsuperscript{8,11,24,25} cobalt-60,\textsuperscript{26} gold-198,\textsuperscript{27,28} iridium-192,\textsuperscript{11,29,30} cesium-137,\textsuperscript{21,26} iodine-125,\textsuperscript{11} and radon–222.\textsuperscript{11,21}

Histologically, squamous epithelial tumors can be sub-divided into four types: squamous papilloma, squamous plaque, squamous cell carcinoma \textit{in situ}, and squamous cell carcinoma. A papilloma is a benign tumor in which there is a proliferation of the overlying squamous epithelium over a connective tissue core. Squamous papilloma is sometimes considered a progression of hyperplasia.\textsuperscript{31} In horses and cattle, squamous plaque is considered a progression of squamous papilloma. A squamous plaque is dysplastic squamous epithelium with varying amounts of hyperkeratosis and dyskeratosis. Squamous cell carcinoma is malignant transformation of squamous epithelial cells. The nuclei of these cells can be either vesicular or hyperchromatic with increased mitotic figures and pleomorphism. There are various grades of squamous cell carcinoma ranging from low grade to highly malignant lesions. Squamous cell carcinomas can be graded as well, moderately, and poorly differentiated based on characteristics such as cellular arrangement, intercellular bridging, invasiveness, and keratinization. Squamous cell carcinoma \textit{in situ} has the characteristics of squamous cell carcinoma, however, it does not
invade across the epithelial basement membrane. These features differentiate it from squamous plaque, and squamous cell carcinoma *in situ* which is thought to be a progression of squamous plaque. It is obvious that histologically, the various forms of squamous epithelial neoplasms represent progressive degrees of malignancy and this progressive nature may present a diagnostic challenge.

Veterinary medicine has become increasingly interested in studying aberrant cellular proliferation and its role in tumor pathogenesis. This had led to an increase in the study of cell cycle markers and proliferation assays. There are multitudes of markers and assays described in both veterinary and human literature. Three commonly utilized are argyrophilic nuclear organizing regions (AgNORs), Ki-67 and p53. AgNORs are loops of DNA in a cell’s nucleoli and are ribosomal RNA transcription sites. An increased number of AgNOR staining sites indicate an increased proliferative response of the cell and may be consistent with malignancy. p53 is a protein that acts as a negative transcription factor and its increased expression occurs after DNA damage to the tissue. Immunohistochemical identification of the over expression of p53 can indicate malignancy. Ki-67 is an antigen expressed in the nuclear matrix of proliferating cells and can be detected by the monoclonal antibody M1B1. Ki-67 has a very short half-life and can only be detected in proliferating cells and, like AgNOR and p53, over expression can also be an indicator of malignancy.

Prior research in humans has shown that AgNORs can be a diagnostic tool to differentiate between normal tissue, dysplasia and squamous cell carcinoma. Secondly, and more importantly, AgNORs have been shown to be a strong prognostic marker for squamous cell carcinoma in humans, particularly in the oral cavity and the lung. p53 and Ki-67 as well have been used to evaluate squamous cell carcinoma in humans. p53 has shown to be valuable
in predicting prognosis especially in squamous cell carcinomas of the head and neck; however, studies evaluating Ki-67 have produced variable results regarding its prognostic value.\textsuperscript{37,38,39,40,41} Studies have been performed on squamous cell carcinoma in dogs, cats, and horses using other proliferative assays such as proliferative cell nuclear antigen (PCNA). These studies demonstrated that PCNA is a successful means of grading malignancy but did not demonstrate a predictive value for survival times.\textsuperscript{42} AgNORs have been evaluated in other canine and equine tumors, most extensively in canine mast cell tumors and malignant lymphoma. These studies have shown that AgNORs, as compared to PCNA, and histological grading has a better prognostic predictive value.\textsuperscript{43,44} p53 expression has been evaluated specifically in conjunctival squamous cell carcinomas in the dog, cat, horse and cow. Although, the small sample size of the study limits the significance of the results, the results demonstrate over expression of p53 in conjunctival squamous cell carcinoma.\textsuperscript{45} p53 over expression has been reported in canine mammary tumors, osteosarcomas and feline hematopoietic tumors suggesting a correlation with tumorigenesis and the possible use of p53 as a diagnostic tool.\textsuperscript{46} Ki-67 has frequently been compared with PCNA in canine and feline melanotic tumors and canine squamous cell carcinoma mammary gland, mast cell tumors, and testicular tumors. In each tumor type, Ki-67 and PCNA proliferation assays demonstrated diagnostic ability, however, only in mast cells, melanotic and testicular tumors did they show any predictive value.\textsuperscript{47,48,49,50,51}

**Purpose of the study**

The first purpose of this study is to characterize the typical presentation of equine patients at The University of Georgia with the ocular squamous cell carcinoma. It is our goal to evaluate
the treatment of these patients; specifically, to determine whether radiation treatments or treatments that do not incorporate radiation achieve less tumor recurrence. The second purpose of this study is to identify an improved means of histologically distinguishing between borderline cases of the various epithelial ocular neoplasms found in the horse. Using the proliferation assays Ki-67, and AgNORs, as well as evaluating p53 immunoreactivity we plan to determine whether they can serve as useful diagnostic tools for borderline cases of ocular squamous epithelial tumors in the horse.

Expected Results

We expect that the population of equine patients at the University of Georgia will have similar characteristics of previously reported studies of horses with ocular squamous cell carcinoma. We therefore expect that middle aged, draft or appaloosa geldings with light coat colors will be over represented in our patient population. We also expect that the optimal treatment, achieving the least recurrence of tumor will involve radiation therapy. Diagnostically, we expect AgNOR, and p53 and Ki-67 immunoreactivity to be useful tools helping discriminate between types of ocular squamous epithelial neoplasia.
CHAPTER 2

A RETROSPECTIVE ANALYSIS OF EQUINE OCULAR SQUAMOUS CELL CARCINOMA: 91 CASES

Introduction

Squamous cell carcinoma (SCC) is the most common tumor of the eye and adnexa in horses,\textsuperscript{3,9} and the second most common tumor of horses.\textsuperscript{2,10} An increased prevalence for squamous cell carcinoma is reported in heavy drafts breeds,\textsuperscript{7} Appaloosas,\textsuperscript{4} Paint Horses, Thoroughbreds, and Quarter Horses.\textsuperscript{9,6,11,12} A sex predisposition for the development of squamous cell carcinoma is reported for geldings.\textsuperscript{4,6,9} An increased exposure to UV light is thought to be a risk factor for the development of squamous cell carcinoma in light colored horses and those with non-pigmented skin surfaces, and in horses with exposure to a higher intensity and duration of solar radiation regardless of coat color. This suggests an actinic solar response in the pathogenesis of the tumor.\textsuperscript{4,13}

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Previous studies only report overall survival rates in comparison of radiation versus non-radiation therapy. Incidence of non-recurrence is reported for individual treatment types; however, only one study compares radiation and non-radiation therapies,\textsuperscript{6} but this study did not compare the non-recurrence rates of individual treatment types with the anatomical location of the tumor. Our study was performed to characterize equine patients diagnosed with ocular squamous cell carcinoma, and to determine the effects of anatomical location and treatment type on tumor recurrence.
Criteria for Selection of Cases

The medical records of equine cases presented to The University of Georgia between the years 1985-2002 were searched for patients with histopathological confirmation of ocular squamous cell carcinoma.

Procedures

For horses meeting the search criteria, gender, breed, and age were recorded. The number of lesions, location of the tumor, and treatment types at the initial examination were recorded. The recurrence of tumor based on biopsy and treatment performed were recorded for subsequent examinations. The data was statistically analyzed using a logistic regression modeled with two discrete variables: non-recurrence versus recurrence of tumor. A likelihood ratio procedure was used to test treatment effect. All statistical testing was performed at a significance level of p<0.05.

Results

Ninety-one cases met the search criteria. The average follow up time was 449.4 days. The mean age was 12.1 years with a range of 2.4 years to 26.7 years. The gender distribution was 61.5% (n=56) gelding, 29.7% (n=27) mare, 6.6 % (n=6) stallion, and 2.2% (n=2) non-recorded gender. The most prevalent breed was the Quarter Horse 26.4% (n=24), followed by the Appaloosa 17.6% (n=16), and Paint 12.1% (n=11). (Table 2.1) The most represented coat color was chestnut/sorrel 39.5% (n=36), followed by brown 11% (n=10), and white 11% (n=10) (Table 2.2). A total of 157 tumors were diagnosed in the 91 cases. Six anatomical tumor locations were identified: eyelid/canthus 28.7% (n=45), third eyelid/nictatans (Figure 2:1)
26.1% (n=41), cornea 19.1% (n=30), limbus/bulbar conjunctiva 17.8% (=28), palpebral conjunctiva 7.6% (n=12), and orbit 0.64% (n=1). Bilateral involvement was noted in 19.8% (n=18) of the cases. Invasive squamous cell carcinoma into the bony orbit, maxillary sinus, frontal sinus, or nasal turbinates was diagnosed in 5.4% (n=4) of the cases. Non-ocular squamous cell carcinoma of the nares or perianal regions was present in 2.2% (n=2) of the cases prior to referral. Both cases were treated by the referring veterinarian and no recurrence was noted during the study’s follow up period. Throughout the course of the study, non-ocular squamous cell carcinoma was not diagnosed in any case after referral. Single treatments or combinations of treatment were used to treat the patients with ocular squamous cell carcinoma. There were two treatment categories: non-radiation therapy and radiation therapy. There were four groups in the non-radiation therapy category: (1) enucleation or exeneration, (2) one or more cytoreductive surgical procedures, (3) surgery and cisplatin, or (4) one or more surgical procedures followed by one or more cryotherapy treatments; and four groups in the radiation therapy treatment category: one or more cytoreductive surgical procedures followed by one or more of either (1) iridium-192, (2) cobalt-60, (3) strontium-90, or (4) cryotherapy and strontium-90.

A total of 231 treatments were performed on 157 tumors. Regardless of treatment group, recurrence was noted 35.9% (n=83) of the time post-treatment. The highest rate of recurrence, regardless of treatment group, was at the sclera/bulbar conjunctiva (n=21/46) 45.8%, followed by nictitans (n=20/60) 33.3%, eyelid/canthus (n=27/69) 29%, cornea (n=12/49) 27.9%, and palpebral conjunctiva (n=3/13) 23%. There were 172 non-radiation treatments and 59 radiation treatments performed on the tumors. Overall, 41.5% (n=96) of the treatment outcomes were lost.
to follow up; 34.9% (n=60) were non-radiation and 61.0% (n=36) were radiation treatments

(Table 2.3)

There was a significant difference of tumor recurrence between radiation and non-radiation therapy independent of anatomical location (p<0.0001). Non-radiation treatments had a recurrence rate of 44.1% (n=76), and radiation treatments had a recurrence rate of 11.9% (n=7). The recurrence rate of non-radiation versus radiation treatment was significant at the anatomical locations: eyelid/canthus, cornea, and limbus/bulbar conjunctiva. A significant difference was not found at the nictitans (p=0.1581). Statistical analysis was not performed for the palpebral conjunctiva location due to inadequate sample size.

Tumors of the eyelid/canthus received 69 treatments. The recurrence rate of non-radiation versus radiation treatment was significant (p=0.0062). Non-radiation treatments (n=50) had a 54% recurrence rate (n=27); 28.0% (n=14) were lost to follow-up. Radiation treatments (n=19) had a 0.0% (n=0) recurrence rate; 73.7 % (n=14) were lost to follow-up. (Table 2.4)

Tumors of the cornea received 43 treatments. The recurrence rate of non-radiation versus radiation treatment was significant (p=0.0057). The non-radiation treatments (n=34) had a 35.3% (n=12) recurrence rate; 50.0 % (n=17) were lost to follow-up. The radiation treatments (n=9) had a 0.0% (n=0) recurrence rate; 55.6 % (n=5) were lost to follow-up. (Table 2.5)

Tumors of the limbus/bulbar conjunctiva received 46 treatments. The recurrence rate of non-radiation versus radiation treatment was significant (p=0.0079). Non-radiation treatments (n=33) had a 51.5% recurrence rate (n=17); 27.3% (n=9) were lost to follow-up. Radiation treatments (n=13) had a 31.8% recurrence rate (n=4); 46.2% (n=6) were lost to follow-up. (Table 2.6)

Tumors at the nictitans received 60 treatments. The recurrence rate of non-radiation versus radiation treatments was not significant (p=0.1581). Non-radiation treatments (n=45) had a 40%
(n=18) recurrence rate; 35.6% (n=16) were lost to follow-up. Radiation treatments (n=15) had a 13.3% (n=2) recurrence rate; 60.0% (n=9) were lost to follow-up. (Table 2.7)

Tumors at the palpebral conjunctiva received 13 treatments. Statistical analysis was not performed at this location due to inadequate sample size. Non-radiation treatments (n=10) had a 20% (n=2) recurrence rate; 40% (n=4) were lost to follow-up. Radiation treatment (n=3) had a 33.3% (n=1) recurrence rate; 66.7% (n=3) were lost to follow up. (Table 2.8)

Regardless of treatment performed, the recurrence rate of tumors for horses ages 5-10 years (n=30/84), 10-15 years (n=26/73), and 15-20 years (n=22/60) were all 36%. The recurrence rate for horses between 20-25 years (n=1/2) was 50%. Geldings had the highest tumor recurrence rate, (n=49/143) 76.6%. The Thoroughbred had the highest recurrence rate (n=8/13), 61.5%. The coat color with the highest recurrence rate was bay, (n=14/25) 56 %. Coat color (p=0.0278), breed (p=0.0441), and interaction of age and breed (p<0.0001) had a significant effect on tumor recurrence regardless of treatment type and anatomical location. Coat color had a significant effect on tumor recurrence on the cornea regardless of the treatment type (p=0.0033).

Discussion

The mean age of 12.1 years is consistent with previous reports that range from 8.0 years to 11.8 years.\cite{2,6,7,9,52,53,54} The increased incidence of squamous cell carcinoma in geldings is similar to previous studies.\cite{4,6,9} Geldings were the most frequently represented gender diagnosed with squamous cell carcinoma (n=56) 61.5%; however, in the total hospital population, the most frequent gender present for examination was the mare (42.7%) followed by the gelding (38.2%) and stallion (18.4%). Dugan et al (1991)\cite{4} and Lall (1953)\cite{55} proposed that intact males and
females are significantly less likely to develop horn squamous cell carcinoma due to increased circulating sex hormones than castrated males, which could explain the increased frequency in geldings.\textsuperscript{4,55}

The prevalence of ocular squamous cell carcinoma is reported to be higher in the draft breeds,\textsuperscript{7} Appaloosa, \textsuperscript{4} Thoroughbred, Quarter Horse, and Paint.\textsuperscript{9,6,11,12} The increased incidence of ocular squamous cell carcinoma in the Quarter Horse at The University of Georgia may be a factor of the breed distribution in the total hospital population (n=17,214) in which the Quarter Horse (n=5,750)(33.8\%) was the most frequently examined breed during the course of the study.

Horses with light coat colors or areas of skin hypopigmentation have a higher incidence of ocular squamous cell carcinoma,\textsuperscript{4,6,9} which is hypothesized to be secondary to an actinic solar response.\textsuperscript{4,13} Our results indicate that the most prevalent color associated with SCC is sorrel/chestnut (n=36) (39.5\%). However, facial markings and the incidence of periorbital hypopigmentation were not retrospectively available.

The frequency of bilateral ocular involvement is consistent with previous studies. King et al. (1981)\textsuperscript{6} and Lavach and Severin (1977)\textsuperscript{9} reported 11.65 (n=5) and 16.3\% (n=8) bilateral involvement rate respectively. Previously reported metastasis rates range from 0.3\% - 18.6\%.\textsuperscript{6,7,8,9,52,56} The absence of post mortem necropsies on the euthanized cases in our study (n=4) could account for the absence of documented metastatic disease. However, 5.4\% (n=4) of cases were found to have “invasive” squamous cell carcinoma in surrounding periorbital tissues.

The most prevalent tumor location was the eyelid/canthus followed by the third eyelid/canthus, cornea, limbus/bulbar conjunctiva, palpebral conjunctiva, and orbit, which is consistent with previous reports.\textsuperscript{2,4,6,7,9,52,57} Similar to previous reports, the recurrence rate was 35.9\% post treatment independent of the treatment group and tumor site.\textsuperscript{21} Radiation therapy had
a significantly lower recurrence rate when compared to non-radiation therapy independent of tumor location. The specific tumor sites: cornea, limbus/bulbar conjunctiva, and eyelid/canthus exhibited the same significant difference with less recurrence of squamous cell carcinoma with the use of radiation therapy. Non-radiation treatments resulted in a higher recurrence rate at the nictatans when compared to radiation therapies; this result was not significant. The data set for the palpebral conjunctiva was too small to statistically analyze. Radiation treatments for squamous cell carcinoma at the cornea, limbus/bulbar conjunctiva and eyelid/canthus, resulted in a lower tumor recurrence rate than non-radiation treatments. This significant difference was not demonstrated for tumors of the third eyelid. Possibly since small tumors of the third eyelid are more likely to be completely resected, then radiation treatment at the third eyelid may not be a necessary adjunct treatment. Regardless of treatment type or category, tumors of the sclera/bulbar conjunctive had the highest recurrence rate.

Of the radiation treatment outcomes, 61% were lost to follow-up and of the non-radiation treatment outcomes, 34.9% were lost to follow-up. The reason for this difference is unknown. It is possible that the incidence of tumor recurrence in the radiation group was lower than in the non-radiation group; therefore, owners failed to present the animal for follow-up examination. It is possible that the owner’s failed to present the horses for follow-up visits due to expenses associated with radiation therapy compared to non-radiation therapies.

We choose to examine recurrence rates in place of progression free survival times (non-recurrence rate) or over all survival rates. Gross visualization in judging absence of tumor is at greater risk for missing recurrence of the neoplasia than biopsy. Overall survival rate would over estimate the non-recurrence rate since SCC is a locally invasive tumor without a high rate of metastasis. Dugan (1991)\textsuperscript{21} and Schwink (1987)\textsuperscript{7} examined overall survival rates and compared
radiotherapy versus non-radiotherapy; neither found a correlation with survival and treatment type.\textsuperscript{7,21} Our study examined whether there is a significantly statistical difference between radiation and non-radiation therapy at specific anatomical locations. The radiation recurrence rate of 11.9\% independent of all locations is lower than other studies when non-recurrence rates are converted to recurrence rates. King et al in 1991\textsuperscript{6} reported a converted 25\% recurrence rate for beta (strontium–90) and gamma (iridium-192) radiation, and Walker et al.\textsuperscript{11} reported with a converted recurrence rate of 17.5\% for strontium-90 and 20\% for interstitial radiation (Radon-222, Iodine-125 and Iridium-192). Rehbun (1990)\textsuperscript{25} and Fraundfelder et al. (1982)\textsuperscript{8} using strontium-90 as the sole treatment type reported converted recurrence rates of 16.7 \% and 11\%, respectively. In our study, strontium therapy resulted in a 15.1\% recurrence rate independent of the anatomical location. The low recurrence rate reported by Fraundfelder et al.\textsuperscript{8} may be accounted for by the high dosage (25,000 cGY) and limited number of cases treated (n=19).

Two studies examining iridium-192 treatment reported a converted 18.2 \% recurrence rate\textsuperscript{30} and a 0.0\%\textsuperscript{29} recurrence rate compared to 10.5\% recurrence rate independent of the location in our study. Wyn-Jones (1983)\textsuperscript{29} reported a 0.0\% recurrence rate; however, multiple tumor types were treated and only 2 of the 27 horses treated had squamous cell carcinoma.\textsuperscript{29}

Non-radiation therapies in our study had a higher recurrence rate (44.4\%) than the radiation therapies (11.9\%). Surgery alone had the highest recurrence rate at all locations except the eyelid/canthus. King et al (1991)\textsuperscript{6} reported a higher converted recurrence rate for cryotherapy (66.7\%) compared to surgery alone (44.4\%) independent of locations; however, only two case of cryotherapy were performed compared to 18 cases of surgery alone.

Differences between study recurrence rates may be affected by treatment options at specific locations, which are dependent on size and depth of the tumor, and differences in dose. There are
specific indications and limitations for the use of radiotherapy. Strontium-90 is only indicated for superficial tumors that exist or are to debulked to a tumor depth of no greater than 3mm since 80% of the radiation dose is absorbed in the first 2mm of the tumor.\textsuperscript{57} For larger tumors, iridium-192 interstitial implants are a more appropriate treatment. The radiation penetrates a depth of approximately 1-1.5 cm; however, due to the delivery mechanism the treatment is most often used for eyelid/canthus and peri-ocular tumors.\textsuperscript{27,30,58} Cobalt- 60 teletherapy has the greatest penetration depth and is used for more invasive tumors, particularly as a palliative therapy.\textsuperscript{59} The dose range for strontium in our study was a surface dose of 8,000 to 10,000 cGy for cornea, 10,000-12,000 cGy for conjunctiva, and 12,000 to 20,000 cGy for eyelid/canthus. The dose of iridium-192 was between 5,800 and 6,500 cGy. The cobalt-60 dose was between 3,200 cGy and 3,600 cGy divided into 4 weekly treatments. All cryotherapies were performed as a double freeze thaw method. The cisplatin dose used was 1mg/cm\textsuperscript{3}. The absence of exact tumor location and measurements, as well as radiation dosages used in previous studies, makes comparison difficult.

Potential complications should also be considered when choosing a treatment type. Theon and Pascoe (1994) reported the secondary complications of corneal and palpebral ulcerations in 10.3% of cases treated with iridium-192.\textsuperscript{30} However, the use of iridium-192 at doses used in our study have yielded minimal complications, such as skin and hair dyspigmentation and infection.\textsuperscript{11,29}

In humans, strontium-90 is widely used to treat pterygia, and has been closely examined in humans to justify its use in pterygia treatment. Telangiectasia of the conjunctiva is a frequently reported late onset complication of strontium radiation.\textsuperscript{60,61} Other complications reported are punctate corneal ulceration, corneal edema, corneal neovascularization and scarring, iris atrophy,
symblepharon, and ptosis. More severe complications secondary to strontium radiation reported in humans are cataracts, endophthalmitis, and scleral or corneal thinning or rupture. Studies have proposed that secondary scleral ulcerations or ruptures result from exposed sclera without conjunctival coverage post-surgery. To date, there is only one report of strontium induced keratopathy in a horse. This diagnosis was based on the late onset, exclusion of other disease processes, and consistent histopathological findings. Two possible contributing factors was the large keratectomy site (20mm X 16 mm) and the high dose of strontium (20,000cGy). Overall, the associated risk of strontium treatment is low, making its use for a neoplastic disease process justifiable.

Further follow-up studies may include examining tumor locations and size in relationship to treatment modality and tumor recurrence. It may also be beneficial to evaluate length of non-recurrence and its correlation with treatment type.
Figure 2:1 Photograph of equine ocular squamous cell carcinoma of the third eyelid
Table 2.1: Most Prevalent Breeds with Equine Ocular Squamous Cell Carcinoma

<table>
<thead>
<tr>
<th>Breed</th>
<th>% Cases Presented</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quarter Horse</td>
<td>26.5% (n=24)</td>
</tr>
<tr>
<td>Appaloosa</td>
<td>17.6% (n=16)</td>
</tr>
<tr>
<td>Paint Horse</td>
<td>12.1% (n=11)</td>
</tr>
<tr>
<td>Tennessee Walking Horse</td>
<td>9.9% (n=9)</td>
</tr>
<tr>
<td>Belgian</td>
<td>6.6% (n=6)</td>
</tr>
<tr>
<td>Mixed or cross breed</td>
<td>6.6% (n=6)</td>
</tr>
<tr>
<td>Thoroughbred</td>
<td>5.5% (n=5)</td>
</tr>
<tr>
<td>Morgan</td>
<td>3.3% (n=3)</td>
</tr>
<tr>
<td>Pony of the Americas</td>
<td>3.3% (n=3)</td>
</tr>
<tr>
<td>Arabian</td>
<td>2.2% (n=2)</td>
</tr>
<tr>
<td>Clydesdale</td>
<td>2.2% (n=2)</td>
</tr>
<tr>
<td>Paso Fino</td>
<td>1.1% (n=1)</td>
</tr>
<tr>
<td>Standardbred</td>
<td>1.1% (n=1)</td>
</tr>
<tr>
<td>Racking Horse</td>
<td>1.1% (n=1)</td>
</tr>
<tr>
<td>Missouri Fox Trotter</td>
<td>1.1% (n=1)</td>
</tr>
</tbody>
</table>
Table 2.2: Most Prevalent Coat Colors of Horses with Ocular Squamous Cell Carcinoma

<table>
<thead>
<tr>
<th>Coat color</th>
<th>% Cases Presented</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chestnut/Sorrel</td>
<td>(n=36) 39.6%</td>
</tr>
<tr>
<td>Brown or Brown/White Pinto</td>
<td>(n=10) 11%</td>
</tr>
<tr>
<td>White</td>
<td>(n=10) 11%</td>
</tr>
<tr>
<td>Red Roan</td>
<td>(n=8) 8.8%</td>
</tr>
<tr>
<td>Grey</td>
<td>(n=7) 7.7%</td>
</tr>
<tr>
<td>Bay</td>
<td>(n=6) 6.6%</td>
</tr>
<tr>
<td>Palomino/Blonde</td>
<td>(n=6) 6.6%</td>
</tr>
<tr>
<td>Unlisted</td>
<td>(n=5) 5.5%</td>
</tr>
<tr>
<td>Black or Black and White Pinto</td>
<td>(n=3) 3.3%</td>
</tr>
</tbody>
</table>
Table 2.3: Percent Recurrence of Ocular Squamous Cell Carcinoma after Radiation versus Non-Radiation Treatment Regardless of Location

<table>
<thead>
<tr>
<th>Treatment Category</th>
<th>Total % recurrence</th>
<th>Total % lost to Follow-up</th>
<th>Treatment type</th>
<th>Percentage Recurrence</th>
<th>Percentage lost to follow up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-radiation</td>
<td>44.1% (n=76)</td>
<td>34.9% (n=60)</td>
<td>Cytoreductive Surgery (n=81)</td>
<td>61.7% (n=50)</td>
<td>22.2% (n=18)</td>
</tr>
<tr>
<td>Treatment (n=172)</td>
<td></td>
<td></td>
<td>Surgery and Cryotherapy (n=75)</td>
<td>30.75 (n=23)</td>
<td>38.7% (n=29)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Enucleation (n=13)</td>
<td>0.0% (n=0)</td>
<td>100.0% (n=13)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Surgery and Cisplatin (n=3)</td>
<td>100.0% (n=3)</td>
<td>0.0% (n=0)</td>
</tr>
<tr>
<td>Radiation</td>
<td>11.9% (n=7)</td>
<td>61.0% (n=36)</td>
<td>Surgery and Strontium (n=33)</td>
<td>15.1% (n=5)</td>
<td>60.6% (n=20)</td>
</tr>
<tr>
<td>Treatment (n=59)</td>
<td></td>
<td></td>
<td>Surgery and Iridium (n=19)</td>
<td>10.5% (n=2)</td>
<td>63.2% (n=12)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Surgery and Cobalt (n=2)</td>
<td>0.0% (n=0)</td>
<td>0.0% (n=0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Surgery, Cryotherapy and Strontium (n=5)</td>
<td>0.0% (n=0)</td>
<td>80.0% (n=4)</td>
</tr>
</tbody>
</table>
Table 2.4: Outcome of Treatment on Equine Squamous Cell of the Eyelid/Canthus

<table>
<thead>
<tr>
<th>Treatment Category</th>
<th>Total % recurrence</th>
<th>Total % lost to follow up</th>
<th>Treatment Type</th>
<th>% recurrence</th>
<th>% lost to follow up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Radiation</td>
<td>35.5% (n=27)</td>
<td>28.0% (n=14)</td>
<td>Cyto-reductive Surgery (n=22)</td>
<td>68.2% (n=15)</td>
<td>18.2% (n=4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Surgery and Cryotherapy (n=25)</td>
<td>40.0% (n=10)</td>
<td>36.0% (n=9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Surgery and Cisplatin (n=2)</td>
<td>100.0% (n=2)</td>
<td>0.0% (n=0)</td>
</tr>
<tr>
<td></td>
<td>0.0% (n=0)</td>
<td>73.7% (n=14)</td>
<td>Enucleation (n=1)</td>
<td>0.0% (n=0)</td>
<td>100.0% (n=1)</td>
</tr>
<tr>
<td>Radiation</td>
<td></td>
<td></td>
<td>Surgery, Cryotherapy &amp; Strontium (n=3)</td>
<td>0.0% (n=0)</td>
<td>66.7% (n=2)</td>
</tr>
<tr>
<td></td>
<td>0.0% (n=0)</td>
<td></td>
<td>Surgery and Iridium (n=12)</td>
<td>0.0% (n=0)</td>
<td>75.0% (n=9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Surgery and Strontium (n=3)</td>
<td>0.0% (n=0)</td>
<td>100.0% (n=3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Surgery and Cobalt (n=1)</td>
<td>0.0% (n=0)</td>
<td>0.0% (n=0)</td>
</tr>
</tbody>
</table>
### Table 2.5: Outcome of Treatment of Equine Ocular Squamous Cell Carcinoma of the Cornea

<table>
<thead>
<tr>
<th>Treatment Category</th>
<th>Total % recurrence (n)</th>
<th>Total % lost to follow up (n)</th>
<th>Treatment Type</th>
<th>% recurrence (n)</th>
<th>% lost to follow up (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Radiation</td>
<td>35.3% (n=12)</td>
<td>50.0% (n=17)</td>
<td>Cyto-reductive Surgery (n=11)</td>
<td>63.6% (n=7)</td>
<td>27.3% (n=3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Surgery and Cryotherapy (n=14)</td>
<td>28.6% (n=4)</td>
<td>42.9% (n=6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Enucleation (n=8)</td>
<td>0.0% (n=0)</td>
<td>100.0% (n=8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Surgery and Cisplatin (n=1)</td>
<td>100.0% (n=1)</td>
<td>0.0% (n=0)</td>
</tr>
<tr>
<td>Radiation</td>
<td>0.0% (n=0)</td>
<td>55.6% (n=5)</td>
<td>Surgery and Strontium (n=9)</td>
<td>0.0% (n=0)</td>
<td>100.0% (n=5)</td>
</tr>
</tbody>
</table>

### Table 2.6: Outcome of Treatment on Equine Squamous Cell Carcinoma of the Limbus/ Bulbar Conjunctiva

<table>
<thead>
<tr>
<th>Treatment Category</th>
<th>Total % recurrence (n)</th>
<th>Total % lost to follow up (n)</th>
<th>Treatment Type</th>
<th>% recurrence (n)</th>
<th>% lost to follow up (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Radiation</td>
<td>51.5% (n=17)</td>
<td>40.9% (n=9)</td>
<td>Cyto-reductive Surgery (n=12)</td>
<td>83.3% (n=10)</td>
<td>0.0% (n=0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Surgery and Cryotherapy (n=20)</td>
<td>35.0% (n=7)</td>
<td>40.0% (n=8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Enucleation (n=1)</td>
<td>0.0% (n=0)</td>
<td>100.0% (n=1)</td>
</tr>
<tr>
<td>Radiation</td>
<td>31.8% (n=4)</td>
<td>46.2% (n=6)</td>
<td>Surgery and Strontium (n=13)</td>
<td>30% (n=4)</td>
<td>46.2% (n=6)</td>
</tr>
</tbody>
</table>
Table 2.7: Outcome of Treatment on Equine Squamous Cell Carcinoma of the Third Eyelid

<table>
<thead>
<tr>
<th>Treatment Category</th>
<th>Total % recurrence</th>
<th>Total % lost to follow up</th>
<th>Treatment Type</th>
<th>% recurrence</th>
<th>% lost to follow up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Radiation</td>
<td>40% (n=18)</td>
<td>46.7% (n=21)</td>
<td>Cyto-reductive Surgery (n=35)</td>
<td>51.4% (n=18)</td>
<td>31.4% (n=11)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Surgery and Cryotherapy (n=9)</td>
<td>0.0% (n=0)</td>
<td>44.4% (n=9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Enucleation (n=1)</td>
<td>0.0% (n=0)</td>
<td>100.0% (n=1)</td>
</tr>
<tr>
<td>Radiation Therapy</td>
<td>13.3% (n=2)</td>
<td>60.0% (n=9)</td>
<td>Surgery and Strontium (n=8)</td>
<td>12.5% (n=1)</td>
<td>75.0% (n=6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Surgery and Iridium (n=6)</td>
<td>16.7% (n=1)</td>
<td>50.0% (n=3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Surgery and Cobalt (n=1)</td>
<td>0.0% (n=0)</td>
<td>100.0% (n=0)</td>
</tr>
</tbody>
</table>
### Table 2.8: Outcome of treatment Of Equine Squamous Cell Carcinoma of the Palpebral Conjunctiva

<table>
<thead>
<tr>
<th>Treatment Category</th>
<th>Total % recurrence</th>
<th>Total % lost to follow up</th>
<th>Treatment Type</th>
<th>% recurrence</th>
<th>% lost to follow up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Radiation</td>
<td>20% (n=2)</td>
<td>40.0% (n=4)</td>
<td>Cyto-reductive Surgery (n=1)</td>
<td>0.0%</td>
<td>0.0% (n=0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Enucleation (n=2)</td>
<td>0.0%</td>
<td>100.0% (n=2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Surgery and Cryotherapy (n=7)</td>
<td>28.6%</td>
<td>28.6% (n=2)</td>
</tr>
<tr>
<td>Radiation</td>
<td>33.3% (n=3)</td>
<td>66.7% (n=2)</td>
<td>Surgery and Iridium (n=1)</td>
<td>100.0%</td>
<td>0.0% (n=0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Surgery, Cryotherapy &amp; Strontium (n=2)</td>
<td>0.0%</td>
<td>100.0% (n=2)</td>
</tr>
</tbody>
</table>
DETECTION OF ARGYROPHILIC NUCLEOLAR ORGANIZER REGIONS (AgNORs) AND, p53, AND KI-67 IMMUNOREACTIVITY IN OCULAR SQUAMOUS EPITHELIAL TUMORS IN THE HORSE

Introduction

Squamous epithelial neoplasms are the result of neoplastic transformation of squamous epithelial cells. These neoplasms can be sub-divided into four types: squamous papilloma, (benign epithelial neoplasia), squamous plaque, (pre-malignant epithelial dysplasia), squamous cell carcinoma \textit{in situ}, (malignant epithelial transformation that has not broken through the basement membrane), and invasive squamous cell carcinoma. There is ample evidence that the various forms of epithelial neoplasms represent a continuum and this progressive nature can present a diagnostic challenge. Benign squamous papillomas can progress to pre-malignant squamous plaques, and squamous plaques can progress to squamous cell carcinoma \textit{in situ} and eventually to invasive squamous cell carcinoma. Each of these forms of squamous epithelial neoplasia represents a different prognosis making accurate diagnosis essential. Although these tumor types appear clearly defined, identifying borderline cases histologically can be quite challenging. Histologic samples of tissue taken from a horse with an early squamous cell carcinoma may be difficult to differentiate from a squamous plaque. Inadequate sampling of an invasive squamous cell carcinoma may result in a diagnosis of squamous cell carcinoma \textit{in situ}.
or even squamous plaque. In this study, the objective was to find ancillary methods to accurately diagnose squamous epithelial neoplasia.

In human medicine, proliferation assays and cell cycle markers are utilized as diagnostic tools to help discriminate between tumor types and veterinary medicine has become increasingly interested in studying aberrant cellular proliferation and its role in tumor pathogenesis. This had led to an increase in the study of cell-cycle markers and proliferation assays. There is a vast array of indices described in both veterinary and human literature. Three common methods are measuring argyrophilic nuclear organizing regions (AgNORs), as well as Ki-67 and p53 immunoreactivity.

AgNORs appear as dark staining foci in the nuclei of a cell. AgNORs are proteins that are easily located due to their argyrophilia (sulphur dioxide bonds that adhere silver stain). The AgNOR staining technique was first applied and simplified by Ploton et al. in 1986 and later popularized by Crocker and his colleagues in 1989. AgNORs correspond to nonhistone proteins that are associated with loops of ribosomal DNA that serve as binding sites for RNA polymerase and thereby DNA transcription. In humans they are found on chromosome 13,14,15,21, and 22.

The number of AgNORs per cell is closely related to the degree of cellular proliferation. An increased number of AgNOR staining sites indicates an increased proliferative response of the cell and may be consistent with malignancy. AgNORs have been used to compare cells in normal, hyperplastic, benign, and malignant conditions.

Downfalls of the technique include potential lack of reliability and reproducibility, background staining and precipitates as well as fading of the stain over time. Even though there is existing controversy over the usefulness of AgNOR scores, there are large number of
studies that support the conclusion that a high AgNOR count indicates malignant tissue while a lower count indicates benign or normal tissue. AgNOR staining can be performed not only on histological specimens but also on cytological aspirates.

In humans, AgNORs have been shown to be a strong diagnostic aid and prognostic marker for squamous cell carcinoma, particularly in the oral cavity and the lung. In the veterinary literature, AgNORs have been used to investigate both feline and canine tumors; however no studies to date have used AgNORs to evaluate any equine tumor. In the dog, AgNORs have been used to evaluate neuroendocrine tumors, lymphoma, and transmissible venereal tumors, but have been studied most often in mammary tumors and mast cell tumors. A study by Bratulic et al. (1996), demonstrated a significant difference in the mean AgNOR score between benign and malignant canine mammary tumors. Bostock et al. (1989) found that there was a significant difference between AgNOR scores for different grades of canine mast cell tumors. Bostock et al. also determined that the mean AgNOR score served as a prognostic indicator.

The p53 gene has been shown to be the most commonly altered gene in human cancers. Reportably 50-55% of all human neoplasms have evidence of p53 mutation. The p53 gene produces a phosphoprotein that serves as a negative regulator of cell proliferation; a negative transcription factor that has increased expression after DNA damage occurs. The function of p53 is to act as an inducible transcription factor after DNA damage. A transcription target of p53 protein is P21WAF1, a protein that binds to and inhibits the cyclin-cyclin dependent kinase system and therefore results in cell arrest, or G1 cell cycle arrest. p53 can also affect apoptosis by inducing Bax or p53-regulated apoptosis inducing protein 1 gene (p53AIp1). Lastly, p53 plays a role in G2 checkpoint control. For these reasons the p53 gene has become known as the
“Guardian of the Genome”. Wild type p53 protein has a very short half life and prevents detection. Mutations in the p53 gene result in amino acid substitutions and a longer half-life which enable the protein to be detected immunohistochemically. Mutations in the p53 gene result in loss of normal regulatory function of the gene and thereby result in uncontrolled cellular proliferation. The loss of the p53 gene is associated with tumorigenesis due to this lack of regulatory control of the cell cycle and loss of apoptosis. Immunohistochemical identification of the over expression of p53 can therefore indicate malignancy.

p53 over expression has been evaluated in many types of neoplasms in the dog, and cat, as well as the horse. In the dog, it has been widely studied in reference to soft tissue sarcomas, melanomas, mast cell tumors, osteosarcomas, lymphomas, mammary tumors, and in the cat, hematopoietic tumors. In the horse, p53 has been studied in cholangiocarcinoma, melanomas, and sarcoids with poor results in demonstrating the role of the gene in carcinogenesis. In the dog, cat, and horse, the role of p53 and the pathogenesis of squamous cell carcinoma has been specifically studied. Intense immunoreactivity (>50% of tumor cell staining) has been reported in canine and feline cutaneous squamous cell carcinomas. One study by Tiefke et al. (1998) compared p53 immunoreactivity between canine oral papillomas and squamous cell carcinomas. This study found that there was significantly less p53 staining of the papillomas when compared to squamous cell carcinoma. In the horse, the p53 gene mutations have been evaluated in cutaneous squamous cell carcinoma. Pazzi et al. (1996) found that in eight samples they evaluated of cutaneous squamous cell carcinoma the mutation occurred most often at exon 5, 7, and 8 with mutations at hotspots on codons 2445, 247/248, 273, 278, and 285/286. The missense mutations that were identified were both C to T transitions at dipyrimidine sites. These mutations are consistent with the distinct changes that UV light cause,
predominately the C to T transition at dipyrimidine sites, and therefore support the etiology of an actinic solar response in the pathogenesis of equine squamous cell carcinoma. Two previous studies evaluated p53 specifically in ocular squamous cell carcinoma. In the first study by Sironi et al. (1999), they found that five equine samples of ocular conjunctival squamous cell carcinoma exhibited p53 immunostaining, while normal tissue did not. In the second study, Tiefke and Lohr (1996) described increased aberrant P53 expression in ocular squamous cell carcinoma in horses and cattle as compared to other tumor locations. These findings are consistent with what has been reported in human pterygia and other limbal tumors that show preferentially higher amounts of mutant p53 expression. This further supports the theory that UV light exposure plays a pivotal role in both the pathogenesis of human epithelial limbal tumors and pterygium as well as ocular epithelial neoplasia in the horse. It also indicates that p53 may be a useful diagnostic and even prognostic tool in the histological diagnosis of these tumors.

Ki-67 is a nuclear non histone protein with two protein subunits each with a molecular mass of 345 and 395 KD respectively. The Ki-67 protein is highly protease sensitive and is one of the most widely used proliferation probes. The Ki-67 protein is expressed in cycling cells during late G1, G2, S phase and M phase. After mitosis of the cell, the expression of Ki-67 rapidly drops off during G0 and early G1. Ki-67 is an antigen expressed in the nuclear matrix of proliferating cells and can be detected by the monoclonal antibody MIB1. Ki-67 has a very short half-life and can only be detected in proliferating cells, and like AgNORs and p53, can also be an indicator of malignancy.

Ki-67 has been widely used to evaluate squamous cell carcinoma in humans with variable results regarding its prognostic value. In veterinary medicine Ki-67 has most been
most widely studied in reference to canine and feline tumors; specifically melanotic tumors, squamous cell carcinoma, mammary gland, mast cell, lymphoma, extramedullary plasmacytomas, and testicular tumors. In each tumor type Ki-67 proliferation indices demonstrated diagnostic ability, however only in mast cell, melanotic and testicular tumors did they show any predictive value.

In the horse, Ki-67 is a less successful proliferation assay. For example, Ki-67 was not successful in discriminating between different histological forms of equine sarcoid. In a study by Kelly and Mahaffey (1998), only seven out of thirty cases of equine T and B cell lymphoma were positive for Ki-67. Conversely, a study by Roles et al, (2000) examined six equine melanocytic tumors finding a significantly higher Ki-67 immunoreactivity in the tumors than those found in normal tissue. It was therefore proposed that positive staining could not be merely associated with Ki-67 reactivity of normal tissue. The use of Ki-67 as a cell proliferation assay has therefore been limited. The hypothesis of this study is that AgNOR score, as well as p53 and Ki-67 immunoreactivity scores can be utilized in the histological diagnosis of ocular squamous epithelial tumor of the horse. These have been chosen as having the strongest diagnostic potential based on previous studies in not only horses or squamous epithelial tumors but rather a variety species and tumor types. It is the goal of this study to have these assays as available tools assisting in the often times challenging histological diagnosis between squamous cell carcinoma, squamous cell carcinoma in situ, and squamous plaque.
Experimental Methods and Design

Tissue:

Laboratory reports from the biopsy and mail in services in the Department of Pathology at the University of Georgia were reviewed to identify ocular surgical biopsy submissions diagnosed as squamous papilloma, squamous plaque, squamous cell carcinoma in situ, and invasive squamous cell carcinoma in horses. The locations included the cornea, limbus/ bulbar conjunctiva, nictitans, palpebral conjunctiva, eyelid/canthus. The hematoxylin and eosin (H&E) stained tissue sections were reviewed for the presence of adequate tissue and accuracy of diagnosis. Paraffin-embedded tissue blocks subsequently were obtained from the archival storage for further study. Any newly diagnosed cases from the clinic that were identified during the course of the study were also used as samples. An AgNOR stain, Ki-67 and p53 immunohistochemistry were performed on the samples.

Tissue preparation and processing:

Formalin-fixed, paraffin embedded tissue blocks were sectioned at 3 um. Replicate tissue sections were sectioned on Super Frost/Plus Slides (Fisher Scientific, Pittsburg, PA) and underwent deparaffinization. This process was performed in the DAKO automated stainer. Deparaffinization consisted of ten minutes in a 60C oven followed by two xylene washes, rehydration in a series of graded alcohols (100%, 95%, 70%) and rehydration in distilled water.

AgNOR staining:

Following deparaffinization and rehydration of the slides, the tissues were stained with the AgNOR working solution. This solution is a 2:1 50% nitrate to gelatin solution. The slides remained in the working solution for forty-five minutes and then were washed with deionized water and mounted with Permount media. The AgNOR score was obtained by counting the
number of AgNOR “foci” per cell in 100 cells. The total count was divided by 100 to achieve a mean AgNOR score per cell. This was repeated twice and the three means were averaged to achieve a final mean AgNOR score.

p53 Immunohistochemistry:

After deparaffinization, the slides were placed in a citrate buffer (0.01M, Ph 6.0) (Biogenex, San Ramon CA) and then heated in a microwave pressure cooker for fifteen minutes at full power and then fifteen minutes at 40% power for antigen retrieval. The sections were then allowed to cool for at least twenty minutes. In the automated stainer (DAKO, Carpinteria, CA), the sections were rinsed with Tris buffer solution (TBS) (DAKO, Carpinteria, CA). Then a universal power block (Biogenex, San Ramon, CA) was applied to the section for ten minutes and followed by a rinse with TBS solution. The primary antibody (p53, mouse, monoclonal) (BD Biogenex, San Diego, CA) in a 1:100 dilution with Antibody Plain Diluent (DAKO, Carpinteria, CA) was added to all the slides except for the negative control which received only antibody diluent. The tissue slides were allowed to incubate for one hour at room temperature and then rinsed with TBS. The secondary biotinylated antibody (Supersensitive multilinked, mouse rabbit and rat anti-immunoglobulin) (Biogenex, San Ramon, CA) was placed on all tissue sections and incubated for twenty-five minutes, and then rinsed with TBS. Then the supersensitive label, alkaline phosphatase conjugated streptavidin (Biogenex, San Ramon CA) was applied for twenty-five minutes. Fast Red stain (Biogenex, San Ramon CA) was placed on the tissue sections for two ten minute periods and then rinsed with distilled water. The sections were then counterstained with Gill II Hematoxylin stain with five dips in the solution. The tissue slides then were placed in a series of graded ethanols (95%, & 100%) and then two xylene rinses. The tissue sections were then cover slipped with Permount media. The percentage of P53
staining cell per one hundred cells was counted three times and the final percentage was an average of the three counts.

**Ki-67 Immunohistochemistry:**

After deparaffinization, the slides were placed in a citrate buffer (0.01M, Ph 6.0) (Biogenex, San Ramon CA) and then heated in a microwave pressure cooker for fifteen minutes at full power and then fifteen minutes at 40% power for antigen retrieval. The sections were then allowed to cool for at least twenty minutes. In the automated stainer (DAKO, Carpinteria, CA) the sections were rinsed with TBS). Then a universal power block (Biogenex, San Ramon, CA) was applied to the section for ten minutes and followed by a rinse with TBS solution. The primary antibody, monoclonal MIB1 antibody, (DAKO Carpinteria, CA), in a 1:100 with Antibody Plain Diluent (DAKO, Carpinteria, CA) was added to all the slides except for the negative control which received only antibody diluent. The tissue slides were allowed to incubate for one hour at room temperature and then rinsed with TBS. Secondary biotinylated antibody (Supersensitive multilinked, mouse rabbit and rat anti-immunoglobulin) (Biogenex, San Ramon, CA) was placed on all tissue sections and incubated for twenty-five minutes, and then rinsed with TBS. Then the supersensitive label, alkaline phosphatase conjugated streptavidin stain (Biogenex, San Ramon CA) was applied for twenty-five minutes. Fast Red stain (Biogenex, San Ramon CA) was placed on the tissue sections for two ten minute periods and then rinsed with distilled water. The sections were then counterstained with Gill II Hematoxylin stain with five dips in the solution. The tissue slides were then placed in a series of graded ethanols (95% & 100%) and then two xylene rinses. The tissue sections were then cover slipped with Permount media. The percentage of Ki-67 staining cells per one hundred cells was counted three times and the final percentage was an average of the three counts.
Statistical Analysis:

AgNOR scores and p53 and Ki-67 percentages were statistically analyzed to study their effectiveness as diagnostic tools and whether they are adequate indicators of malignancy. The analysis was performed using a one-way analysis of variance. A Kolmogorov-Smirnov test was used to check normality. Logarithmic transformation was applied to the AgNOR score and Ki-67 percentages. Pairwise comparisons were made between average score or percentage for different tumor types and correlation studies were performed. Statistical comparisons were conducted at 5% significance level. All analyses were performed using SAS software version 8.02. Statistical analyses were performed by the Department of Statistics Veterinary Consultants.

Results

107 archived samples of equine ocular squamous cell epithelial neoplasms were retrieved. There were 18 squamous plaques, 12 squamous cell carcinoma \textit{in situ}, and 77 invasive squamous cell carcinomas. No squamous papillomas were found. The sample population (n=48) consisted of 16 squamous plaques, 10 squamous cell carcinoma \textit{in situ} and 22 squamous cell carcinomas. The other samples were excluded either due to missing tissue block, inadequate sample size or borderline histological diagnosis.

All 22 samples of squamous cell carcinoma had both AgNOR staining as well as p53 immunohistochemistry performed, however, only 19 of the samples had Ki-67 immunohistochemistry performed due to inadequate tissue. All 10 of the squamous cell carcinoma \textit{in situ} samples had AgNOR staining performed, however, only 9 had p53 immunohistochemistry performed and 8 had Ki-67 immunohistochemistry performed due to
inadequate tissue. All of the 16 squamous plaques had AgNOR staining performed however only 15 had p53 immunohistochemistry performed and 11 had Ki-67 immunohistochemistry performed due to inadequate remaining tissue.

The mean AgNOR score for squamous cell carcinoma (n=22) was 3.02 +/- 0.52 (Figure 3.1). The mean AgNOR score for squamous cell carcinoma in situ (n=10) was 1.95 +/- 0.19 (Figure 3.2), and the mean AgNOR score for squamous plaque (n=16) was 1.58 +/- 0.25 (Figure 3.3).

The mean percentage of p53 positive staining cells for squamous cell carcinoma (n=22) was 50.8% +/- 34.9 (Figure 3.4) The mean percentage of p53 positive staining cells for squamous cell carcinoma in situ (n=9) was 49.2% +/- 37.5. (Figure 3.5) and the mean percentage of p53 positive staining cells for squamous plaque (n=15) was 4.4% +/- 5.9. (Figure 3.6) The mean percentage of Ki-67 positive staining cells for squamous cell carcinoma (n=19) was 27.4% +/- 34.5. (Figure 3.7) The mean percentage of Ki-67 positive staining cell for squamous cell carcinoma in situ (n=8) was 18.7% +/- 11.2 (Figure 3.8), and the mean percentage of Ki-67 positive staining cells for squamous plaque (n=11) was 7.6% +/- 7.34. (Figure 3.9)

The mean AgNOR score was significantly different between squamous cell carcinoma and squamous plaque (p<0.0001) and squamous cell carcinoma in situ (p<0.0001). Mean AgNOR score was also significantly different between squamous cell carcinoma in situ and squamous plaque (p=0.0005). The percentage of p53 positive staining cells was significantly different between squamous cell carcinoma and squamous plaque (p<0.0001) and squamous cell carcinoma in situ and squamous plaque (p=0.0006). The percentage of Ki-67 positive staining cells was significantly different between squamous cell carcinoma and squamous plaque (p<0.0004). (Table 3.1)
Using both Pearson (0.38247) and Spearman (0.36302) correlation coefficients there was a positive correlation between p53 and Ki-67 values for the tumor types respectively. There was a significant correlation between the p53 and the AgNOR score (p=0.0014) but not between the AgNOR score and Ki-67 staining (p=0.0648).

**Discussion**

Squamous epithelial tumors are ubiquitous neoplasms of the equine eye and adnexa. In the horse, these neoplasms can undergo progressive malignant change ranging from the benign papilloma to the pre-neoplastic plaque to squamous cell carcinoma (*in situ* and invasive). This continuum can create diagnostic challenges especially in borderline cases. Human medicine has utilized proliferation assays in a wide spectrum of tumor types. Human medicine has not only established indices to aid in the diagnosis and “quantification of malignancy” but also to gain predictive and prognostic capabilities. Veterinary medicine has more recently started to embark on exploring proliferation assays and correlating them to degree of malignancy and prognosis of tumors.

In general, the majority of proliferation assays have been studied in reference to canine and feline tumors. To our knowledge, this is the first report of AgNOR scoring in equine neoplasm. There have been few studies on the use of Ki-67 as a proliferation assay in the horse. To date, Ki-67 has only been evaluated in equine lymphoma, melanoma, and sarcomas. Ki-67 and AgNOR in humans however, have been studied in other non-epithelial tumors and in squamous epithelial tumors.

Although AgNORs have never been used to evaluate squamous epithelial tumor in the horse, nor any tumor of the horse, the studies of its use in human medicine and more limited use
in veterinary medicine justified our use to evaluate ocular squamous epithelial tumor of the horse. A few studies, such as that performed by Bratulic et al. (1996), evaluated canine mammary tumors (some being squamous cell carcinoma) and demonstrated a significant difference in the mean AgNOR score between benign and malignant tumors, as well as, prognostic value.\textsuperscript{74,75,76} These were justified based on the expansive array of human literature exploring the use of AgNORs in human mammary carcinomas and squamous cell carcinoma of the lung,\textsuperscript{36,105,106,107,108} oral cavity,\textsuperscript{109} and skin\textsuperscript{69}. It is well proven that AgNORs in human medicine are able to aid in the histologic diagnosis and have prognostic predictive value for various types of squamous epithelial neoplasms.

The results of our study supported our hypothesis that mean AgNOR scores between all types of squamous epithelial tumors are significantly different. The results of our study therefore show that AgNORs can be used as a helpful diagnostic tool in assisting discrimination between squamous epithelial tumors. Our recommendation is that, in borderline cases, an AgNOR score of 2.59 -3.53 suggests squamous cell carcinoma and an AgNOR score of 1.76- 2.14 suggests squamous cell carcinoma \textit{in situ}. An AgNOR score of less than 1.60 is suggestive of squamous plaque. It should be noted that since this is the only reported use of AgNOR scoring in equine tumors, the strength of these recommendations needs to be supported by additional studies. These studies should include both squamous epithelial tumors in horses evaluated by different investigators using different populations.

Ki-67 has limited use in equine tumors and has only exhibited poor and unreliable results. Even so, we proposed Ki-67 to be a potentially powerful assay for squamous epithelial tumors in the horse based on use in human head and neck squamous cell carcinoma,\textsuperscript{41} and limited use in canine and feline squamous epithelial tumors such as squamous cell carcinoma of the skin,\textsuperscript{95,96}
and mammary gland tumors. These studies showed that Ki-67 was a valuable proliferation assay for these tumors and in certain circumstances showed prognostic predictive power. Our results, however, were not promising for Ki-67 as a discriminative pathological diagnostic aid. There was only a significant difference in percentage of staining between the malignant squamous cell carcinoma and the squamous plaque which is usually histologically obvious and not a challenge. In the process of establishing an immunohistochemical protocol with limited previously established protocols in the horse, a variety of different monoclonal antibodies were tried before any immunostaining was achieved. Subsequently we used paired canine tissue control samples with the failed clones and achieved successful staining. This may indicate an inability of the commercially available monoclonal antibodies to react with equine tissue rather than the poor results being a reflection of the low proliferative activity of the equine neoplasms.

Unlike AgNORs and Ki-67, p53 has been utilized extensively to assess not only squamous epithelial tumors in humans, but also to a limited degree in the horse and specifically the conjunctiva of the equine eye. This discrepancy is most easily explained by the role that the p53 gene plays in the actinic solar response, which is thought to be implicated in the carcinogenesis of squamous epithelial tumors and therefore a logical choice to assess equine squamous cell carcinoma. Mutations of the p53 gene are found in 50-55% of all tumors in humans. A high rate of p53 positive cases have been reported in human pterygia and limbal tumor suggesting that the increased p53 in the limbal cells indicate p53 mutation caused by UV light radiation.

Both in horses and in dogs, p53 gene mutations have been investigated. In a study of mammary tumor in dogs, Muto et al. (2000) showed that when analyzing the exons 5-8 for the
p53 gene there were four (11%) missense mutations found in the thirty-eight benign tumors and five (20%) mutations in the twenty-five malignant tumors. A study by Gamblin et al. (1997) demonstrated nuclear over expression of p53 in numerous canine tumors, with the greatest over expression in squamous cell carcinoma, nasal adenocarcinomas, and perianal adenocarcinomas. In the horse, the role of p53 has been evaluated in sarcoids, melanocytic tumors and squamous cell carcinoma. Analysis of exons 5-9 in both equine melanocytic tumors and sarcoids did not demonstrate p53 gene mutations and suggested that the degree of p53 gene mutation in the pathogenesis of equine sarcoi and melanocytic tumors is low. Conversely, studies of equine cutaneous and ocular squamous cell carcinoma evaluating p53 over expression, as well as, mutations of exons 5-9 demonstrate the role of p53 in the tumorgenesis of squamous cell carcinoma. The single study by Sironi et al. evaluating p53 expression specifically in the conjunctiva of horses, cats and dogs revealed higher (greater than 10%) staining in moderately differentiated squamous cell carcinomas versus well-differentiated tumors. These results were suggestive of p53 mutations in that they utilized pAb 240 and DO12 antibodies which are specific for mutant not wild type p53, however, the study, only included eight samples, 5 of which were equine.

In our study, we evaluated three different types of squamous epithelial tumors (squamous plaque, squamous cell carcinoma in situ and squamous cell carcinoma), whereas previous studies have focused on SCC alone. There is a significantly higher p53 immunostaining of invasive squamous cell carcinoma (50.8%) and squamous cell carcinoma in situ (49.2%) as compared to squamous plaque (4.4%). In our study, any tumor with less than 10% p53 immunoreactivity is likely not a squamous cell carcinoma. The p53 data did little to differentiate between invasive squamous cell carcinoma and carcinoma in situ. Our data suggests that the greatest degree of
mutagenesis has occurred once the tumor has reached the malignant form of squamous cell carcinoma *in situ* and does not significantly increase during progression to invasive squamous cell carcinoma. The next step in the study of the role of p53 in the pathogenesis of ocular squamous cell carcinoma in the horse would be to evaluate the presence of mutations in exons 5-9.

In conclusion, we have shown that the selected assay can be helpful in determining the histological diagnosis of borderline cases of squamous epithelial neoplasia in horse. Both AgNORs and p53 immunostaining are useful in distinguishing between pre-malignant and malignant squamous epithelial tumor. In addition, AgNOR scores may be able to help differentiate cases of invasive and *in situ* squamous cell carcinomas. Ki-67 however was not found to be a useful indicator in our studies. Further studies should be undertaken to develop Ki-67 antibodies that are more suited for equine tissue as well as to determine whether AgNORs and p53 have the potential to serve as a prognostic indicators of long term survival and outcome.
Figure 3.1: Photomicrograph of a squamous cell carcinoma with mean AgNOR score of 3.37
Figure 3.2: Photomicrograph of a squamous cell carcinoma *in situ* with mean AgNOR score of 1.93
Figure 3.3: Photomicrograph of a squamous plaque with mean AgNOR score of 1.56
Figure 3.4: Photomicrograph of a squamous cell carcinoma with 84.7% p53 immunoreactivity
Figure 3.5: Photomicrograph of a squamous cell carcinoma *in situ* with 21.7% p53 immunoreactivity
Figure 3.6: Photomicrograph of a squamous plaque with 1% p53 immunoreactivity
Figure 3.7: Photomicrograph of a squamous cell carcinoma with 87.7% Ki-67 immunoreactivity
Figure 3.8: Photomicrograph of a squamous cell carcinoma *in situ* with 15.7% Ki-67 immunoreactivity
Figure 3.9: Photomicrograph of a squamous plaque with 3.3% Ki-67 immunoreactivity
Table 3.1: Significant Differences Between Squamous Epithelial Neoplasms for AgNOR Score, and p53 and Ki-67 Immunoreactivity

<table>
<thead>
<tr>
<th>Tumor Comparisons</th>
<th>AgNORs</th>
<th>p53</th>
<th>Ki67</th>
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<tr>
<td>Squamous cell carcinoma vs Squamous plaque</td>
<td>Significant</td>
<td>Significant</td>
<td>Significant</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.0001</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0004</td>
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<tr>
<td>Squamous cell carcinoma in situ vs Squamous plaque</td>
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<td>Not Significant</td>
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<td></td>
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<td>P=0.0006</td>
<td>P=0.0845</td>
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<tr>
<td>Squamous cell carcinoma vs Squamous cell carcinoma in situ</td>
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<td>Not Significant</td>
<td>Not Significant</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.0001</td>
<td>P=0.9141</td>
<td>P=0.0886</td>
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CHAPTER 4
CONCLUSION

Equine ocular squamous cell carcinoma is a commonly identified neoplasm of the equine eye and adnexa. Even though ocular squamous cell carcinoma is so prevalent, the diagnosis of the tumor can be quite challenging. Squamous epithelial tumors represent a continuum from the benign squamous plaque through the malignant squamous cell carcinoma and often borderline cases (e.g. squamous plaque and early squamous cell carcinoma in situ) may be a diagnostic challenge. It was our goal to achieve histopathological tools to assist in the diagnosis of these borderline cases of squamous epithelial neoplasms. The first part of the study, retrospectively determined the typical equine patient that presents to the University of Georgia with equine ocular squamous cell carcinoma and its response to treatment. The second part of the study evaluated the ability of AgNORs, p53 and Ki-67 as proliferation assays and immunohistochemical tools to assist in the histopathologic diagnosis of borderline cases of equine ocular squamous epithelial tumors.

Many important findings were elucidated in the retrospective study. The most significant however, is the superiority of radiation treatment for squamous cell carcinomas in any location as compared to treatments that did not include radiation. This information elucidates the effectiveness of radiation therapy such as strontium-90, and the importance of accessibility of such therapies in veterinary medical practices. This study was limited to the treatments used at the University of Georgia, and did not evaluate other modalities such as photodynamic therapy, 5-Flurouracil, or CO2 laser ablation. It would be useful to compile data from other institutions.
with different patient populations and treatments modalities and compare results. The most
typical presentation of a patient to the University of Georgia with ocular squamous cell
carcinoma was a Quarter Horse, chestnut, gelding approximately 12 years of age with the tumor
located on the eyelid or third eyelid. The most prevalent breed as the Quarter Horse was unique
to our study, and may be biased by the composition of the general population of patients seen at
the University of Georgia.

The second study demonstrated that AgNOR scoring and to a lesser degree p53
immunoreactivity can be useful tools to aid in the histological diagnosis of squamous epithelial
tumors in the horse. AgNOR scores can differentiate between invasive squamous cell carcinoma
and squamous plaque, squamous cell carcinoma in situ and squamous plaques, as well invasive
squamous cell carcinoma and squamous cell carcinoma in situ. Further studies to evaluate the
usefulness of this tool should include applying the AgNOR score to actual borderline cases and
prospective studies of cases of squamous plaque, squamous cell carcinoma in situ, and invasive
squamous cell carcinoma diagnosed using our scoring system. p53 immunoreactivity was useful
in differentiating between squamous cell carcinoma (both invasive and in situ) and the pre-
malignant squamous plaque. This immunostain, however, could not help distinguish between
invasive squamous cell carcinoma and squamous cell carcinoma in situ. Finally, we believe that
although Ki-67 was not a useful diagnostic assay in this study, further work to develop a better
equine anti-Ki-67 antibody would be beneficial.
REFERENCES


