CANINE LOWER URINARY TRACT UROTHELIAL CARCINOMA: RELEVANCE AS AN ANIMAL MODEL

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ABSTRACT

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Lower urinary tract urothelial carcinomas are commonly diagnosed neoplasms in humans and dogs. Similarities between human and canine urothelial carcinomas have been well described in terms of histomorphology and clinical progression. Further due to strong breed predispositions for tumor development, there are likely heritable factors that regulate carcinogenesis in the lower urinary tract of subsets of dogs. This has led to the suggestion that canine urothelial carcinomas could be used as a naturally occurring animal model. However, it is unclear what features in canine urothelial carcinomas are associated with prognostic significance or treatment response. Further, while some similarities between human and canine urothelial carcinomas are known, what similarities or differences may exist in terms of molecular features that drive carcinogenesis are largely unknown. This dissertation first examines correlations of common urothelial carcinoma markers to histologic classification, grading, and degree of bladder wall invasion in dogs relative to the histologic classification scheme accepted for humans in order strengthen the stance that there are biologic differences between proliferative urothelial lesions and histologic grades. Then, specific carcinogenesis pathways that have been suggested to play roles in epithelial-to-mesenchymal transition and that have prognostic significance in human urinary bladder urothelial carcinomas were evaluated. These included pathways that govern prostaglandin E2 regulation, cadherin swithching, and Wnt signaling. Finally,

the role of defective DNA mismatch repair (MMR) was examined. In urothelial carcinomas, evidence of MMR repair dysfunction was found and was correlated with genetic background of the dogs from which tumors originated. Additionally, canine lower urinary tract urothelial carcinoma cell lines were established which differential MMR proficiency and which had also had differential response to treatment similar to that described in humans. These studies combine to show that while there are many similarities between urothelial carcinomas in dogs and humans; there are also many differences suggesting that while further study of canine urothelial carcinoma is warranted, canine urothelial carcinoma do not perfectly recapitulate similar disease in humans

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KEY TO ABBREVIATIONS

CK7: Cytokeratin 7

COX-2: Cyclooxygenase-2

DAB: 3,3'-Diaminobenzidine

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

HPGD:15-hydroxyprostaglandin dehydrogenase

IHC: Immunohistochemistry

MMR: DNA mismatch repair

MS: Microsatellite

MSI: Microsatellite instability

PAP: Prostatic acid phosphatase

PGE_2: Prostaglandin E_2

PUNLMP: Papillary urothelial neoplasm of low malignant potential

qPCR: quantitative real-time PCR

SD: Standard deviation

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SE: Standard error

TBST: Tris-buffered saline with 0.1% Tween 20

UPIII: Uroplakin III

XTT: 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide

CHAPTER 1

Introduction and Literature Review

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Introduction: The dog as a model for urothelial carcinogenesis

Bladder cancer is a significant problem in humans. Based on current trends, 2.4% of people in the United States are predicted to develop bladder cancer during the course of their life.[1] An estimated 74,690 people have or will be diagnosed and 15,580 are expected die due to the disease in the United States during 2014.[1] In 2008, bladder cancer was reported as the fourth most common cancer diagnosis in men, and the ninth most common in women.[2] Although the majority (70-80%) of bladder cancers in humans are organ-confined and considered "superficial," the recurrence rate may be as high as 50%, and as many as 25% of these patients will progress to invasive disease, in spite of treatment.[3] Another 20-30% of patients have advanced bladder cancer at diagnosis.[4] Therapies for invasive bladder cancer are minimally effective. Even with treatment, the median survival time is less than 2 years from initial diagnosis.[4] Little improvement in survival has been observed in the past 30 years, as evidenced by slowly increasing numbers of new cases and deaths per 100,000 people over this time period.[1] From a financial view, bladder cancer is among the costliest cancers to manage and treat.[5] For these reasons, an improved understanding of urothelial carcinogenesis and novel, cost-effective therapeutic approaches are urgently needed.

Establishment and utilization of preclinical animal models beyond the typical rodent model that reflect the biology of human cancers would have great impact on drug development. Chemically induced, transgenic, and xenograft rodent models have been well described and are commonly used for the study of cancer; however, such models of often fail predict treatment success and to guide the rational development of early phase human trials.[6-8] In general, dogs offer a cost-effective, naturally occurring model for cancer in

which clinical trials are easily preformed. Among other factors, dogs are attractive for such studies because the general types and histomorphology of canine tumors are similar to those of humans, cancer is naturally occurring in dogs, drug metabolism is similar, dogs share environments with their owners and often have similar general lifestyles, the larger size of dogs in comparison to rodents allows for easier performance of many clinical procedures, and lifespans of dogs are longer than rodents, yet still relatively short in comparison to humans.[9, 10] In addition, the genetic diversity of dogs more closely mirrors that of humans than that seen in laboratory rodents, while individual breeds provide genetic similarities and often pedigree information.[11] Further, findings of studies in dogs can be applied to improving clinical outcome not only in human medicine, but also can be used in veterinary medicine.

Many features make canine lower urinary tract urothelial carcinomas attractive for study. Similar to humans, bladder cancer is common in dogs. A reported 2% of malignant tumors of the dog originate from the bladder, and most of these are derived from the urothelium.[10, 12] Such tumors occur spontaneously rather than necessitating exposure to carcinogens or genetic manipulation.[13, 14] Clinical signs including hematuria, increased frequency of urination, and stranguria are common to both dogs and humans, as are secondary bacterial urinary tract infections.[15] Macroscopic and microscopic findings are similar between dogs and humans.[16, 17] Canine bladder cancer typically follows a similar clinical course to that reported in invasive bladder carcinomas of humans suggesting that the pathways involved in the development and progression of these tumors may be homologous.[13, 15] In addition, there are strong breed predispositions for development of these cancers in dogs with Scottish terriers, West Highland white terriers, beagles, and

Shetland sheepdogs being overrepresented.[13, 14] This suggests a potential hereditary component to the development of these tumors in a subset of dogs and thus, a common molecular pathogenesis for cancer development.

Studies of molecular features associated with carcinogenesis are severely lacking in the dog; however, few studies in canine urothelial carcinomas evaluating potential cancer markers have yielded results similar to that in humans. COX-2 overexpression is common in both human and canine bladder cancer, as is the expression of prostaglandin E_2.[10, 15, 18] High levels of p53 expression has been reported in a small number of canine urothelial carcinomas, similar to reports in humans.[10] Basic fibroblast growth factor, a proangiotic factor, has been reported in the urine of affected dogs and is expressed in human urothelial carcinomas.[10] Nuclear expression of survivin, an apoptosis-inhibiting protein, is described in many human and canine urothelial carcinomas, but is not expressed in normal bladder.[19, 20] Telomerase activity is reported in 90% of human urothelial carcinomas, and has been described in canine urothelial carcinoma cell lines and in urine samples from affected dogs.[10] Expression of the receptor tyrosine kinase, epidermal growth factor receptor, which is commonly observed in human urinary bladder cancer, was significantly higher in canine urothelial carcinomas in comparison to normal urotheluim and cases of polypoid cystitis.[21] β-catenin and p63 expression were significantly lower and Ki67 expression higher in urothelial carcinomas in comparison to normal urinary bladder and cases of polypoid cystitis.[22]

While there are numerous similarities in dogs and humans in terms of urothelial carcinomas, there are also differences. In humans, men develop urothelial carcinomas twice as often as women; however in dogs, females are nearly twice as likely to be affected

as males.[1, 10, 12, 15] In dogs, the vast majority of bladder cancers occur within the trigone area, while in humans occurrence is more randomly distributed throughout the bladder.[15] This difference is interesting, as it has classically been proposed that the trigone is embryologically distinct from the remainder of the urinary bladder originating from the mesoderm derived Wolffian ducts; however, more recent studies have suggested that the trigone develops from endoderm similar to the rest of the bladder.[23, 24] More importantly, *in situ* carcinomas are only rarely identified in dogs, while they are by far the most commonly recognized form of urothelial carcinoma in humans.[13, 14, 25] This fact limits the dog as a model suggesting that not all pathways leading to urothelial carcinomas in humans are active in the dog; however, the dog can still serve as an excellent model for invasive human urothelial carcinomas.

Prognostication in canine lower urinary tract urothelial carcinomas

Naturally occurring lower tract urothelial carcinomas comprise a significant proportion of canine neoplasms; however, discrete criteria for prognostic evaluation and targeted treatment options are lacking. Up to 90% of canine urothelial tumors of the urinary bladder are diagnosed as malignant, most are highly invasive, and many metastasize to regional and distant sites.[26] Radical cystectomy is considered the treatment of choice for invasive human urothelial carcinomas, which are similar to those occurring in the dog.[10] This, however, is rarely feasible in pet dogs. Because complete surgical resection is rarely achievable in dogs and 30-50% of these cancers metastasize, most animals die of their disease.[14] Although affected dogs are frequently treated with chemotherapy, these cancers respond inconsistently. However, the choice of chemotherapeutic for urothelial carcinomas has traditionally been empirical rather than being targeted and based on scientific evidence.[14, 27] Most dogs are treated with chemotherapy and nonsteroidal anti-inflammatory drugs, which tend to be palliative and not curative. Recently, multimodality, bladder-preserving strategies for invasive human bladder cancer have been shown to be very effective in certain patients, although such approaches have not been optimized.[28]

The prognostic significance of clinico-demographic features has been evaluated in multiple studies. In general, correlations of individual with prognostic measures in such studies have been inconsistent and sometimes contradictory. This, however, may be due to low sample sizes within individual studies, differences in treatment, differences in what prognostic measure was used, or differences in grouping of cases for analysis. Most commonly, studies have employed survival time as the main prognostic indicator, but

survival time can be highly influenced by treatment, euthanasia, and other factors. Few studies have examined other measures of prognosis such as progression free interval.

In general, basic demographic information such as age, sex, and breed are unlikely to influence prognosis; however, reports vary. In one study, sex was associated with survival time with spayed females surviving significantly longer than castrated males.[29] In another study, there was no difference in survival time when sex.[30] One study found a negative correlation between breed and survival time with at-risk breeds having shorter survival.[31] Other studies have found no significant association between survival time and breed.[15, 29, 32] Age at diagnosis has been suggested to not be associated with survival time in multiple studies. [29, 30] In addition, patient weight at the time of diagnosis was not associated with survival in at least one study.[32]

Features such as tumor location as it relates to potential uretheral obstruction, clinical stage, and surgical respectability are likely to influence clinical outcome, but correlation of prognostic measures with such factors has varied between study. Ultrasonographic evidence of wall involvement, heterogeneity of masses, and location of tumors within the trigone had significant negative associations with survival time.[33] In addition, urethral involvement has been suggested to have a significant negative association with survival in multiple studies.[31, 34] However, other studies have found no association between survival time and location of the tumor or urethral involvement. [35, 36] One study found negative associations between progression free interval and presence of metastasis at the time of presentation and negative associations between survival time and the presence of distant metastasis.[31] Other studies in which clinical staging was preformed found no statistical association between survival time and variable features including T

stage, N stage, M stage, or presence of nodal or other metastasis.[15, 30, 32, 37] In terms of surgical resection, a statistically significant longer survival time was associated with complete resection compared to only incomplete resection, and in another study, surgical debulking prolonged survival, but did not prolong progression free survival.[34, 38]

Multiple histologic grading schemes have been proposed largely based on systems developed for use in humans. In 1995, Valli, et al., described a classification and grading system for use in canine bladder and urethral cancer. [17] This scheme was based on the 1986 version of the World Health Organization classification and grading scheme for proliferative urothelial in human bladder cancer.[39] In 2006, Patrick, et al., showed that the canine urothelial carcinomas could be classified according to the updated World Health Organization/International Society for Urologic Pathology consensus system used for classifying and grading proliferative urothelial lesions in humans, as reported in 1998 and updated in 2004.[16, 40, 41] More recently, Knapp et al., suggested a simplified two-tier version of grading classifying urothelial carcinomas as either high or low grade.[15]

While histologic classification and grading likely have relevance, the prognostic significance is unclear. In application, Valli et al. found that there were significant correlations with tumor grade and depth of invasion, tumor grade and presence of metastases, and peritumoral desmoplasia and metastases.[17] Regarding survival time, Valli, et al., also found significant correlations between survival and tumor grade when comparing grade 2 and 3 to grade 1 tumors, but no significant difference in survival between grades 2 and 3.[17] Further, there were also no significant differences between survival and type of invasion (tentacular or broad) or macroscopic tumor architecture (flat or papillary).[17] Two independent studies employing Valli's system of grading found no

significance between histologic grades of urothelial carcinoma; however, overall architecture was not specifically examined in either study, analysis in one study compared a combination of grades 1 and 2 to grade 3, and the other study only included and thus, only compared grades 2 and 3.[29, 35] No studies have specifically evaluated the prognostic significance of histologic classification and grading as described by Patrick, et al., or Knapp, et al.

Aside from grading, individual histologic factors have rarely been evaluated with respect to prognostic significance. The presence of necrosis within urothelial carcinomas was negatively statistically associated with survival time in one study.[33] Other studies have found no significance correlation between survival time and mitotic index or lymphatic invasion [29, 33]

Few studies have evaluated the prognostic significance of molecular markers with regards to canine urothelial carcinomas, and the few such studies that have attempted to correlate molecular markers with prognosis have often not found statistical significance. The expression of p63 is an exception as low p63 levels have been associated with vessel invasion, metastasis, and short survival time.[22] No statistical significance was detected between survival time and immunoreactivity for the chemotherapy resistance markers P-glycoprotein and glutathione-S-transferase π, or factor VIII-related antigen, which was used as a marker for angiogenesis.[29] Expression of epidermal growth factor receptor was not significantly associated with vessel invasion, lymph node metastasis, or survival time in canine urothelial carcinomas.[21]

Based on the relatively sparse and disparate results of studies examining prognosis with regards to demographic, clinical, histopathologic and molecular features, it is clear

that additional studies are needed. Prognostication of cancer, especially in veterinary medicine, from a diagnostic and pathologic perspective has classically relied on classification and grading based on histopathologic criteria. More recently, there has been a paradigm shift to move beyond H&E, and to improve general prognostication and prediction of treatment response by exploiting the molecular constitution of a given tumor. Molecular features that could be exploited to better prognosticate canine urothelial carcinomas likely exist; however, a better understanding of the pathways that drive carcinogenesis is obviously required.

Outline of studies

Optimization of therapy for canine urothelial carcinomas and acceptance of the dog as a model for urothelial carcinogenesis is hampered by a lack of understanding of the inherent features that affect prognosis and knowledge of the molecular constitution of individual tumors. Numerous studies have examined associations of canine urothelial carcinomas with risk factors for development and response to specific treatments; however, relatively few have examined markers of prognostication or molecular features that underlie carcinogenesis of these cancers. While it is clear that similarities between lower urinary tract urothelial carcinomas in humans and dogs exist in terms of general histomorphology and clinical progression, it is less clear whether the molecular pathways that drive carcinogenesis are the same. An understanding of the pathologic basis for lower urinary tract cancer in dogs is needed from a prognostic and treatment perspective in veterinary medicine and to further establish the dog as a relevant model for human disease.

Given these facts the main aims of the studies presented in this dissertation are as follows:

- To demonstrate biologic and prognostic significance for histomorphologic classification and grading of canine lower urinary tract urothelial carcinomas
- To evaluate components of specific carcinogenesis pathways in canine lower urinary tract urothelial carcinomas with the goal of identifying molecular prognostic markers and therapeutic targets

- To compare and contrast urothelial carcinogenesis in the lower urinary tract of dogs to that reported in humans with the goal of better establishing the dog as a relevant model for human disease

Studies are laid out in four chapters followed by a conclusion that presents a summary of findings and discussion of limitations and future directions. The background and goals of each chapter are briefly described here, and expanded upon within the chapters themselves.

Chapter 2

Chapter 2 examines the biologic significance of the histologic classification and grading of canine lower urinary tract urothelial carcinoma with respect to the distribution of a set of immunohistochemical markers that have been associated with differentiation and/or prognosis in humans. The central hypothesis for this work was that there are significant differences in the expression of the evaluated markers between histologic classifications and grades of proliferative urothelial lesions. The goal in examining this hypothesis was to provide rationale for use of this classification and grading system in dogs; however, this study was limited by the fact that follow-up information regarding outcome was not available.

The World Health Organization (WHO)/International Society of Urologic Pathology (ISUP) Consensus Classification System published in 1998 and updated in 2004 has been demonstrated to be significantly associated with clinical outcome in humans.[42-50] While Patrick, et al., demonstrated that canine proliferative urothelial lesions could easily be

classified according to the system based on histomorphology, the significance of this system in terms of prognostication in dogs remains unclear.[16] To further characterize differences between histologic classification and grade beyond histomorphology, we examined the expression of uroplakin III, cytokeratin 7, COX-2, and caspase 3 in a set of canine proliferative urothelial carcinomas including neoplastic and non-neoplastic lesions.

Uroplakin III and cytokeratin 7 are expressed by urothelium and used as differentiation markers for diagnostic purposes in dogs.[51-60] In humans, loss of UPIII expression is frequently seen in metastatic sites of high-grade tumors and is associated with lymphovascular invasion, stage, and grade.[60, 61] No such associations have previously been found in dogs; however, studies have not focused on the distribution pattern of expression as was evaluated in this case series.

The expression of the inducible enzyme COX-2 and the subsequent production of prostaglandin E_2 have significant roles in carcinogenesis, including immunosuppression, inhibition of apoptosis, increasing the metastatic potential of neoplastic epithelial cells, promoting drug resistance, and stimulating angiogenesis.[62-67] Numerous studies have variably shown significant correlations between COX-2 expression and tumor grade, invasion, metastasis, and survival.[68-75]

Caspase-3 is an executioner protein that is activated by both the intrinsic and extrinsic pathways of apoptosis.[76-78] Due to the fact that this protein is activated late in the apoptotic pathway, immunohistochemical detection of activated caspase-3 has been used to evaluate apoptotic rate.[79, 80] In human bladder cancers, expression of caspase-3 has been suggested as to have prognostic significance.[81-83]

Chapter 3

Chapter 3 investigates the potential relationship between mechanisms governing prostaglandin E_2 regulation and epithelial-to-mesenchymal transition in terms of cadherin expression and Wnt signaling in canine lower urinary tract urothelial carcinomas. As canine urothelial carcinomas are often highly invasive and metastasis is common, it is likely that these tumors often undergo epithelial-to-mesenchymal transition during progression, gaining the ability to invade. Based on studies in human urothelial carcinomas, it has been suggested that loss expression of 15-hydroxyprostaglandin dehydrogenase (HPGD) is associated with loss of E-cadherin expression and development of an invasive phenotype. Our central hypothesis for this study was that altered expression of COX-2 and PGDH in canine urothelial carcinomas is associated with aberrant expression of adhesion-associated cadherin proteins, higher histologic grade, invasion, and shorter survival times.

Prostaglandins are inflammatory mediators implicated in avoidance of apoptosis, angiogenesis, cellular proliferation, invasion and metastasis in cancer.[66, 84-88] COX-2 is an inducible enzyme that is up regulated in a variety of inflammatory and neoplastic processes and that acts in the production of prostaglandins. Increased expression of COX-2 by neoplastic cells has been associated with worsening prognosis.[66, 89] As numerous COX inhibitors are available, COX-2 is a prime therapeutic target in many cancers. Unfortunately, COX-2 inhibition has been associated with side effects including life-threatening cardiovascular effects, best recognized in association with the infamous drug, Vioxx.[90] HPGD, on the other hand, inactivates prostaglandin E_2.[10] Decreased expression of HPGD results in increased amounts of prostaglandin within tissues and has been associated with worsening prognosis in cancers.[91, 92] Further, a recent study showed specific single-

nucleotide polymorphisms within the HPGD gene were associated with both increased risk of colon cancer development and decreased expression of HPGD in the colon overall.[93] Developing a better view of the prostaglandin pathways, the consequences of altered expression, and how this impacts treatment response could lead to the development of better clinical strategies to address canine urothelial cancer.

The cadherins are a group of membrane-associated molecules involved in cell-cell adhesion. As such, these proteins play integral roles in embryogenesis and development, cellular polarity, and carcinogenesis.[94] E-cadherin is a primary mediator of cell-cell adhesion in epithelial cells and is strongly expressed in the urothelium of the bladder. N-cadherin is predominately expressed by mesenchymal cells in adults, but is expressed by epithelial cells during embryogenesis. P-cadherin is most prominently expressed in the placenta, but is also expressed by basal cells of stratified epithelia. Decreased expression of E-cadherin and increased expression of other cadherins (termed cadherin switching) result in decreased strength of cell-cell adhesions and an increased propensity for cellular migration.[94-96] In terms of carcinogenesis, cadherin switching is observed in epithelial to mesenchymal transitions and has been associated with increased invasiveness and metastasis. [94-97]

Because cadherins form associations with other proteins within cells, they are integrally linked to intracellular signaling and trafficking. β-catenin binds to the cytoplasmic tail of cadherin molecules. In this capacity it functions in cell-cell adhesion, but it is also involved in a variety of signaling pathways including some that are involved in carcinogenesis, such as the Wnt pathways.[95, 98, 99] While loss of cadherin expression alone has not been shown to directly result in intracellular signaling, altered expression of

cadherins has been shown to amplify or buffer the effects of such pathways.[95, 98, 99] This is thought to be due to loss of the association and sequestration of β-catenin in cadherin junctional complexes as cadherin expression is lost.

It has recently been shown that cadherin expression is integrally linked to HPGD. HPGD expression was shown to increase in expression with urothelial differentiation and inhibition of PGDH expression resulted in disruption of E-cadherin expression at cell-cell junction in cell lines.[100] In non-small cell lung cancer of humans, it was shown that exogenous prostaglandin can decrease E-cadherin expression, and that such changes are mediated by the specific transition repressors, ZEB1 and Snail.[101] In squamous cell carcinomas, administration of prostaglandin E2 or prostaglandin receptor agonists lead to decreased expression of E-cadherin and internalization of this molecule into the cytoplasm.[102]

Changes in the expression of all of the above mentioned molecules have been reported in urothelial carcinomas and many are targets for treatment making them of particular interest for further study. COX-2 over expression has been described in a significant number of urothelial carcinomas of humans and increasing expression is associated with invasiveness, metastasis, and increased mortality.[84, 89] Previous studies of COX-2 expression have yielded similar results in dogs.[86] Treatment studies with COX-2 inhibitors, however, have met mixed results. *In vitro* studies have suggested that treatment of urothelial carcinoma cell lines with such compounds can result in decreased invasiveness and decreased tumor grade.[103] Results of i*n vivo* studies vary with only a proportion of studies showing a decreased risk for tumor development.[87, 103-105] PGDH has been shown to be important in urothelial differentiation and its expression is decreased in malignancies.

E-cadherin and B-catenin expression is often down regulated in urothelial carcinomas.[106, 107] Such down regulation is associated with higher degrees of neoplastic infiltration.[107] Mutations in E-cadherin genes are rarely implicated in carcinogenesis.[98] Rather, silencing of E-cadherin expression is most often an epigenetic change. As such, numerous classes of drugs have been suggested to up regulate E-cadherin expression.[98] N-cadherin expression has been shown to be up regulated in urothelial carcinomas and in bladder cancer cell lines.[85] The compound (-)-epigallocatechin-3-gallate, which is found in green tea, has been associated with down regulation of N-cadherin and decreased migration in bladder cancer cell lines.[85] Increased P-cadherin expression has been associated with a significantly worse bladder cancer-specific survival and a more malignant and invasive cancer phenotype in humans.[106]

Chapter 4

Chapter 4 examines the potential role of DNA mismatch repair (MMR) in carcinogenesis of canine lower urinary tract urothelial carcinomas through evaluation of microsatellite instability and expression of MMR proteins. Hereditary deficiency of MMR in humans is associated with syndromes of cancer development throughout the body, but MMR is often defective in sporadic cancers as well. Based on the fact that MMR repair deficiency is hereditary in humans and that there are strong breed predispositions for cancer in dogs, we hypothesized that deficiencies in MMR contributed to carcinogenesis of hereditary cancers in dogs including urothelial carcinomas. To evaluate MMR in dogs, we examined the prevalence of microsatellite instability (MSI) in a set of cancer types that have breed predispositions including urothelial carcinomas. After demonstrating MSI in

urothelial carcinomas, we subsequently evaluated MMR protein expression.

The DNA mismatch repair (MMR) system participates in a variety of cellular processes. Most notably, this system is responsible for post replication recognition and repair of base-base mismatches and the resolution of insertion and deletion loops that can occur in repetitive regions of DNA.[108-112] Accordingly, deficiencies in this system can lead to increased rates of point and frame shift mutations throughout the genome. Such mutations can lead to loss of function changes in tumor suppressors or gain of function changes in tumor oncogenes.[113-117] In addition, the MMR system also participates in a variety of other processes including DNA damage recognition signaling, promoting cell cycle arrest and apoptosis, homologous recombination, meiotic recombination, and other DNA repair pathways.[108, 109, 111, 118] Given the varied roles played by MMR in maintenance of DNA integrity and signaling, it is not surprising that defects in MMR facilitate carcinogenesis and affect response to treatment.

MMR function has most often been evaluated through analysis of microsatellites. Microsatellites are regions of nucleotide repeats located throughout the genome. These areas are prone to polymerase slippage during replication leading to formation of small insertion and deletion loops.[119] If not recognized and repaired by MMR, buildup of frame shift mutations occurs within microsatellites.[112, 118] In cancers with defects in the MMR system, a high percentage of microsatellites have recognizable mutations. Such buildup of mutations within microsatellites is termed microsatellite instability (MSI) and is considered a "signature" for MMR dysfuntion.[118, 120]

In terms of prognosis, MMR deficient cancers are generally associated with a more favorable clinical outcome than those that are MMR proficient.[121-125] In colorectal

carcinomas, for example, MMR deficient tumors are associated with longer survival times and a decreased risk of metastasis compared to those that are proficient.[124] The reason for this difference in prognosis is likely multifactorial. Inherently, cancers with defects in MMR are genetically less stable that those with intact MMR leading to an increase in DNA lesions, which promotes cell cycle arrest and apoptosis signaling.[126] Also, there is evidence that MMR deficient cancer cells are often highly immunogenic. This is proposed to occur due to production of atypical proteins generated through frameshift mutations resulting in T cell mediated immune responses directed against the cancer cells.[127] Additionally, it has been shown that several genes associated with antitumor immune responses are over expressed in MMR deficient cancers and cell lines.[126]

Urinary carcinomas of the urinary bladder represent one of the many cancer types in which a significant percentage of tumors have been reported to show defects in MMR in humans. Development of urothelial carcinomas of the urinary bladder has been associated with both hereditary and spontaneously developing MMR deficiency. High MSI and a particular form of MSI known as elevated microsatellite instability at selected tetranucleotide repeats (EMAST) have been described in urothelial carcinomas.[121] Differential expression of MMR proteins including MSH2, MSH3, and MLH1 has been associated with urothelial carcinoma grade and clinical outcome in humans.[121, 125, 128]

Chapter 5

Chapter 5 describes the characterization of canine lower urinary tract urothelial carcinoma cell lines and the evaluation of their MMR capacity. Further, chapter 4 also describes the assessment of sensitivity to chemotherapeutic therapy *in vitro* as influenced

by variable MMR proficiency.

The potential application of MMR as a therapeutic target is highlighted by the differences observed in prognosis and the response to treatment between cancers of the same type that differ in MMR capacity. Having shown in chapter 3 that MSI and decreased expression of the MMR protein, MSH2, is common in canine lower urinary tract urothelial carcinomas, we hypothesized that variance in MMR would be reflected by differences in response to chemotherapeutics. To investigate this hypothesis, four canine urothelial carcinoma cell lines were established and characterized with the goal of generating *in vitro* canine urothelial carcinoma models. We were able to show that one such cell line had decreased relative expression of MMR proteins and genes, and considered this line MMR deficient. Based on this, we preformed survival assays exposing MMR proficient and deficient cell lines to a panel of chemotherapeutics to show that there are differences in response to treatment, thus highlighting MMR proficiency as a target for selective therapy.

The effects of MMR deficiency on chemotherapeutic response are complex. MMR deficiency is capable of conferring either drug resistance or sensitivity according to the drugs mechanism of action.[123, 129-137] For example, MMR deficiency results in drug resistance to the fluorinated pyrimidine analog 5-FU as well as certain alkylating agents including the SN1 methylators, temozolomide and dacarbazine.[123, 129, 134, 135, 137] In contrast, MMR deficient cells are highly sensitive to many of the interstrand cross-linking alkylators, including CCNU and mitomycin C.[129, 137] There have also been reports of differences in response of cancers with varying MMR capacity to some drugs within the same class. Resistance has been reported to platinum containing compounds cisplatin and carboplatin in MMR deficient cancers, while no such resistance has been reported to oxaliplatin.[123, 134]

The differential effect of the MMR system in response to drugs probably reflects the ability of the MMR machinery to participate in a number of alternative DNA damage processing/signaling pathways. In the case of simple methylated bases in the DNA molecule, it has been proposed that MMR may be involved in triggering apoptosis through either futile repair attempts or through conversion of the alkylated base to a lethal lesion such as a double strand break.[137] In contrast, an increased sensitivity to cytotoxins can be observed if defective MMR results in failure to repair certain types of DNA lesions. For example, certain interstrand cross-links are recognized by the MMR system and repair is thought to occur through MMR mediated homologous recombination.[129] Cells deficient in MMR do not effectively repair these cross-links and are extremely sensitive to agents that induce such these lesions.

Additionally, there has been a great deal of recent attention to the targeting of DNA repair defective tumor cells through inhibition of coordinating DNA repair systems. Such an approach has been termed synthetic lethality.[133] The basis for this paradigm is that due to molecular redundancies, cells may tolerate loss of function of a single pathway that participates in DNA repair. However, when another coordinating pathway is inhibited, the result is cell death. New evidence suggests that inhibition of particular DNA polymerases is synthetically lethal in cells that have MMR defects.[133] Also, drugs that are known to cause oxidative damage such as methotrexate have been suggested to have a potential synthetic lethal relationship with deficiencies in MMR.[123]

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CHAPTER 2

Differences in Expression of Uroplakin III, Cytokeratin-7, and COX-2 in Canine Proliferative Urothelial Lesions of the Urinary Bladder

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Abstract

The expression of immunohistochemical markers that have been used in diagnosis and/or prognostication of urothelial tumors in humans (uroplakin III (UPIII), cytokeratin-7 (CK7), cyclooxygenase-2 (COX-2), and activated caspase 3) was evaluated in a series of 99 canine proliferative urothelial lesions of the urinary bladder and compared to the lesion classification and grade as defined by the WHO/ISUP Consensus System. There were significant associations between tumor classification and overall UPIII pattern ($P=1.49x10^{-18}$), loss of UPIII ($P=1.27x10^{-4}$), overall CK7 pattern ($P=4.34x10^{-18}$), and COX-2 pattern ($P=8.12x10^{-25}$). In addition, there were significant associations between depth of neoplastic cell infiltration into the urinary bladder wall and overall UPIII pattern ($P=1.54x10^{-14}$), loss of UPIII ($P=2.07x10^{-4}$), overall CK7 pattern ($P=1.17x10^{-13}$), loss of CK7 expression ($P=0.0485$), and COX-2 pattern ($P=8.23x10^{-21}$). There were no significant associations between tumor classification or infiltration and caspase-3 expression pattern.

Introduction

Proliferative lesions of the urothelium of the canine urinary bladder range from benign polyps and papillomas to carcinomas with varying metastatic potential. Also, inflammatory conditions such as polypoid cystitis can form tumor-like masses within the urinary bladder that can be confused with urothelial neoplasms. Classification of urothelial proliferative lesions and histologic grading of urothelial carcinomas is key to accurate prognostication and treatment selection. A classification and grading scheme based on the 1986 World Health Organization classification scheme for human urinary bladder and urethral cancer was proposed in 1995 for use in evaluating urothelial neoplasms in dogs based on pattern of growth, nuclear atypia, and degree of infiltration into the urinary bladder wall.[50] In the initial description of this system's use in dogs, significant correlations between tumor grade and depth of infiltration, the presence of metastases, and survival time were found. However, a subsequent smaller study failed to find a significant correlation between grade and prognosis.[41] To our knowledge, this scheme is not widely used in dogs and has been replaced in human medicine by more modern classification schemes. There remains a need for a clear, reproducible, and well-accepted classification and grading scheme for urothelial proliferative lesions in dogs.

Currently, the most widely accepted scheme for classification and grading of proliferative urothelial lesions in humans is the World Health Organization (WHO)/International Society of Urologic Pathology (ISUP) Consensus Classification System published in 1998 and updated in 2004.[6,7] In multiple studies, the WHO/ISUP Consensus Classification system has been demonstrated to be significantly associated with clinical outcome.[2,32-34,37,42,43,51,59] It has long been known that canine urothelial neoplasms are

similar to urothelial neoplasms in humans in terms of morphology, biologic behavior and response to chemotherapy.[14,18,19,27,28,59] Recently, Patrick et al. examined the potential use of the WHO/ISUP Consensus Classification System in classifying canine proliferative urothelial lesions.[36] In that study, the authors demonstrated that the histomorphology of proliferative urothelial lesions was homologous between dogs and humans, and that canine lesions could easily be classified according to the system; however, data regarding the prognostic relevance of classification system remains lacking. Additional study of the biologic differences between classifications and grades of proliferative urothelial lesions in dogs is needed.

Uroplakins comprise a group of membrane-associated proteins expressed by urothelial cells that are important for cell-to-cell adhesion and maintenance of water impermeability.[57,58] These proteins form a plaque-like complex along the apical membrane of the umbrella cells that form the most superficial layer of the urothelium.[57] Cytokeratin 7 (CK7) is a cytokeratin expressed by simple epithelium as well as differentiated urothelial cells and a variety of carcinomas in humans including those of urothelial, pancreatic, cholangiolar, and ovarian origin.[49,52] Uroplakin III (UPIII) and CK7 are used in both dogs and humans as diagnostic markers of urothelial differentiation in primary tumors and metastases.[8,13,31,35,39,47] In humans, loss of UPIII has been associated with several prognostic features.[13,25]

The inducible enzyme COX-2 and the resulting production of prostaglandin E_2 have been ascribed significant roles in carcinogenesis, including immunosuppression, inhibition of apoptosis, increased metastatic potential of neoplastic epithelial cells, promotion of drug resistance, and stimulation of angiogenesis.[9,11,23,54-56] Numerous studies have shown

significant correlations between COX-2 expression and tumor grade, infiltration, metastasis, and survival.[21,24,30,40,44-46,53] Concordantly, a decreased risk for urinary bladder cancer development has been seen in humans undergoing long-term non-steroidal anti-inflammatory therapy and in vitro and in vivo studies have suggested potential use of COX-2 inhibitors in treatment.[3,5,10,29,53] In humans and dogs, COX-2 is not expressed by normal urothelium of the urinary bladder.[17,24] Substantial expression of COX-2, however, has been observed in transitional cell carcinomas.[17,20,22]

Caspase 3 is an effector or executioner caspase that is activated by both intrinsic and extrinsic apoptosis signaling pathways to cleave multiple cellular structural and repair proteins.[4,12,38] Due to the fact that this protein is activated late in the apoptotic pathway, immunohistochemical detection of activated caspase-3 has been used to evaluate apoptotic rate.[1,48] In human urinary bladder cancers, expression of caspase-3 has been suggested to have prognostic significance.[15,16,26]

The goals of the current study were twofold: 1) to evaluate the expression of UPIII, CK7, COX-2, and caspase 3 in non-neoplastic and neoplastic proliferative lesions of the canine urothelium of the urinary bladder, and 2) to correlate the observed patterns of expression of each of these markers with specific lesion classification and grade as defined by the WHO/ISUP Consensus Classification System.

Materials and methods

Selection of Cases and Histologic Classification

A series of 99 formalin-fixed, paraffin-embedded proliferative urothelial lesions from 99 dogs that had been submitted as diagnostic cases were selected from the tissue archives of the Michigan State University, Diagnostic Center for Population and Animal Health (DCPAH). Of these 99, 93 had previously been classified and where applicable graded using the WHO/ISUP Consensus Classification System.[36] Six additional diagnostic cases that presented to DCPAH, including additional low-grade neoplasms, were included in the study set. For grading of these additional samples, 5 μm sections of all samples were routinely processed and stained with hematoxylin and eosin for microscopic examination. Proliferative urothelial lesions were categorized according to the WHO/ISUP Consensus Classification System as previously described and summarized in Table 1.[36] For urothelial carcinomas, the degree of infiltration into the urinary bladder wall was scored as no infiltration, infiltration into the substantia propria, or infiltration into the tunica muscularis.

Of the 99 proliferative urothelial lesions examined, 44 were non-neoplastic and categorized as either urothelial polyps or polypoid cystitis. Of the 55 neoplasms, there were two urothelial papillomas and one papillary urothelial neoplasm of low malignant potential (PUNLMP). The remainders of the urothelial neoplasms were papillary urothelial carcinomas of varying grade. Low-grade (grade 1) papillary urothelial carcinomas were rare with only 2 being included in the set. Both of these had some degree of infiltration into the urinary bladder wall with one sample having infiltrative clusters of neoplastic

urothelial cells within the muscularis. Papillary carcinomas infiltrated at least into the substantia propria in 49/52 (94%) and into the muscularis in 21/52 (40%) of cases.

Immunohistochemistry

Five μm sections of all samples were processed for immunohistochemistry and labeled with a mouse monoclonal anti-UPIII antibody (1:5, RDI, Fitzgerald Industries Intl, Concord, MA, USA), a mouse monoclonal anti-CK7 antibody (1:75, Dako Cytomation, Carpentaria, CA, USA), a rabbit polyclonal anti-COX-2 antibody (1:100. Cayman Chemical Company, Ann Arbor, MI, USA), or rabbit polyclonal anti-activated caspase-3 antibody (1:5,000, RDI, Fitzgerald Industries Intl, Concord, MA, USA). Deparaffinization, antigen retrieval, immunohistochemical labeling with 3,3'-diaminobenzidine (DAB) chromogen and counterstaining with hematoxylin were performed on the Bond maXTM Automated Staining System (Vision BioSystemsTM, Leica, Bannockburn, IL, USA) using the BondTM Polymer Detection System (Vision BioSystemsTM, Leica, Bannockburn, IL, USA). Sections of normal canine urothelium were similarly labeled as positive controls for UPIII and CK7. A canine squamous cell carcinoma known to express COX-2 and a lymph node with large numbers of activated caspase-3-positive cells were respectively used as positive controls for these antibodies. For negative controls, homologous non-immune sera or buffer replaced primary antibodies.

Immunoreactivity for UPIII and CK7 was scored according to overall pattern within the urothelium and partial loss of immunoreactivity. Immunoreactivity for UPIII and CK7 in positively labeled cells was variably perimembranous, predominately cytoplasmic without distinct perimembrane labeling, or associated with both the membrane the cell membrane and the cytoplasm. Specifically, for overall pattern of UPIII and CK7 immunoreactivity

within the urothelium: pattern 1 was defined by labeling limited to the most superficial layer of cells (Fig. 1 and 4), pattern 2 was defined by labeling extending to the middle layer of the urothelium (Fig. 2), pattern 3 was defined by cells labeling throughout the full thickness of the urothelium (Fig. 5), and pattern 4 was defined by immunoreactivity that was patchy and randomly distributed (Fig. 3 and 6). Partial loss of immunoreactivity was noted when greater than 50% of epithelial cells were immunonegative and there were areas within the proliferative urothelium with no immunopositive cells in at least 2 contiguous high power (400X) fields.

Immunoreactivity for COX-2 and activated caspase 3 was classified according to overall pattern within the urothelium. Cells immunoreactive for COX-2 had diffuse cytoplasmic to perinuclear labeling. Cells immunoreactive for activated caspase 3 had diffuse or, more often, finely granular labeling within the cytoplasm and/or nucleus. In pattern 1 for these markers, immunoreactivity was limited to the superficial 1-3 cell layers (Fig. 7). In Pattern 2, cells were labeled throughout the full thickness of the urothelium (Fig. 8). In pattern 3, immunoreactivity was patchy and randomly distributed throughout the proliferative urothelium, but greater than 15% of neoplastic cells were positively labeled (Fig. 9).

For each marker, any section that completely lacked immunoreactivity was excluded from analysis, as it could not be determined whether this was true loss of expression or was artifactual.

Statistical Analysis

Statistical Analysis Software (SAS) version 9.1.3 (2002, SAS Institute Inc, Cary, NC) was used for the data analysis. Fisher exact test was used to test the association between

grade or degree of infiltration and the pattern of immunoreactivity for all evaluated markers and loss of immunoreactivity for UPIII and CK7. For all statistical analyses, lesions were categorized as follows: non-neoplastic lesions (urothelial polyps and polypoid cystitis), low-grade neoplasms (urothelial papillomas, papillary urothelial neoplasms of low malignant potential, and grade 1 urothelial carcinomas), grade 2 urothelial carcinomas, and grade 3 urothelial carcinomas. Significance was set at P=0.05.

Results

Immunohistochemistry: UPIII and CK7

The urothelium of control urinary bladders and areas of normal urothelium in tumor samples demonstrated strong expression of UPIII and CK7 diffusely throughout the superficial layers (umbrella cells). This distribution of immunolabeling typified UPIII and CK7 pattern 1.

In the hyperplastic urothelium of polyps and polypoid cystitis, expression of UPIII and CK7 was generally limited to the superficial (umbrella) cell layers (UPIII pattern 1; CK7 pattern 1) or extended only to the mid-portion of the urothelium (UPIII pattern 2). Only one urothelial polyp had UPIII pattern 4 and none had CK7 pattern 4. The PUNLMP and urothelial papillomas demonstrated UPIII pattern 3 and CK7 pattern 2 or 3. With few exceptions, papillary carcinomas of all grades demonstrated patchy randomly distributed UPIII expression consistent with pattern 4. Partial loss of UPIII expression was not detected in any grade I papillary carcinoma and in only one grade II papillary carcinoma. In contrast, 14/33 (42%) grade III papillary carcinomas had partial loss of expression of UPIII. More grade II and III carcinomas had a CK7 pattern 3, than had a UPIII pattern 3 (11/50 CK7 pattern 3 cases compared to 3/50 UPIII pattern 3 cases). The majority (71%) of grade II and III urothelial carcinomas, however, had a CK7 pattern 4 similar to that observed for UPIII. Only grades II and III urothelial carcinomas had significant loss of CK7 expression (Tables 2 and 3).

Immunohistochemistry: COX-2

The squamous cell carcinoma from the digit of a dog used as a positive control demonstrated strong positive cytoplasmic and mainly perinuclear immunoreactivity for

COX-2 in 30% of neoplastic cells. There was no positive immunoreactivity for COX-2 in any part of the normal urothelium from control urinary bladders; however, in the superficial layers of non-proliferative urothelium adjacent to proliferative lesions, there were often a small percentage (less than 10%) of COX-2 positive cells.

In all proliferative lesions, at least 10% of proliferative urothelial cells exhibited positive COX-2 immunoreactivity. In urothelial polyps and polypoid cystitis, COX-2 expression in the proliferative urothelium was usually restricted to the superficial layers (COX-2 pattern 1) or was less commonly full thickness (COX-2 pattern 2). Only one polyp had randomly distributed and patchy expression of COX-2 (COX-2 pattern 3). The PUNLMP and the two papillomas demonstrated COX-2 pattern 1 while the grade I papillary carcinomas exhibited either pattern 2 or 3. All high-grade (grades II and III) papillary urothelial carcinomas in which COX-2 expression was detected exhibited COX-2 pattern 3 (Table 4).

Immunohistochemistry: Activated caspase 3

Approximately 10% of cells in the lymph node used as a positive control for activated caspase 3 had positive finely granular labeling of the cytoplasm and/or nucleus. In the normal urothelium of control urinary bladders and adjacent to proliferative lesions, immunoreactive cells comprised less than 10% of the total urothelium and were largely limited to the superficial 1-2 cell layers. The total percentage of caspase 3 immunoreactive cells ranged from 10-40% in all other proliferative lesions; however, there was no appreciable variation in the percentage of positive cells between different tumor types. Immunoreactivity for activated caspase 3 was most commonly noted in the superficial most cell layers of all proliferative lesions (caspase 3 pattern 1), but was also seen both

diffusely throughout the urothelium (caspase 3 pattern 2) and in a randomly distributed patchy distribution (caspase 3 pattern 3)(Table 5).

A summary of the predominant patterns of immunoreactivity for UPIII, CK7, and COX-2 observed in each proliferative urothelial lesion classification is presented in Table 6.

Statistical Analysis

Using Fisher's exact test there were significant associations between tumor classification and overall UPIII pattern ($P=1.49x10^{-18}$), loss of UPIII ($P=1.27x10^{-4}$), overall CK7 pattern ($P=4.34x10^{-18}$), and COX-2 pattern ($P=8.12x10^{-25}$). Also by Fisher's exact test, there were significant associations between depth of neoplastic cell infiltration and overall UPIII pattern ($P=1.54x10^{-14}$), loss of UPIII ($P=2.07x10^{-4}$), overall CK7 pattern ($P=1.17x10^{-13}$), loss of CK7 expression ($P=0.0485$), and COX-2 pattern ($P=8.23x10^{-21}$). There were no significant associations between tumor classification and loss of CK7 or caspase-3 pattern. There were also no significant associations between depth of infiltration and caspase-3 expression.

Discussion

In the current study, strong differences in expression of UPIII, CK7, and COX-2 were observed between urothelial polyps and cases of polypoid cystitis, urothelial papillomas and papillary urothelial neoplasms of low malignant potential, and papillary carcinomas. However, no association was observed between the expression of activated caspase-3 and tumor classification, grade, or depth of infiltration into the urinary bladder wall.

Overall, urothelial polyps and cases of polypoid cystitis predominately had expression of UPIII and CK7 that either was limited to the superficial-most cell layers (UPIII/CK7 pattern 1) or extended to the middle-most cell layer (UPIII/CK7 pattern 2). Urothelial papillomas and a papillary urothelial neoplasm of low malignant potential had UPIII and CK7 expression throughout the full thickness of the urothelium or extending at least up to the middle-most cell layer. The majority of papillary carcinomas, in contrast, had randomly distributed patchy immunoreactivity for UPIII and CK7 (UPIII/CK7 pattern 4). In addition, there was often partial loss of UPIII and CK7 expression in high-grade carcinomas that invaded into the urinary bladder wall.

Based on the association with infiltration into the urinary bladder wall, loss of UPIII and CK7 in urothelial carcinomas may suggest a lack of differentiation or epithelial-mesenchymal transition in that favors infiltration. In humans, loss of UPIII expression is frequently seen in metastases of high-grade tumors and has been associated with lymphovascular infiltration, stage, and grade.[13,25] Loss of cell membrane adhesion molecules may also result in an increased propensity for metastasis in dogs; however, information on clinical outcome including presence of metastasis and survival was not available for the examined canine tumor set. The observed significant loss of UPIII and CK7

in many high-grade carcinomas suggests that when used as diagnostic markers on small specimens, some urothelial carcinomas might not be positively labeled.

There was a clear difference in the location of COX-2-positive cells within the urothelium between non-neoplastic and neoplastic lesions as well as between papillary urothelial carcinomas with different degrees of infiltration into the urinary bladder wall. Urothelial polyps, cases of polypoid cystitis, urothelial papillomas, and the one PUNLMP exhibited expression of COX-2 in the superficial layers of the urothelium (COX-2 pattern 1) or rarely diffusely throughout the urothelium (COX-2 pattern 2). In contrast, papillary carcinomas had a randomly distributed, patchy pattern of COX-2 expression (COX-2 pattern 3).

Previous studies of COX-2 in canine urinary bladder urothelium have focused on the difference in COX-2 expression between normal urinary bladder and urothelial carcinomas, and reported differences as a percentage of COX-2 positive cells. In the current study, COX-2 immunoreactivity was observed in the majority of non-neoplastic and neoplastic lesions, often in a relatively high percentage of cells, precluding the use of percentage of positive cells as a differentiating criterion. In one previous immunohistochemical study, only 30/52 (58%) canine TCC demonstrated COX-2 expression by IHC.[22] In another study of 18 canine TCC, all tumors expressed COX-2 and three showed more than 30% of cells to be positive for COX-2.[20] The reasons for discrepancy between these studies in terms of the numbers of cases and number of cells within a given case that are positively labeled for COX-2 may be due to the low number of cases examined in each study, differing methods of fixation and processing of the tissues, differences in immunohistochemical methods including the anti-COX-2 antibody used, or inter-observer variation.

To our knowledge, COX-2 expression has not been previously evaluated in non-neoplastic proliferative urothelial lesions of the urinary bladder in dogs. In contrast to normal urothelium from urinary bladders used as controls, all urothelial polyps and cases of polypoid cystitis had expression of COX-2. Urothelial polyps and polypoid cystitis are often associated with mucosal irritation such as might occur with cystoliths. Expression of COX-2 often throughout the superficial portion or full thickness of the proliferative urothelium of non-neoplastic lesions observed in the current study might represent a consequence of such surface irritation.

Overall, there were distinct differences in the patterns of UPIII, CK7, and COX-2 expression in canine urothelial proliferative lesions of the urinary bladder as defined and categorized by the WHO/ISUP Consensus Classification System. As the cases included in the study set were comprised of diagnostic samples, reliable follow-up data regarding clinical outcome was not available. Definitive prospective studies of the clinical outcome using these markers and the WHO/ISUP Consensus Classification System in dogs remain lacking, but this study encourages the continued prognostic evaluation of the WHO/ISUP Consensus Classification system and of these immunohistochemical markers in canine proliferative urothelial lesions.

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APPENDIX

Figure 1: *Urinary Bladder; Dog. Hyperplastic urotheluim with Uroplakin III (UPIII) Pattern 1*: Immunolabeling (brown) is limited to the superficial 1-2 cell layers. 3,3'-Diaminobenzidine (DAB) chromogen, hematoxylin counterstain.

Figure 2: *Urinary Bladder; Dog. Hyperplastic urothelium with UPIII IHC Pattern 2*: UPIII is expressed strongly by all but the most basal cell layers. DAB chromogen, hematoxylin counterstain.

Figure 3*: Urinary Bladder; Dog. Papillary urothelial carcinoma grade II with UPIII IHC Pattern 4*: UPIII expression is randomly distributed throughout the neoplasm. Individual or small clusters of neoplastic cells strongly express UPIII, while numerous islands of neoplastic cells lack expression. DAB chromogen, hematoxylin counterstain.

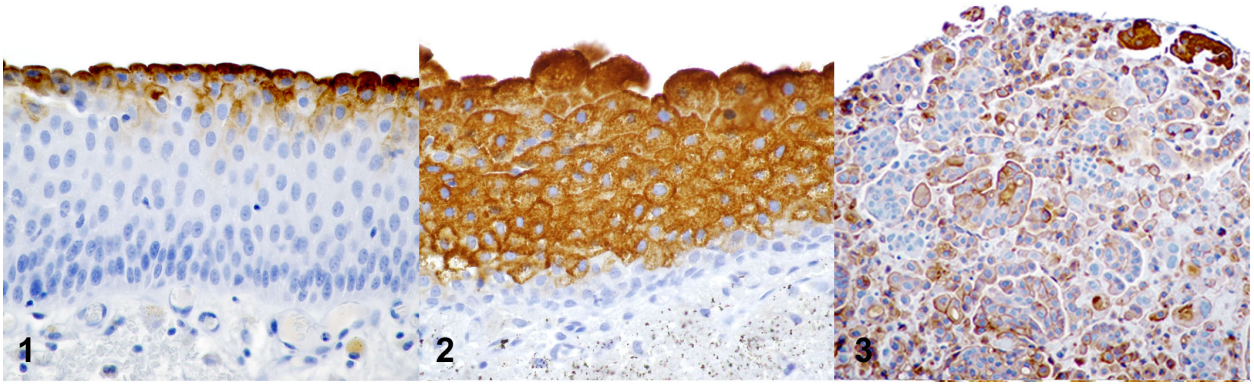

Figure 4*: Urinary Bladder; Dog. Hyperplastic urotheluim with Cytokeratin 7 (CK7) IHC Pattern 1*: Strong expression of CK7 is limited to the superficial 1-2 cell layers. DAB chromogen, hematoxylin counterstain.

Figure 5: *Urinary Bladder; Dog. Papillary urothelial carcinoma grade II with CK7 IHC Pattern 3*: CK7 is strongly expressed by all cell layers. DAB chromogen, hematoxylin counterstain.

Figure 6: *Urinary Bladder; Dog. Papillary urothelial carcinoma grade II with CK7 IHC Pattern 4*: CK7 expression is patchy. While most cells have strong expression of CK7, individual or small groups of neoplastic cells randomly distributed throughout the mass lack expression. DAB chromogen, hematoxylin counterstain.

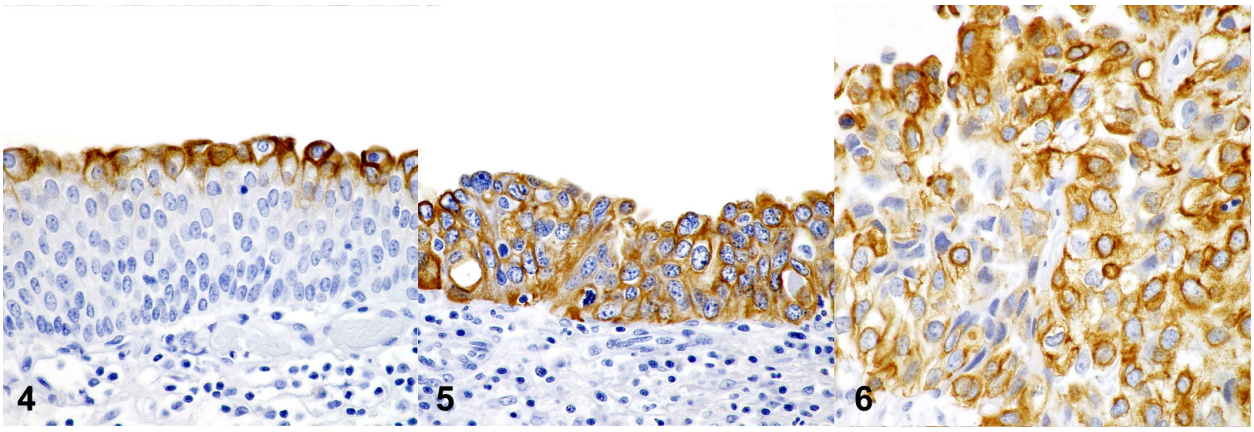

Figure 7: *Urinary Bladder; Dog. Hyperplastic urotheluim with Cyclooxygenase-2 (COX-2) IHC Pattern 1*: Immunolabeling for COX-2 is limited to the superficial 1-2 cell layers. DAB chromogen, hematoxylin counterstain.

Figure 8: *Urinary Bladder; Dog. Hyperplastic urotheluim with COX-2 IHC Pattern 2*: COX-2 is strongly expressed by cells throughout all cell layers. DAB chromogen, hematoxylin counterstain.

Figure 9: *Urinary Bladder; Dog. Papillary urothelial carcinoma grade II with COX-2 IHC Pattern 3*: Cells that strongly express COX-2 are randomly distributed throughout the neoplastic cell population. DAB chromogen, hematoxylin counterstain.

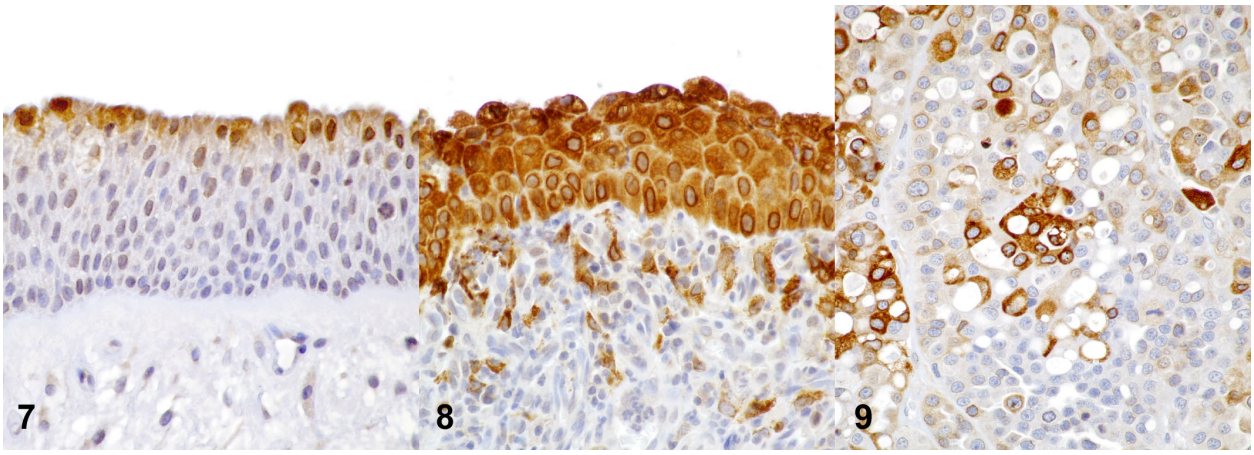

Table 1: *Histologic Features of Proliferative Urothelial Lesions according to the WHO/ISUP Consensus Classification System**

Classification	**Mitoses**	**Histologic Characteristics**
Non-neoplastic Lesions		
Polyp/Polypoid Cystitis	Rare and confined to the basal cell layers	Exophytic protrusions of mucosa and supporting fibrovascular stroma lacking true papillary fronds; often associated with stromal edema and inflammation; may occur as single fibroepithelial polyp or in multiples as polypoid cystitis
Neoplastic Lesions		
Urothelial Papilloma	Rare and confined to the basal cell layer	<6 cell layers lining papillary fronds; orderly arrangement of cells; nuclei are uniform in size, shape, and chromatin staining
PUNLMP**	Rare and confined to the basal cell layer	>6 cell layers lining papillary fronds; orderly arrangement of cells; nuclei are uniform in size, shape, and chromatin staining
Papillary carcinoma		
Grade I	Infrequent and limited to the basal 1/2 of the epithelium	Orderly appearance with recognizable variation in architectural or cytological features at low magnification; mild anisokaryosis with variable chromatin staining
Grade II	Low to moderate numbers throughout all levels of the urothelium with possible atypia	Overall disorderly appearance with retainment of some degree of polarity; irregular clustering and disorganization of cells; moderate anaplasia; moderate anisokaryosis with prominent nucleoli and clumped chromatin
Grade III	High numbers throughout all levels with common atypia	Complete loss of polarity; irregular clustering and disorganization of cells; marked pleomorphism, anisocytosis, and anisokaryosis; prominent nucleoli and clumped chromatin

**Adapted from descriptions made by Patrick et al. 2006[36]*

***Papillary urothelial neoplasm of low malignant potential*

Table 2: *Immunohistochemical Scoring of Uroplakin III Expression in Canine Proliferative Urothelial Lesions by Overall Pattern*

Classification	Pattern 1	Pattern 2	Pattern 3	Pattern 4	Loss
Polyp/Polypoid Cystitis	19/44 (43%)	20/44 (46%)	3/44 (7%)	1/44 (2%)	1/44 (2%)
Urothelial Papilloma	0	0	1/1 (100%)	0	0
PUNLMP*	0	0	1/1 (100%)	0	0
Papillary Carcinoma					
Grade I	0	0	0	2/2 (100%)	0
Grade II	1/17 (6%)	0	1/17 (6%)	15/17 (88%)	1/17 (6%)
Grade III	2/33 (6%)	0	2/33 (6%)	27/33 (82%)	14/33 (42%)

**Papillary urothelial neoplasm of low malignant potential*

Table 3: *Immunohistochemical Scoring of Cytokeratin 7 Expression in Canine Proliferative Urothelial Lesions by Overall Pattern*

Classification	Pattern 1	Pattern 2	Pattern 3	Pattern 4	Loss
Polyp/Polypoid Cystitis	16/44 (36%)	25/44 (57%)	3/44 (7%)	0	0
Urothelial Papilloma	0	1/2 (50%)	1/2 (50%)	0	0
PUNLMP*	0	0	1/1 (100%)	0	0
Papillary Carcinoma					
Grade I	0	1/2 (50%)	0	1/2 (50%)	0
Grade II	2/17 (12%)	0	5/17 (29%)	10/17 (59%)	2/17 (12%)
Grade III	0	1/33 (3%)	6/33 (18%)	25/33 (76%)	1/33 (3%)

**Papillary urothelial neoplasm of low malignant potential*

Table 4: *Immunohistochemical Scoring of COX-2 Expression in Canine Proliferative Urothelial Lesions by Overall Pattern*

Classification	**Pattern 1**	**Pattern 2**	**Pattern 3**
Polyp/Polypoid Cystitis	32/44 (73%)	11/44 (25%	1/44 (2%)
Urothelial Papilloma	1/2 (50%)	1/2 (50%)	0
PUNLMP*	1/1 (100%)	0	0
Papillary Carcinoma			
Grade I	1/2 (50%)	0	1/2 (50%)
Grade II	0	0	17/17 (100%)
Grade III	0	0	33/33 (100%)

**Papillary urothelial neoplasm of low malignant potential*

Table 5: *Immunohistochemical Scoring of Activated Caspase-3 in Canine Proliferative Urothelial Lesions by Overall Pattern*

Classification	**Pattern 1**	**Pattern 2**	**Pattern 3**
Polyp/Polypoid Cystitis	27/44 (61%)	13/44 (30%)	4/44 (9%)
Urothelial Papilloma	1/2 (50%)	0	1/2 (50%)
PUNLMP*	1/1 (100%)	0	0
Papillary Carcinoma			
Grade I	1/2 (50%)	0	1/2 (50%)
Grade II	10/16 (63%)	3/16 (19%)	3/16 (19%)
Grade III	22/30 (73%)	4/30 (13%)	4/30 (13%)

**Papillary urothelial neoplasm of low malignant potential*

Table 6: *Summary of Patterns of Immunoreactivity of Uroplakin III, Cytokeratin 7, and COX-2 in Proliferative Urothelial Lesions*

Classification	Uroplakin III	Cytokeratin 7	COX-2
Polyp/Polypoid Cystitis	89% had expression limited to cells in the superficial-most cell layer (pattern 1) or extending to the middle-most layer (pattern 2)	93% had expression limited to cells in the superficial-most cell layer (pattern 1) or extending to the middle-most layer (pattern 2)	98% had expression limited to the superficial 1/3 of cell layers (pattern 1) or throughout all cell layers (pattern 2)
Urothelial Papilloma/PUNLMP*	All cases had expression throughout all cell layers (pattern 3)	All cases had expression that extending to the middle-most layer of the urotheluim (pattern 2) or throughout all cell layers (pattern 3)	All cases had expression limited to the superficial 1/3 of cell layers (pattern 1) or throughout all cell layers (pattern 2)
Papillary Carcinoma	83% had randomly distributed, patchy expression (pattern 4)	21% had expression throughout all cell layers (pattern 3); 68% had randomly distributed, patchy expression (pattern 4)	98% had randomly distributed and patchy expression (pattern 3)
Grade I	No significant loss of expression was observed	No significant loss of expression was observed	50% had expression limited to the superficial-most 1/3 of cell layers (pattern 1); 50% had randomly distributed, patchy expression (pattern 3)
Grade II	6% had significant areas of expression loss	12% had significant areas of expression loss	All cases had randomly distributed, patchy expression (pattern 3)
Grade III	42% had significant areas of expression loss	3% had significant areas of expression loss	All cases had randomly distributed, patchy expression (pattern 3)

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CHAPTER 3

Evaluation of 15-hydroxyprostaglandin dehydrogenase (HPGD), cyclooxygenase-2 (COX-2), cadherin, and β-catenin expression in canine urinary bladder urothelial carcinomas

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Abstract

There likely are links between prostaglandin regulation, cadherin switching, and Wnt signaling in the carcinogenesis of urinary bladder urothelial carcinomas. Cadherin switching is described and decreased expression of 15-hydroxyprostaglandin dehydrogenase (HPGD), which metabolizes prostaglandin E2, is associated with loss of E-cadherin expression in human urothelial carcinomas. Aberrant expression of β-catenin, an integral part of Wnt signaling, is associated with changes in prostaglandin E2 and cadherin expression in other carcinomas. Using the dog as a model, the expression of HPGD, cyclooxygenase-2 (COX-2), β-catenin, and E-, P-, and N-cadherin was evaluated in canine normal urinary bladders and urothelial carcinomas, and urothelial carcinoma cell lines. Using immunohistochemistry, normal canine urinary bladders and low grade, noninvasive canine urothelial carcinomas expressed HPGD in superficial epithelial cells, lacked COX-2, and expressed E- and P-cadherin and β-catenin along cell membranes. In comparison, a significant proportion of high grade, infiltrative urothelial carcinomas exhibited loss of HPGD, increased COX-2, decreased P-cadherin, and aberrant localization of β-catenin expression within neoplastic cells. E-cadherin was expressed in all canine urothelial carcinomas regardless of HPGD expression. N-cadherin was not expressed in normal or neoplastic canine urothelium. Western blots demonstrated that none of the canine urothelial carcinoma cell lines expressed HPGD or N-cadherin, but all expressed E- and P-cadherin. In contrast, human urothelial carcinoma cell lines examined in parallel had loss of HPGD had loss of E- and P-cadherin expression and gain of N-cadherin expression. These data suggest that while these pathways may be related in both canine and human urothelial carcinomas, regulation is somewhat different between dogs and humans.

Introduction

Increased expression of prostaglandin E_2 (PGE_2) has been associated with carcinogenesis in many epithelial tissues and has specifically been implicated in avoidance of apoptosis, angiogenesis, cellular proliferation, invasion, and metastasis in cancer.[1-6] The overall expression of PGE_2 within a given tissue is dependent on the rates of its synthesis from arachadonic acid and its metabolism by 15-hydroxyprostaglandin dehydrogenase (HPGD). Carcinogenesis research concerning PGE_2 has most intensely focused on the synthesis side of expression. Specifically, cyclooxygenase-2 (COX-2), an inducible enzyme that metabolizes arachidonic acid to the PGE_2 precursor prostaglandin H_2, has been shown to be upregulated in carcinomas arising in numerous tissues including the urothelium of the urinary bladder.[2, 7] More recently, a loss of HPGD has been documented in various carcinomas suggesting that a decrease in PGE_2 degradation also plays a role in carcinogenesis.[8, 9] The exact mechanisms that drive PGE_2 associated carcinogenesis are unclear and likely multifactorial, but there is some evidence that changes in PGE_2 regulation are related to cadherin expression and the Wnt signaling pathway through changes in β-catenin expression.[10-12]

Cadherins are membrane-associated proteins that mediate cell-cell and/or cell-matrix interactions. Loss of cadherins typically expressed by epithelial cells such as E- and P-cadherin and/or switching of the expression of cadherins, often to N-cadherin, have been associated with increased invasiveness, increased metastatic potential, and an overall epithelial-mesenchymal transition in many types of carcinomas.[13-16] Regulation of cadherin expression has recently been linked to the regulation of PGE_2 in some types of carcinomas including, but not limited to urothelial carcinomas, squamous cell carcinomas, and non-

small cell lung cancer.[17-19]

In addition to the potential effects altered metabolism of PGE_2 has on cadherin expression, alterations in prostaglandin regulation may affect urothelial carcinogenesis through the Wnt signaling pathway. While numerous factors can affect Wnt signaling, the end result of Wnt signaling is the accumulation and translocation of β-catenin into the nucleus where it interacts with transcription factors resulting in the expression of cancer associated genes. β-catenin is normally sequestered along the cell membrane due to its association with intracellular portions of cadherins. Although loss of cadherin expression alone cannot lead to Wnt signaling, changes in cadherin expression can free β-catenin from its normal membrane localization favoring its accumulation within the cytoplasm and nucleus.[3,6] Thus, aberrant expression of PGE_2 due to either increased production or decreased degradation may lead to increased Wnt pathway signaling in association with changes in cadherin expression.

In urothelial carcinomas of humans, it has recently been shown that decreased expression of HPGD and increased PGE_2 expression are associated with a more invasive phenotype and worse prognosis making this neoplasm ideal for the study of the role of aberrant prostaglandin regulation in carcinogenesis.[12, 19] Interestingly, decreased expression of HPGD in neoplastic cells has been associated with loss of E-cadherin. Given such loss of E-cadherin in urothelial carcinomas, it is reasonable to postulate that cadherin switching driven by alterations in prostaglandin regulation occurs in the development of urothelial carcinomas. Also, it is plausible that changes in both prostaglandin expression and cadherin expression would affect Wnt signaling through changes in β-catenin expression.

Dogs are recognized as outstanding models for many human diseases including some cancers.[20] Specific to the current study, dogs spontaneously develop bladder cancers that histologically resemble human bladder cancers and that have a similar clinical course.[21, 22 23] Given their similarity to human urothelial carcinomas in both morphology and progression, it is plausible that canine urothelial carcinomas have features of carcinogenesis homologous to those in humans. As such, dogs may prove pivotal in studies into specific pathways of urothelial carcinogenesis as well as provide a nonhuman model for therapeutic manipulation. To investigate the significance of prostaglandin regulation pathways, cadherin switching, and Wnt signaling in canine urothelial carcinomas and potential applicability of the dog as a model for these specific carcinogenesis pathways, we evaluated the expression of HPGD, COX-2, E-cadherin, P-cadherin, N-cadherin, and β-catenin in canine urothelial carcinoma cell lines and in ex vivo tissues.

Materials and methods

Selection of Cases and Histologic Classification

A series of 36 dogs that were diagnosed with urothelial carcinomas at either the Michigan State University Veterinary Teaching Hospital or the University of Minnesota Veterinary Teaching Hospital through biopsy were selected for inclusion in the study based on owner consent, availability of clinical history, and availability of paraffin-embedded, formalin-fixed diagnostic samples. For each urothelial carcinoma case, descriptive information was obtained from medical records and periodic follow-up questionnaires including age at diagnosis, breed, sex, survival time from the date of diagnosis, the reported cause of death, and any treatment employed. In addition, bladder samples from 10 healthy dogs that were used for veterinary student teaching purposes were harvested in 10% buffered formalin and routinely processed for histologic examination. Five μm sections of all samples were routinely processed and stained with hematoxylin and eosin. Urothelial proliferative lesions were classified and graded according to the WHO/ISUP Consensus Classification System as previously described in canine urothelial tumors.[22] In addition, the degree of invasion of all carcinomas into the wall of the urinary bladder was scored as no invasion, invasion into the substantia propria, or invasion into the muscularis.

Immunohistochemistry

Immunohistochemistry (IHC) was used to evaluate the expression of HPGD, COX-2, β-catenin, and E-, N-, and P-cadherin in normal canine urinary bladder and canine urothelial carcinomas. Five μm sections of all formalin-fixed, paraffin-embedded tissues were processed for immunohistochemistry and labeled with a rabbit monoclonal anti-HPGD antibody (1:100, Sigma-Aldrich, St. Louis, MO, USA), a rabbit polyclonal anti-COX-2

antibody (1:100. Cayman Chemical Company, Ann Arbor, MI, USA), a rabbit monoclonal anti-β-catenin antibody (1:1000, Abcam, Cambridge, MA, USA), a mouse polyclonal anti-E-cadherin antibody (1:300, BD Biosciences, San Jose, CA, USA), a mouse monoclonal anti-N-cadherin (1:100, Invitrogen, Life Technologies, Grand Island, NY, USA), or a mouse monoclonal anti-P-cadherin antibody (1:100, NovoCastra, Leica Biosystems, Buffalo Grove, IL USA). For HPGD, β-catenin, and E-, N-, and P-cadherin, deparaffinization, antigen retrieval, immunohistochemical staining and counterstaining was performed on a Bond maX™ Automated Staining System (Leica Biosystems, Buffalo Grove, IL, USA) using the Bond™ Polymer Refine Detection System (Leica Biosystems, Buffalo Grove, IL, USA), which employs a 3,3' diaminobenzidine tetrahydrochloride (DAB) chromogen detection system. For COX-2, deparaffinization, antigen retrieval, immunohistochemical staining and counterstaining was preformed on a Benchmark XT™ autostainer (Ventana, Tucson, AZ, USA) using and an Enhanced Alkaline Phosphatase Red Detection Kit (Ventana) that uses an indirect biotin streptavidin and Fast Red chromogen detection system. Retrieval for HPGD was accomplished by incubation for 20 minutes with EnVision™ FLEX Target Retrieval Solution, Low pH (Dako, Carpinteria, CA). Retrieval for β-catenin and E- and N-cadherin was accomplished using heat induced epitope retrieval and incubation with Bond™ Epitope Retrieval Solution 1 (Leica Biosystems) for 20 minutes. Retrieval for P-cadherin was accomplished using heat induced epitope retrieval and incubation with Bond™ Epitope Retrieval Solution 1 (Leica Biosystems) for 20 minutes. Positive controls using appropriate canine tissues were ran in parallel to cases for each of the IHC protocols as follows: normal bladder for HPGD, squamous cell carcinoma for COX-2, haired skin and normal bladder for β-catenin and E-cadherin, heart for N-cadherin, and uterus for P-

cadherin. For negative controls, homologous non-immune sera or buffer replaced primary antibodies.

For each case, immunoreactivity for HPGD was scored by the percentage of positively labeled cells and the location of immunoreactivity within urothelium (Fig. 10). Specifically, percentage of urothelium expressing HPGD was categorized as none, <5%, 5-15%, 15-30% or >30%. Location of HPGD was categorized as Pattern 1 when limited to the superficial 1-3 cell layers and diffuse throughout these cell layers; Pattern 2 when superficial, but patchy within the urothelium with <80% of the total superficial urothelium being labeled; Pattern 3 when diffuse throughout the full thickness of urothelium; or Pattern 4 when randomly distributed patchy areas of immunoreactivity with <80% of the total urothelium being labeled. Immunoreactivity for COX-2 was scored by percentage of positive cells with expression being categorized as none, <5%, 5-15%, 15-30% or >30% (Fig. 11). For E- and P-cadherin, immunoreactivity was scored by location within urothelium and within cells, intensity of immunolabeling, and degree of loss (Fig. 12 and 13). Location of E- and P- expression was categorized as membrane associated, cytoplasmic only, or mixed cytoplasmic with strong membrane labeling. Intensity was subjectively categorized as weak, moderate, or strong. Loss of E- and P-cadherin expression was categorized as no loss, patchy loss, or extensive loss. For analysis, aberrant P-cadherin expression was defined as extensive loss of expression or cytoplasmic expression. For N-cadherin, only presence or absence of immunoreactivity within the urothelium was evaluated. β-catenin expression was scored by percentage of cells within each lesion with membrane, cytoplasmic, mixed membrane and cytoplasmic, and nuclear expression (Fig.

14). Aberrant β-catenin expression within a given case was defined as > or = to 40% of cell showing cytoplasmic labeling or > or = to 10% of cells showing nuclear labeling.

Statistical Analysis

Chi square tests performed with SPSS statistical software (Somers, NY, USA) was used to evaluate correlations between histologic classifications, degree of anaplasia, depth of infiltration, and immunohistochemical expression of examined markers. SPSS statistical software (Somers, NY) was also used to analyze correlations between survival time of canine urinary carcinomas and clinico-demographic data, histologic classification and grading, and immunohistochemical expression of examined markers using Kaplan-Meier estimators with log rank tests, and univariate and multivariate Cox proportional hazard modeling. For all statistical analysis, significance was defined as $p<0.05$.

Western blots

In order to 1) validate antibody use in dogs and 2) establish an *in vitro* model for future studies, IHC and Western blotting were used to evaluate expression of these molecules in a variety of normal canine tissues and canine and human urothelial carcinoma cell lines. Eight canine urothelial carcinoma cell lines (ANGUS, AXA, AXC, KINSEY, K9TCC, NK, TYLER1, TYLER2) and seven human urothelial carcinoma cell lines (RT4, UC2, UC3, UC6, UC12, UC14, 5637) were cultured in 100mm plates in 1:1 DMEM/F12 media with 10% fetal bovine serum, l-glutamine, and penicillin/streptomycin. Cells were harvested using a cell scraper and protein was subsequently isolated through incubation with RIPA buffer. Total protein for each was quantitated using Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA), a Victor X3 microplate reader (PerkinElmer, Shelton, CT USA) reading absorbance at 750nm, and comparison to a dilution series of bovine serum

albumin. Equally loaded proteins isolates were separated using SDS-PAGE. For PGDH, COX-2 blots, proteins were separated using 12% gels (BioRad, Hercules, CA, USA). For E-, N, and P-cadherin and ß-catenin blots, proteins were separated using 7.5% gels (BioRad, Hercules, USA). Percision Plus Protein™, Dual Color (BioRad, Hercules, USA) was used for protein standards. Proteins were transferred to nitrocellulose by electroblotting. Following washes in TBST, blots were blocked using 3% bovine serum albumin. Blots were incubated overnight with the primary antibodies used for IHC at respective concentrations of 1:000, 1:500, 1:200, 1:5000, 1:1000, 1:200 for HPGD, COX-2, ß-catenin, and E-, N- and P-cadherin in 3% powdered milk. Following washes in TBST, blots were accordingly incubated for 2 hours with either 1:2000 goat anti-rabbit IgG or 1:7500 goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA). Following washes in TBST, blots were developed using a chemiluminescence detection system (Thermo Scientific, Rockford, IL, USA) and using Amersham Hyperfilm™ MP autoradiography film (GE Healthcare, Little Chalfont, BM, UK). Blots were subsequently stripped for 1 hour using Western blot stripping solution (Thermo Scientific, Rockford, IL, USA). Following washes in TBST and blocking in 3% BSA, blots were incubated overnight with goat anti-GAPDH primary antibody. Following washes in TBST, blots were incubated for 2 hours with donkey anti-goat secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA). Following washes in TBST, blots were developed using a chemiluminescence detection system (Thermo Scientific, Rockford, IL, USA) and chemilumiescence was detected using Amersham Hyperfilm™ MP radiographic film (GE Healthcare, Little Chalfont, BM, UK).

Results

Demographic information, histologic classification, and grading of urothelial carcinomas

For dogs diagnosed with urothelial carcinomas, the mean age at initial diagnosis was 10.5 years of age (2.0 SD) with a range of 6.7 to 17.1 years of age. Affected dogs included 4 intact females, 21 spayed females, and 11 castrated males. Represented breeds included 5 Scottish Terriers, 2 West Highland White Terriers, 7 Beagles, 7 Shetland Sheepdogs, and 14 dogs of other breed or mixed breed. For the 32 cases in which information regarding treatment was available, 7 had no treatment, 2 had only local resection of the mass, 17 were treated with only piroxicam, and 6 were treated with piroxicam, surgical resection, and systemic chemotherapy which variably included adriamycin, cyclophosphamide, and/or mitoxantrone. Two animals were alive at the end of study, 1 animal died from causes unrelated to the urinary carcinoma, 30 reportedly died or were euthanized as a result of progressive disease related to the urinary bladder tumors, and 3 were lost to follow-up.

Of the 36 urothelial carcinomas, 8 were diagnosed as infiltrating carcinomas, which had no appreciable exophytic papillary component, but instead primarily infiltrated into and expanded the bladder wall. The remaining 28 cases were papillary carcinomas, the vast majority of which (24 of 28) were high grade (grade II or III). Invasion of high-grade papillary carcinomas into the bladder wall was common with 9 of 24 having invasion into the substantia propria and 7 of 24 invading into the muscularis. For all carcinomas, the mean survival time was 326 days with a standard deviation of 341 and range of 0 to 1225 days. For infiltrative carcinomas, the mean survival time was 173 days with a standard deviation of 223 days and range of 0 to 365 days. For papillary carcinomas, the mean

survival time was 370 days with a standard deviation of 359 days and range of 0 to 1225 days.

Immunohistochemistry: HPGD

There was diffuse to rarely patchy superficial immunoreactivity for HPGD consistent with a HPGD Pattern 1 or 2 in all but one of ten examined control bladders from normal dogs. The percentage of urothelial cells that were immunoreactive for HPGD was highly variable between control bladders ranging from <5 to >30%. The vast majority of urothelial carcinomas had either extensive lack of immunoreactivity (14/36) or had Pattern 4, patchy immunoreactivity randomly scattered throughout the tumors (14/36) with the total percent of cells expressing HPGD ranging from <5 to >30%, similar to that seen in control bladders (Table 7).

Comparing urothelial carcinomas and urothelium from normal controls, there was no significant difference between the overall percent of cells within the urothelium expressing HPGD ($p=0.205$), but there was a significant difference between the patterns of HPGD expression ($p<0.0001$). Examining only urothelial carcinomas, there was no significant correlation between percentage of cells within the urothelium expressing HPGD and classification as infiltrative or papillary ($p=0.094$), degree of anaplasia ($p=0.70$), or depth of invasion into the bladder wall ($p=0.547$). There was also no significant correlation between pattern of HPGD expression and classification as infiltrative or papillary ($p=0.121$), degree of anaplasia ($p=0.484$), or depth of invasion into the bladder wall ($p=0.109$).

Immunohistochemistry: COX-2

Less than 5% of cells within the urothelium expressed COX-2 in all of the normal control urinary bladders and 8 of 10 had no appreciable immunoreactivity for COX-2. In contrast, 25 of 36 urothelial carcinomas had 5% or greater urothelial cells with expression of COX-2 and 14 of these had more than 30% of urothelial cells with immunoreactivity.

Comparing urothelial carcinomas and urothelium of normal controls, there was a significant difference between the overall percent of cells within the urothelium expressing COX-2 ($p<0.001$). Examining only urothelial carcinomas, there were statistically significant associations between COX-2 expression and degree of anaplasia ($p=0.048$) and depth of invasion into the urinary bladder wall ($p=0.017$), with COX-2 often being expressed in high percentages of neoplastic urothelial cells in urothelial carcinomas with anaplasia grades 2 and 3, and in carcinomas with invasion into the muscularis of the urinary bladder. There was no significant correlation between percentage of cells within the urothelium expressing COX-2 and classification of the urothelial carcinomas as infiltrative or papillary ($p=0.481$).

Immunohistochemistry: Cadherins

All normal control bladders had strong to rarely moderate (1/10 cases), membranous immunoreactivity for E-cadherin and no significant loss of expression. The majority (25/36) of urothelial carcinomas had membranous immunoreactivity for E-cadherin, 5/36 had predominately cytoplasmic immunoreactivity, and 2/36 had both membranous and cytoplasmic immunoreactivity. Twenty-nine of 36 urothelial carcinomas had moderate to strong immunoreactivity, and 3 had only weak immunoreactivity. Six urothelial carcinomas had significant loss of E-cadherin expression that was most often patchy (5/6 cases), and rarely extensive (1/6 cases).

All ten normal control urinary bladders had strong membranous immunoreactivity for P-cadherin and no significant loss of expression. The majority (26/36) of urothelial carcinomas had membranous immunoreactivity for P-cadherin, 1/36 had predominately cytoplasmic immunoreactivity, and 4/36 had both membranous and cytoplasmic immunoreactivity. Twenty-four of 36 urothelial carcinomas had moderate to strong immunoreactivity, and 7 had only weak immunoreactivity. Twenty-one urothelial carcinomas had significant loss of P-cadherin expression that was most often patchy (14/21 cases with significant loss), and rarely extensive (7/21 cases). Defining P-cadherin as aberrant when there was extensive loss of expression or cytoplasmic expression, no normal control urinary bladders had aberrant P-cadherin expression (Table 8). Four of 7 (57%) infiltrating urothelial carcinomas had aberrant P-cadherin expression compared to 2/23 (9%) of papillary urothelial carcinomas with aberrant expression.

There was no expression of N-cadherin in urothelial carcinomas, within tumor adjacent well-differentiated urothelium, or within the urothelium of normal urinary bladder control samples.

Comparing urothelial carcinomas and normal urothelium, there was no significant difference between the overall E-cadherin pattern ($p=0.26$), E-cadherin strength ($p=0.24$), degree of E-cadherin expression loss ($p=0.38$), P-cadherin pattern ($p=0.31$), or aberrant P-cadherin expression ($p=0.139$). There were, however, significant differences between urothelial carcinomas and urothelium of normal controls in terms of P-cadherin expression strength ($p<0.001$) and degree of P-cadherin expression loss ($p=0.013$).

Examining only urothelial carcinomas, there were no significant associations between histologic classification of urothelial carcinomas as infiltrative or papillary and E-

cadherin expression pattern (p=0.622), strength of E-cadherin immunoreactivity (p=0.629), or degree of E-cadherin expression loss (p=0.829); degree of anaplasia and E-cadherin expression pattern (p=0.684), strength of E-cadherin immunoreactivity (p=0.489), or degree of E-cadherin expression loss (p=0.563); or depth of invasion into the urinary bladder wall and E-cadherin expression pattern (p=0.431), strength of E-cadherin immunoreactivity (p=0.060), or degree of E-cadherin expression loss (p=0.273). There were statistically significant associations between histologic classification of urothelial carcinomas as infiltrative or papillary and P-cadherin expression pattern (p=0.014), degree of P-cadherin expression loss (p=0.011), and aberrant P-cadherin expression pattern (p=0.012); however, there were no significant associations between histologic classification of urothelial carcinomas as infiltrative or papillary and strength of P-cadherin immunoreactivity (p=0.280); degree of anaplasia and P-cadherin expression pattern (p=0.659), strength of P-cadherin immunoreactivity (p=0.333), or degree of P-cadherin expression loss (p=0.334); or depth of invasion into the urinary bladder wall and P-cadherin expression pattern (p=0.148), strength of P-cadherin immunoreactivity (p=0.416), or degree of P-cadherin expression loss (p=0.357).

Immunohistochemistry: β-catenin

None of the ten normal urinary bladder control samples had aberrant expression of β-catenin, while 18/33 urothelial carcinomas had aberrant expression (Table 8). This difference in aberrant labeling for β-catenin between normal urinary bladder controls and urothelial carcinomas was statistically significant (p=0.002). Evaluating only urothelial carcinomas, there were significant differences in aberrant β-catenin expression between

carcinomas classified as infiltrating or papillary (p=0.032), but no significant correlations with anaplasia grade (0.102) or invasion into the bladder wall (p=0.084).

Survival analysis

Correlations between survival time and clinico-demographic data or expression of immunohistochemically examined markers in urothelial carcinomas as defined above were evaluated using Kaplan-Meier statistics, and univariate and multivariate analyses. For clinico-demographic data, treatment groups were defined as none, only local resection, only piroxicam, and combination of piroxicam, surgical resection, and systemic chemotherapy; breed groups were defined as Scottish Terriers, West Highland Whites, Beagles, Shetland Sheepdogs, and other breeds; and sex was defined as female, spayed female, and castrated male (there were no intact males in the data set).

Using Kaplan-Meier estimators and log rank tests, there was no significance difference in survival time between groups receiving different treatment (p=0.578), breed (p=0.739), sex (p=0.497), anaplasia grade (p=0.239), degree of infiltration into the bladder wall (p=0.230), percentage of COX-2 expressing cells within tumors (p=0.231), E-cadherin pattern (p=0.103), E-cadherin strength (p=0.726), E-cadherin loss (p=0.401), P-cadherin strength (p=0.963); P-cadherin loss (p=0.830). There were significant differences in survival time comparing histopathologic classification of urothelial carcinomas as infiltrating or papillary (p=0.017), percentage of HPGD expressing cells (p=0.010), HPGD expression pattern (p=0.042), P-cadherin pattern (p<0.001), aberrant P-cadherin expression (p=0.010), and aberrant β-catenin expression (0.004). Examining those factors that had significant correlations with survival time, Kaplan-Meier estimators showed estimated 50% cumulative survival times of 237 days (58.2 SE) for papillary urothelial

carcinomas compared to 23 days (28.8 SE) for infiltrating urothelial carcinomas; of 482 days (224.4 SE) for urothelial tumors that had any cells expressing HPGD compared to 183 days (74.9 SE) for urothelial carcinomas that had complete lack of HPGD expression; of 618 days for urothelial carcinomas with HPGD Pattern 2 expression and 539 days (330.7 SE) for HPGD Pattern 1 compared to 258 (205.0 SE) for HPGD Pattern 4 and 183 days (73.9 SE) for urothelial carcinomas with lack of HPGD expression; of 267 days (104.4 SE) for urothelial tumors with P-cadherin Pattern 1 compared to 1 day (1.1 SE) for urothelial tumors with P-cadherin pattern 2 or 3; of 258 days (152.7 SE) for urothelial carcinomas with non-aberrant P-cadherin expression compared to 1 day (6.7 SE) for urothelial carcinomas with aberrant P-cadherin expression; and of 618 days (292.2 SE) for urothelial carcinomas with non-aberrant β-catenin expression compared to 62 days (120.9 SE) for urothelial carcinomas with aberrant β-catenin expression.

Using univariate analysis to evaluate correlations between survival time and clinic pathologic information or expression of immunohistochemically examined markers in urothelial carcinomas, there were significant correlations between survival time and histologic classification as infiltrating or papillary, percentage of HPGD expressing cells, pattern of HPGD expression within tumors, percentage of COX-2 expressing cells, pattern of P-cadherin expression, and aberrant expression of β-catenin (Table 9). In multivariate analysis including only the factors listed above that had statistical significance in univariate analysis, only percentage of HPGD expressing cells and pattern of P-cadherin expression retained statistical significance (Table 9).

Western blot analysis of human and canine urothelial carcinoma cell lines

All antibodies yielded bands of expected molecular weight in normal canine tissues demonstrating the applicability of these antibodies for use in the dog. Representative results of Western blots are presented in Figure 6. None of the examined canine urothelial carcinoma cell lines expressed HPGD. While expression varied mildly between canine urothelial carcinoma cell lines, all examined cell lines retained E- and P-cadherin expression and failed to express N-cadherin (Figure 15). Human urothelial carcinoma cell lines that expressed HPGD (RT4, UC14) also expressed E- and P-cadherin, but failed to express N-cadherin. Conversely, human urothelial carcinoma cell lines that did not express HPGD (UC2, UC3, UC6, UC12, 5367) had no expression of E- and P–cadherin expression, but expressed N-cadherin. All canine and human urothelial carcinoma cell lines expressed COX-2 and β-catenin with mild variations in expression between cell lines.

Discussion

Overall, normal canine urinary bladders and low grade, noninvasive canine urothelial carcinomas typically expressed HPGD in superficial epithelial cells, expressed no COX-2, and predominately expressed E- and P-cadherin and β-catenin along cell membranes. In contrast, a significant number of high grade urothelial carcinomas had loss of HPGD and increased COX-2 expression. In such tumors, P-cadherin was occasionally lost or was expressed aberrantly in the cytoplasm and β-catenin was aberrantly localized within the cytoplasm or nucleus, while membrane-associated E-cadherin expression remained. N-cadherin was not expressed by canine normal urothelium or urothelial carcinomas.

In humans, HPGD has been shown to be important in urothelial differentiation and its expression is decreased in urothelial malignancies.[12, 19] In canine urothelial carcinomas of the current study, while expression of HPGD in urothelial carcinomas was not correlated with histologic classification, grading, or depth of invasion into the bladder wall, there was an association between HPGD expression and prognosis in terms of survival time post diagnosis. COX-2 overexpression has been described in a many urothelial carcinomas of humans and increased expression is associated with invasiveness, metastasis, and increased mortality.[1, 7, 24, 25] Previous studies of COX-2 expression have yielded similar results in dogs.[4] Consistent with these reports, there were positive correlations between COX-2 expression and degree of anaplasia and depth of invasion into the urinary bladder wall in urothelial carcinomas; however, COX-2 expression was not correlated with survival time. These data suggest that pathways regulating prostaglandin E_2 are altered in canine urothelial carcinomas, and that such alterations likely play roles in progression.

Decreased expression of E-cadherin and increased expression of other cadherins, or cadherin switching, result in decreased strength of cell-cell adhesions and an increased propensity for cellular migration.[14-16] In terms of carcinogenesis, cadherin switching is observed with epithelial-to-mesenchymal transition and has been associated with increased invasiveness and metastasis.[13-16] Epithelial-to-mesenchymal transition has been well described in human bladder cancers and has been suggested to involve decreased E-cadherin expression and gain of N-cadherin and P-cadherin expression.[26-32] Increased P-cadherin expression, in particular, has been associated with a significantly worse bladder cancer-specific survival and a more malignant and invasive cancer phenotype in humans.[29, 33]

In 5/36 canine urothelial carcinomas of the evaluated tumor set, there was loss of preimembranous association of E-cadherin expression with translocation of E-cadherin expression into the cytoplasm, and in 6/36 cases there were significant areas of E-cadherin expression; however, differences in E-cadherin expression were not statistically significant between normal urothelium and urothelial carcinomas, or between various histologic classification, grading, or depth of invasion into the bladder all. Further, no correlations between E-cadherin expression and survival time were found. In contrast to E-cadherin, differences in P-cadherin expression between normal urotheluim and urothelial carcinomas and between infiltrative and papillary urothelial carcinomas had statistical significance. In addition, loss of P-cadherin expression and aberrant P-cadherin were negatively associated with survival time. The absence of N-cadherin expression in all examined canine urothelial carcinomas and within canine urothelial carcinoma cell lines contrasts with our findings in human urothelial cell lines and previous reports in human

urothelial carcinomas. These data suggest that while there may be some alteration in cadherin expression in canine urothelial carcinomas, cadherin switching as has it been described in human urothelial carcinomas, does not occur. Despite this difference between humans and dogs, aberrant expression or loss of P-cadherin likely plays a role in the clinical outcome of canine urothelial carcinomas.

In human bladder cancer, up-regulation or more specifically altered distribution of expression with nuclear localization of β-catenin has been suggested to be a prognostic indicator which has been variably negatively associated with grade, stage, survival, and/or recurrence.[10, 34-40] Aberrant expression (increased cytoplasmic and/or nuclear expression) of β-catenin was common in canine urothelial carcinomas, and in particular, in infiltrating urothelial carcinomas. Further, aberrant β-catenin expression was strongly associated with decreased survival time of dogs with urothelial carcinomas by univariate statistical analysis, but lost significance with multivariate analysis.

It has recently been shown that cadherin expression in normal urotheluim and urothelial carcinomas is integrally linked to prostaglandin E_2 regulation pathways. In rats, partial bladder outlet obstruction results in increased expression of COX-2 and decreased expression of E-cadherin, which was subsequently remediated by COX-2 inhibitors.[41] In human urothelial carcinomas, a negative correlation between COX-2 and E-cadherin expression has been suggested with high grade tumors often having both high levels of COX-2 expression and decreased E-cadherin expression or reduced membranous association of E-cadherin expression.[10] Further, *in vitro* studies have shown that treatment with COX-2 promoters can result in reduced E-cadherin expression and that knockdown of COX-2 results in increased E-cadherin expression.[10] PGDH expression, on the other hand,

was shown to increase in expression with urothelial differentiation, and inhibition of PGDH expression resulted in disruption of E-cadherin expression at cell-cell junction in cell lines.[12]

In addition, because cadherins form associations with other proteins within cells, they are integrally linked to intracellular signaling and trafficking. β-catenin binds to the cytoplasmic tail of cadherin molecules. In this capacity it functions in cell-cell adhesion, but it is also involved in a variety of signaling pathways including some that are involved in carcinogenesis, such as the Wnt pathways.[14, 42, 43] While loss of cadherin expression alone has not been shown to directly result in intracellular signaling, altered expression of cadherins has been shown to amplify or buffer the effects of such pathways.[14, 42, 43] Given the associations between prostaglandin E_2 regulation pathways and cadherin expression, there are likely also associations between such pathways and β-catenin regulation. In high grade human urothelial carcinomas, expression of β-catenin has been positively correlated with COX-2 expression.[10] Rather than being only a consequence of altered prostaglandin E_2 regulation, altered β-catenin expression and Wnt signaling may also drive alterations in prostaglandin E_2 regulation. Recent work has suggested that β-catenin expression is inversely related to the expression of HPGD at least in intestinal epithelium and within colorectal tumor cell lines.[11]

In canine urothelial carcinoma cell lines, expression of HPGD and COX-2 was not associated with cadherin switching or obvious differences in β-catenin expression. In canine urothelial carcinoma cell lines, there was no appreciable expression of HPGD and variable, but often strong expression of COX-2. E-cadherin and P-cadherin were expressed in all canine cell lines, while there was no appreciable expression of N-cadherin. In contrast

to canine urothelial carcinomas and urothelial carcinoma cell lines, cadherins were differentially expressed in human urothelial cell lines and cadherin expression pattern was correlated with HPGD status. Specifically, in examined human urothelial carcinoma cell lines, those cell lines that expressed HPGH also expressed E-cadherin and P-cadherin. Lack of HPGD expression in human urothelial carcinoma cell lines was consistently associated with gain of N-cadherin expression. Thus, while canine urothelial carcinoma cell lines appear similar to human urothelial carcinomas in terms of the common loss of HPGD expression and expression of COX-2, they are dissimilar in the lack of associated overt cadherin switching.

While the results of this study are intriguing and statistical correlations between many factors and survival time appear strong, this study is limited by the relative low number of cases and, in particular, the low number of low grade urothelial carcinomas. High grade urothelial carcinomas are far more commonly diagnosed in dogs than low grade tumors accounting for the low number of low grade tumors in the examined set. This may be due to an actual lower rate of occurrence in canine populations or diagnosis of urothelial neoplasms only late in the course of disease when larger, more aggressive tumors affect micturition or cause other clinical disease. Further, euthanasia is common in veterinary medicine and is especially common in animals with urothelial carcinomas due to the negative prognosis historically associated with such tumors, the effects on micturition, the common non-respectable nature such tumors at the time of diagnosis, and client choice due to financial or homecare constraints.

Despite the inherent limitations, this work shows that expression of HPGD, COX-2, cadherins, and β-catenin is altered in at least subsets of canine urothelial carcinomas;

however, the patterns of alterations is different than that previously described in humans or observed in human cell lines in the current study. This suggests that while human and canine urothelial carcinoma may be similar in terms of clinico-pathologic features, drivers of carcinogenesis and progression of these tumors may differ between humans and dogs. While COX-2 and HPGD have been suggested to be correlated with features of epithelial-to-mesenchymal transition such as cadherin switching in humans, such may not be the case in dogs. As the molecular pathways which were evaluated in this study have potential as therapeutic targets, developing a better understanding why such there are differences between dogs and humans and how this impacts treatment response through additional study could lead to the development of better clinical strategies to address canine and human urothelial cancers.

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APPENDIX

Figure 10: *Urinary bladder, Dog, 15-hydroxyprostaglandin dehydrogenase (HPGD) immunohistochemistry (IHC).* Immunoreactivity (brown labeling) was defined by cytoplasmic and/or nuclear labeling and categorized by the percentage of positively labeled urothelial cells and the overall pattern of immunoreactivity within the urothelium. Immunoreactivity is distributed diffusely throughout 100% of cells in superficial 1-2 cell layers of the urothelium consistent with HPGD Pattern 1 in the urinary bladder of a healthy control dog consistent with Pattern 1 (A). In a grade I (low grade) papillary carcinoma, there is immunoreactivity in 20% of the total neoplastic urothelial cells and immunoreactivity is patchy and randomly distributed throughout <80% of cells in the superficial most 1-2 cell layers consistent with HPGD Pattern 2 (B). In a grade III (high grade) invasive carcinoma extending into the muscularis, there is lack of appreciable HPGD immunoreactivity (C). 3,3'-Diaminobenzidine (DAB) chromogen, hematoxylin counterstain.

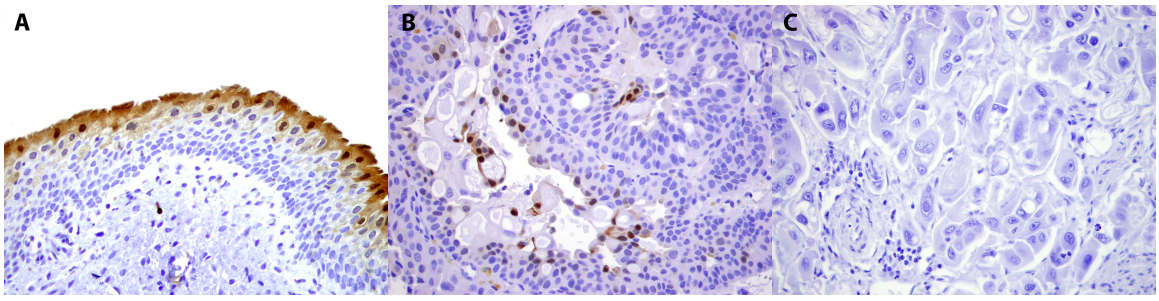

Figure 11: *Urinary bladder, Dog, Cyclooxygenase-2 (COX-2) IHC*. Immunoreactivity (red) was defined by cytoplasmic labeling and categorized by the percentage of positive urothelial cells. Urothelium from the urinary bladder of a healthy control dog (A) and neoplastic urothelium of a grade 1 (low grade) papillary carcinoma (B) lack appreciable immunoreactivity. There is strong immunoreactivity for COX-2 in >30% of neoplastic cells of a grade III (high grade) invasive carcinoma with infiltration of the muscularis (C). Indirect biotin streptavidin and Fast Red chromogen, hematoxylin counterstain.

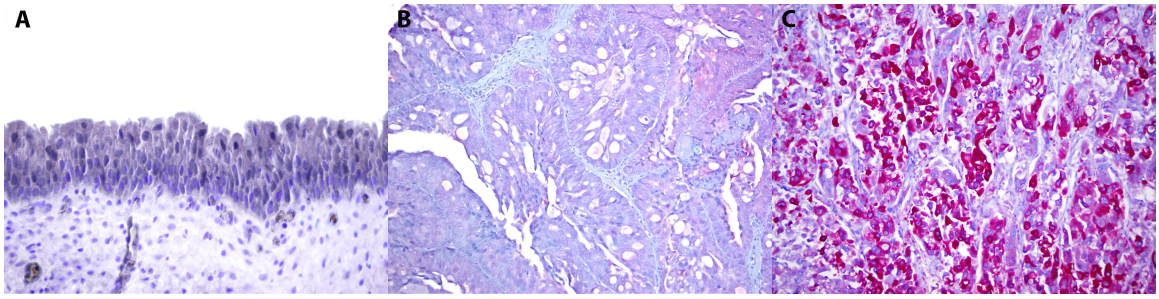

Figure 12: *Urinary bladder, Dog, E-cadherin IHC.* Immunoreactivity (brown) was subjectively categorized as weak, moderate, or strong; assessed according to the predominant pattern within urothelial cells as membrane associated, intracytoplasmic, or mixed membranous and cytoplasmic; and categorized by degree of loss as no loss, patchy loss, or complete loss. In urothelium from the urinary bladder of a healthy control dog, there is strong membrane associated immunoreactivity throughout the full urothelial thickness and no loss (A). While there is strong membrane associated immunoreactivity in superficial cell layers in a grade II (high grade) papillary carcinoma with substantia propria infiltration, the predominant pattern is moderate cytoplasmic immunoreactivity with patchy areas of loss (B). In a grade III (high grade) invasive carcinoma with infiltration into the muscularis, there is strong membrane associated immunoreactivity in 90% of neoplastic cells and patchy loss (C). DAB chromogen, hematoxylin counterstain.

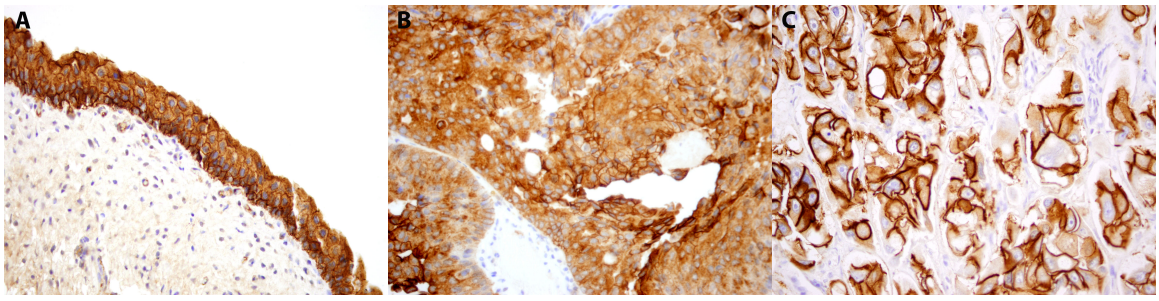

Figure 13: *Urinary bladder, Dog, P-cadherin IHC.* Immunoreactivity (brown) was subjectively categorized as weak, moderate, or strong; assessed according to the predominant pattern within urothelial cells as membrane associated, intracytoplasmic, or mixed membranous and cytoplasmic labeling; and categorized by degree of loss as no loss, patchy loss, or complete loss. In urothelium from the urinary bladder of a healthy control dog, there is strong membrane associated immunoreactivity throughout the full urothelial thickness and no loss (A). In a grade I (low grade) papillary carcinoma, there is moderate to weak membrane associated immunoreactivity and patchy loss in the basal cell layers (B). In a grade III (high grade) invasive carcinoma with infiltration into the muscularis, there is predominant moderate intracytoplasmic immunoreactivity with patchy loss (C). DAB chromogen, hematoxylin counterstain.

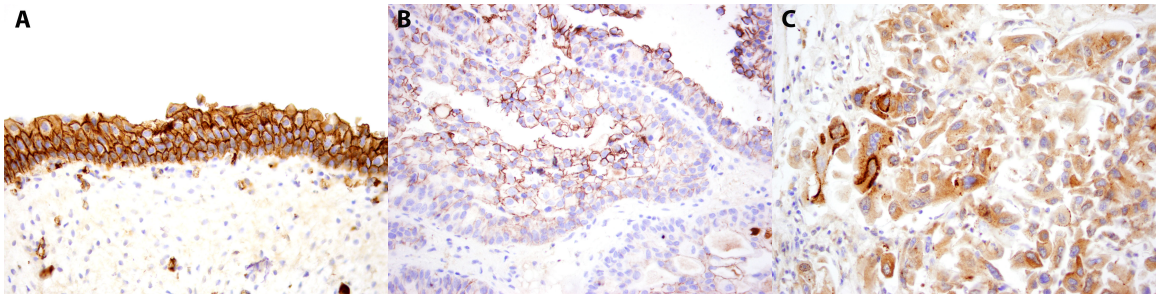

Figure 14: *Urinary bladder, Dog, β-catenin IHC.* Immunoreactivity, the percentage of cells with membrane, cytoplasmic, mixed membrane and cytoplasmic, and nuclear immunoreactivity (brown) was assessed. In urothelium from the urinary bladder of a healthy control dog, there is membrane associated immunoreactivity in 100% of cells and no cytoplasmic, mixed membrane and cytoplasmic, or nuclear immunoreactivity (A). In a grade I (low grade) papillary carcinoma, there is membrane associated immunoreactivity in 90% of cells, cytoplasmic immunoreactivity in 10% of cells, and no mixed membrane and cytoplasmic or nuclear immunoreactivity (B). In a grade III (high grade) invasive carcinoma with infiltration into the muscularis, there is primary cytoplasmic immunoreactivity in 85% of cells and nuclear immunoreactivity in 15% of cells (C). DAB chromogen, hematoxylin counterstain.

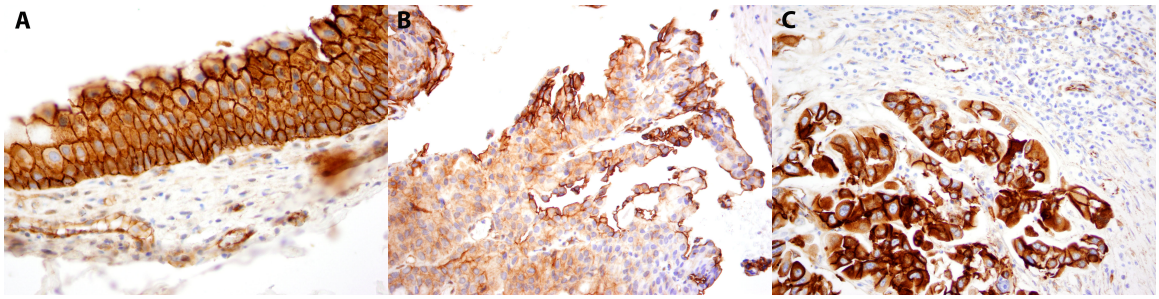

Figure 15: *Western blot comparing expression of HPGD, COX-2, E-cadherin, P-cadherin, N-cadherin, and β-catenin in human (RT4 and UC3) and canine urothelial carcinoma cell lines (AXA, AXB, AXC, and NK).* None of the examined canine urothelial carcinoma cell lines expressed HPGD, all retained E- and P-cadherin expression, and failed to express N-cadherin. Human urothelial carcinoma cell lines that expressed HPGD (RT4) also expressed E- and P-cadherin, but failed to express N-cadherin. Conversely, human urothelial carcinoma cell lines that did not express HPGD (UC3) had no expression of E- and P–cadherin expression, but expressed N-cadherin. While expression varied, COX-2 and β-catenin were expressed by all canine and human urothelial carcinoma cell lines.

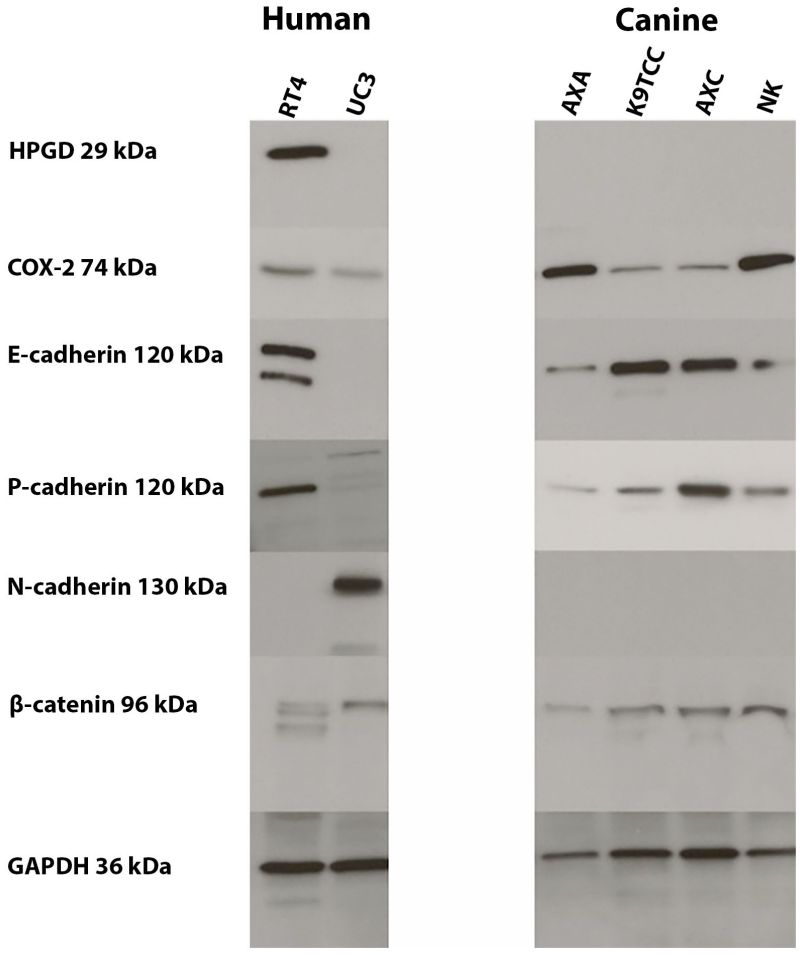

Table 7: *Expression of HPGD in canine urothelial carcinomas and normal urothelium with respect to lesion classification and degree of invasion*

	Percentage of urothelial cells expressing HPGD					**Pattern of HPGD expression**			
	None	<5%	5-15%	15-30%	>30%	Pattern 1	Pattern 2	Pattern 3	Pattern 4
Classification									
Normal urothelium	1/10 (10%)	2/10 (20%)	1/10 (10%)	3/10 (30%)	3/10 (30%)	8/10 (80%)	1/10 (10%)	-	-
Infiltrative carcinoma	5/8 (63%)	3/8 (38%)	-	-	-	-	1/8 (13%)	-	2/8 (25%)
Papillary carcinoma	9/28 (32%)	5/28 (18%)	6/28 (21%)	4/28 (14%)	4/28 (14%)	3/28 (11%)	1/28 (4%)	-	15/28 (54%)
Grade I	1/4 (25%)	-	1/4 (25%)	1/4 (25%)	1/4 (25%)	1/4 (25%)	-	-	2/4 (50%)
Grade II	6/16 (38%)	2/16 (13%)	3/16 (19%)	2/16 (13%)	3/16 (19%)	2/16 (13%)	1/16 (6%)	-	7/16 (44%)
Grade III	2/8 (25%)	3/8 (38%)	2/8 (25%)	1/8 (13%)	-		-	-	6/8 (75%)
Degree of Invasion									
No invasion	3/12 (25%)	3/12 (25%)	2/12 (17%)	2/12 (17%)	2/12 (17%)	3/12 (25%)	3/12 (25%)	2/12 (17%)	4/12 (33%)
Substantia propria	6/12 (50%)	1/12 (8%)	2/12 (17%)	2/12 (17%)	2/12 (17%)	6/12 (50%)	-	-	6/12 (50%)
Muscularis	5/12 (42%)	4/12 (33%)	3/12 (25%)	-	-	5/12 (42%)	-	-	7/12 (58%)

Table 8: *Expression of P-cadherin and β-catenin in canine urothelial carcinomas and normal urothelium with respect to lesion classification and degree of invasion*

	P-cadherin		**β-catenin**	
	Nonaberrant	Aberrant	Nonaberrant	Aberrant
Classification				
Normal urothelium	10/10 (100%)	-	10/10 (100%)	-
Infiltrative carcinoma	3/7 (43%)	4/7 (57%)	1/8 (12%)	7/8 (88%)
Papillary carcinoma	23/25 (92%)	2/25 (8%)	14/25 (56%)	11/25 (44%)
Grade I	4/4 (100%)	-	3/4 (75%)	1/4 (25%)
Grade II	13/14 (93%)	1/14 (7%)	8/14 (57%)	6/14 (43%)
Grade III	6/7 (86%)	1/7 (14%)	3/7 (43%)	4/7 (57%)
Degree of Invasion				
No invasion	12/12 (100%)	-	7/12 (58%)	5/12 (42%)
Substantia propria	8/10 (67%)	2/10 (17%)	6/10 (60%)	4/10 (40%)
Muscularis	6/10 (60%	4/10 (40%)	2/11 (18%)	9/11 (82%)

Table 9: *Significant Correlative Results of Univariate and Multivariate Analysis of Examined Variables with Respect to Survival Time as Determined by p-values less than 0.05*

Univariate Analysis		Multivariate Analysis	
Variable	*p-value*	*Variable*	*p-value*
Histologic Classification	0.018	HPGD: % of positive cells	0.026
HPGD: % of positive cells	0.006	P-cadherin: cellular localization	0.003
HPGD: tissue localization	0.044		
COX-2: % of positive cells	0.042		
P-cadherin: cellular localization	<0.001		
β-catenin: aberrant expression	0.008		

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CHAPTER 4

Evaluation of microsatellite instability and DNA mismatch repair protein expression in canine urothelial carcinomas

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Abstract

Microsatellite Instability (MSI), defined as the accumulation of frameshift mutations in nucleotide repeat sequences of DNA microsatellites, is considered a signature for dysfunction in the DNA mismatch repair (MMR) system. We investigated MSI in canine tumors with breed predispositions, using a panel of 22 microsatellites from 15 different canine chromosomes. Genotypes derived from tumor DNA and normal DNA from blood were compared and the percentage of unstable microsatellites were calculated. Our results demonstrated that MSI is more frequent among canine urothelial carcinomas of the urinary bladder compared to other canine malignancies including gastric carcinoma and mammary tumors, and is common within specific breeds and phylogenic clades of dog suggesting likely genetic predispositions. Further, we investigated the expression of the MMR proteins, MLH1, MSH2, MSH3, and MSH6, in a set of 36 canine urothelial carcinomas of the lower urinary tract and 10 urinary bladders from unaffected dogs using morphometric analysis of immunoreactivity as determined by multispectral imaging. These studies confirmed consistent expression of MMR proteins in normal canine urothelium. While there was no significant decrease in expression of other MMR proteins, expression of MSH2 was significantly decreased in urothelial carcinomas in comparison to that in urothelium of unaffected urinary bladders. These investigations demonstrate likely MMR dysfunction in subsets of canine lower urinary tract urothelial carcinomas.

Introduction

The DNA mismatch repair (MMR) system participates in a variety of cellular processes. Most notably, this system is responsible for post replication recognition and repair of base-base mismatches and the resolution of insertion and deletion loops that can occur in repetitive regions of DNA.[1-5] It has been estimated that the fidelity of DNA replication is improved by the MMR system 50-1000 fold over that seen with the replication machinery alone.[2, 3] Accordingly, deficiencies in this system can lead to increased rates of point and frame shift mutations throughout the genome. Such mutations can lead to loss of function changes in tumor suppressors or gain of function changes in tumor oncogenes.[6-10] In addition, the MMR system also participates in a variety of other processes including DNA damage recognition signaling, promoting cell cycle arrest and apoptosis, homologous recombination, meiotic recombination, and other DNA repair pathways.[1, 2, 4, 11] Given these varied roles played by MMR, it is not surprising that defects in MMR facilitate carcinogenesis. It has long been known in humans that hereditary defects in MMR promote carcinogenesis in a variety of tissues including the colon, endometrium, stomach, skin, prostate, and urinary tract.[11-13] However, defects in MMR have also been reported in a wide number and variety of spontaneously occurring cancers suggesting a wide role in cancer development.[1, 14-16]

The MMR system is composed of a set of highly conserved proteins.[4, 11] Five proteins, including MSH2, MSH3, MSH6, MLH1, and PMS2, provide the backbone of the MMR machinery. Initial DNA damage recognition may be accomplished by either MSH2-MSH6 heterodimers (MutSα) or MSH2-MSH3 heterodimers (MutSβ).[11] The MutS heterodimers are then capable of recruiting the MLH1-PMS2 heterodimer, MutL, which

initiates the assembly of other proteins involved in excising the lesion, re-synthesizing DNA and ligating the gaps. Failure to repair DNA errors may result in signaling for cell cycle arrest and/or apoptosis. Deficient expression or abnormalities in any of these proteins result in a mutator phenotype in which DNA mutations can accumulate in a variety of genes.[6-10]

MMR function has most often been evaluated through analysis of microsatellites. Microsatellites are regions of nucleotide repeats located throughout the genome. These areas are prone to polymerase slippage during replication leading to formation of small insertion and deletion loops.[17] If not recognized and repaired by MMR, buildup of frame shift mutations occurs within microsatellites.[5, 11] In cancers with defects in the MMR system, a high percentage of microsatellites have recognizable mutations. Such buildup of mutations within microsatellites is termed microsatellite instability (MSI) and is considered a "signature" for MMR dysfunction.[11, 18]

Cancer is common in pet dogs. Increased risks for development of specific carcinomas within certain breeds suggests that inheritable defects may underlie carcinogenesis in affected breeds.[19-21] Thus, breed predispositions for specific cancers provide an opportunity to investigate potential differential genetic mechanisms of carcinogenesis, including those associated with MMR. Given the diversity of cancer phenotypes associated with MMR dysfunction, we hypothesized that mutation in MMR could underlie some of the cancer susceptibility in dogs. To investigate this hypothesis, we evaluated for MSI in canine epithelial tumors that may have a genetic basis including gastric, mammary, and urothelial carcinomas. Following identification of a high rate of MSI in canine urothelial carcinomas, the expression of MLH1, MSH2, MSH3, and MSH6 was

evaluated in such tumors using immunohistochemistry and compared to that of normal urothelium, rates of MSI, histomorphologic features of the tumors, and survival time.

Materials and methods

Evaluation of Microsatellite Instability in Canine Tumors

This study was approved by the University of Minnesota Institutional Animal Care and Use Committee. Pet dogs with diagnoses of gastric carcinoma, mammary tumor, or urothelial (transitional cell) carcinoma of the urinary bladder were identified through the University of Minnesota Veterinary Medical Center and Diagnostic Laboratory, and through referring veterinarians.

Upon owner consent, blood samples from each dog were collected in 7.5% EDTA containing tube (Monoject®, Tyco Healthcare, Mansfield, MA) and refrigerated at 4°C. Tumor samples were collected either at the time of diagnostic biopsy, therapeutic surgical excision, or necropsy. DNA was isolated from blood samples and tumor tissue using commercially available DNA extraction kits (Purgene, Gentra Systems, Inc, Minneapolis, MN) using manufacturer recommendations. DNA was stored at -80°C pending microsatellite PCR.

A panel of 22 MS repeats including 10 tetra-, 11 di-, and one mononucleotide repeats, which were located on 15 different canine chromosomes, was used to evaluate MSI. With the exception of the mononucleotide repeat, which we identified, the microsatellite sequences and corresponding PCR primers for their amplification have been reported previously.[22] An 18 nucleotide tail sequence was added to the 5' end of each reverse primer using previously described methods.[23] The microsatellite sequences were PCR amplified from tumor and normal (from blood) DNA in 15 µl reactions including 12.5 ng of DNA, 1.5 mM $MgCl_2$, 1 µM of the forward primer and 0.3 µM of the reverse primer with tail, 156 nM of a fluorescent dye-labeled primer complimentary to the tail sequence,

300 μM of each dNTP (Fisher Scientific, Pittsburg, PA) and 1.5 U Taq Polymerase (HotStarTaq®, Qiagen, Valencia, CA). The PCR program consisted of 20 min at 95°C, 40 cycles of 1 min at 94°C, 1 min at 56°C, 2 min at 72°C, and a final extension step of 10 min at 72°C. PCR products, with DNA size standards, were evaluated using capillary electrophoresis on the CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc, Fullerton, CA) and chromatograms subsequently were analyzed using the system software. Genotypes were manually verified. Any ambiguous results in which PCR products were of poor quality or when genotypes were inconsistent were repeated to confirm instability.

For each tumor MSI was evaluated as %MSI, which was defined for each case as the % of evaluated MS loci in which there was detectable variation in MS length in at least one allele between DNA isolated from tumors and DNA isolated from blood. Tumors with > 25% MSI were classified as high level MSI (MSI-H), tumors with 10% > MSI < 25% were classified as exhibiting low level MSI. Tumors with <10% MSI were considered stable (MSS). For urothelial carcinomas, comparisons of %MSI were made between breeds and phylogenic clade. For statistical evaluation with respect to breed, 4 breeds with a reported high relative risk for development of bladder cancer (Scottish terrier, West Highland white terrier, Shetland sheepdog, and beagle) were evaluated as independent groups while dogs of other breed, which included 17 unique breeds and mixed breed dogs, were categorized into a single group.[20, 24, 25] For statistical evaluation with respect to phylogenetic clade, dogs were of specific breed grouped into phylogenetic clade according to previous description of genetic diversity amongst purebred dogs or evaluated as a separate category if of mixed breed.[26]

Evaluation of MMR protein expression in Canine Urothelial Carcinomas

A series of 36 dogs that were diagnosed with urothelial carcinomas at either the Michigan State University Veterinary Teaching Hospital or the University of Minnesota Veterinary Teaching Hospital through biopsy were selected for inclusion in the study based on owner consent, availability of clinical history, and availability of paraffin-embedded, formalin-fixed diagnostic samples. For each urothelial carcinoma case, descriptive information was obtained from medical records and periodic follow-up questionnaires including age at diagnosis, breed, sex, survival time from the date of diagnosis, the reported cause of death, and any treatment employed. In addition, bladder samples from 10 healthy dogs that were used for veterinary student teaching purposes were harvested in 10% buffered formalin and routinely processed for histologic examination. Five μm sections of all samples were routinely processed and stained with hematoxylin and eosin. Urothelial proliferative lesions were classified, and papillary carcinomas were graded according to the WHO/ISUP Consensus Classification System as previously described in canine urothelial tumors.[27] In addition, the degree of invasion of all carcinomas was scored as no invasion, invasion into the substantia propria, or invasion into the muscularis.

Immunohistochemistry (IHC) was preformed to evaluate MSH2, MSH3, and MSH6, and MLH1 expression in normal canine bladder and canine urothelial carcinomas using commercially available antibodies which were shown be efficacious for use in dog tissues by Western blot (see chapter 4). Five μm sections of all formalin-fixed, paraffin-embedded tissues were processed for immunohistochemistry and labeled with a mouse monoclonal anti-MLH1 antibody (1:200, BD Biosciences, San Jose, CA, USA), a rabbit polyclonal anti-MSH2 antibody (1:100. Santa Cruz Biotechnology, Dallas, TX, USA), a rabbit polyclonal anti-MSH3 antibody (1:100, Abcam, Cambridge, MA, USA), a mouse monoclonal anti-MSH6

antibody (1:500, BD Biosciences, San Jose, CA, USA). For MLH1 and MSH6, deparaffinization, antigen retrieval, immunohistochemical staining and counterstaining was preformed on a Benchmark XT™ autostainer (Ventana, Tucson, AZ, USA) using an Enhanced Alkaline Phosphatase Red Detection Kit (Ventana) that uses an indirect biotin streptavidin and Fast Red chromogen detection system. Retrieval for MLH1 and MSH6 was accomplished with 20 minutes of incubation with Cell Conditioning 1 solution (Ventana). For MSH2, deparaffinization, antigen retrieval, immunohistochemical staining and counterstaining was preformed on a Dako Link 48 Autostainer (Carpinteria, CA, USA) using a LSAB2 kit (Dako), which employs a 3,3' diaminobenzidine tetrahydrochloride (DAB) chromogen detection system. For MSH3, deparaffinization, antigen retrieval, immunohistochemical staining and counterstaining was performed on a Bond maX™ Automated Staining System (Leica Biosystems, Buffalo Grove, IL, USA) using the Bond™ Polymer Refine Detection System (Leica Biosystems), which employs a 3,3' diaminobenzidine tetrahydrochloride (DAB) chromogen detection system. Retrieval for MSH2 and MSH3 was accomplished using heat induced epitope retrieval and incubation with Bond™ Epitope Retrieval Solution 1 (Leica Biosystems) for 20 minutes. Sections of normal urinary bladder used as positive controls were run in parallel to cases for each of the IHC. For negative controls, homologous non-immune sera or buffer replaced primary antibodies.

For all cases, seven randomly picked areas from each slide immunohistochemically labeled for the respective MMR proteins listed above were imaged using a CRi Nuance multispectral imaging system (Woburn, MA, USA). The resulting image cubes were converted to optical density units, then mathematically unmixed to separate chromagen

labeling from counterstain using spectral libraries generated from imaging of only hematoxylin stained or immunohistochemically labeled and hematoxylin counterstained control specimens. Colocalized immunoreactivity was assessed as positive pixels per image using constant thresholds for detection using the multispectral imaging system software. The component images of the image cubes were then pseudocolored, converted to pseudofluorescent format, and unmixed for counting of nuclei, which was done using Imagine morphometric analysis software (Van Andel Research Institute (Grand Rapids, MI, USA) (Figure 16). For statistical analysis results were evaluated for each case as mean number of positive pixels per nucleus.

Statistical analysis

All statistical analysis was preformed using SPSS statistical software (Somers, NY). Permutation F tests were used to evaluate MSI data as a continuous variable. Fisher's exact tests were used to evaluate categorical treatment of MSI with tumors defined as MSI-H, MSI-L or MSS as described above. Mann-Whitney U tests and Kurskal-Wallis tests were used to evaluate correlations between breed, sex, histologic classification, degree of anaplasia, and depth of infiltration, and immunohistochemical expression MMR proteins as morphometrically evaluated by nuance spectral imaging. Regression was used for pairwise comparisons of %MSI and immunohistochemical expression of MMR protein. Correlations between survival time of canine urinary carcinomas and clinico-demographic data, histologic classification and grading, and immunohistochemical expression of MMR proteins were evaluated using ANOVA. For all statistical analysis, significance was defined as $p<0.05$.

Results

Evaluation of MSI

The results of scoring MS aberrations in canine epithelial tumors are depicted in Figure 17 and Table 10. While MS aberrations were uncommon in gastric carcinoma and most mammary tumors, both the percentage of aberrant MS in individual tumors and the frequency of tumors classified as MSI-H were significantly increased in canine urothelial carcinomas of the urinary bladder. Despite a higher prevalence of MSI in canine TCC, the distribution of %MSI was wide, ranging from 0% to 92% of MS loci being affected in individual tumors.

There was variation in the magnitude and frequency of %MSI in canine urothelial within all evaluated breed groups; however, there were differences in overall %MSI between breed groups. Specifically, MSI was greater in urothelial carcinomas from Scottish terriers and West Highland white terriers, less pronounced in Shetland sheepdogs, and uncommon in Beagles (Figure 18 and Table 11). In permutation F tests comparing these 4 breeds and excluding all other breeds, differences in MSI were statistically significant ($p=0.037$); however, statistical significance was lost when MSI instability was compared between the 4 breeds along with a category including all other breeds ($p=0.092$). Using Fisher's exact test to compare the categorical distribution of tumors as MSS, MSI-L, and MSI-H according to breed, there were statistically significant differences whether comparing only the four high risk breeds ($p=0.016$), or all breed categories ($p=0.017$). Further, there were significant differences in MSI expression between dog phylogenetic clades, (Figure 19). Specifically dogs in clade 5 had high levels of MSI in comparison to dogs from other clades.

MSI was observed in tetranucleotide repeats and shorter repeats including mono- and dinucleotide repeats in carcinomas urothelial carcinomas. (Table 12). Permutation F-tests showed significant differences in %MSI for short repeats whether comparing only the four high risk breeds (p=0.016), or all breed categories (p=0.042); however, there was no significant difference between %MSI for tetranucleotide repeats whether comparing only the four high risk breeds (p=0.116), or all breed categories (p=0.158). Fisher exact tests revealed significant differences in category of MSI in the short repeats among breed categories whether comparing only the four high risk breeds (p=0.017), or all breed categories (p=0.050). In contrast, no statistical association could be identified between MSI category in tetranucleotide repeats and breed (p=0.390 for only high risk breeds, p=0.455 including all breed categories).

Demographic information, histologic classification, and grading of urothelial carcinomas

For dogs diagnosed with urothelial carcinomas, the mean age at initial diagnosis was 10.5 years of age (2.0 SD) with a range of 6.7 to 17.1 years of age. Affected dogs included 4 intact females, 21 spayed females, and 11 castrated males. Represented breeds included 5 Scottich terriers, 2 West Highland white terriers, 7 Beagles, 7 Shetland Sheepdogs, and 14 dogs of other purebred or mixed breed. For the 32 cases in which information regarding treatment was available, 7 had no treatment, 2 had only local resection of the mass, 17 were treated with only piroxicam, and 6 were treated with piroxicam, surgical resection, and systemic chemotherapy which variably included adriamycin, cyclophosphamide, and/or mitoxantrone. Two animals were alive at the end of study, 1 animal died from causes unrelated to the urinary carcinoma, 30 reportedly died

or were euthanized as a result of progressive disease related to the urinary bladder tumors, and 3 were lost to follow-up.

Of the 36 urothelial carcinomas, 8 were diagnosed as infiltrating carcinomas, which had no appreciable exophytic papillary component, but instead primarily infiltrated into and expanded the bladder wall. The remaining 28 cases were papillary carcinomas, the vast majority of which (24 of 28) were high grade (grade II or III). Invasion of high-grade papillary carcinomas into the bladder wall was common with 9 of 24 having invasion into the substantia propria and 7 of 24 invading into the muscularis. For all carcinomas, the mean survival time was 326 days with a standard deviation of 341 and range of 0 to 1225 days. For infiltrative carcinomas, the mean survival time was 173 days with a standard deviation of 223 days and range of 0 to 365 days. For papillary carcinomas, the mean survival time was 370 days with a standard deviation of 359 days and range of 0 to 1225 days.

MLH1, MSH2, MSH3, and MSH6 Immunohistochemistry

Nuclear expression was observed in urothelium for all evaluated MMR protein markers. For all cases, mean with standard error, median, and range of %MSI and expression of MMR proteins MLH1, MSH2, MSH3, and MSH6 in terms of positive pixels per nucleus as determined by IHC and Nuance spectral imaging are reported in Table 13. The expression of MLH1, MSH2, MSH3, and MSH6 and %MSI and were not normally distributed amongst cases.

Overall, while there was variation in the expression of MMR proteins in both urothelium of normal urinary bladders and urothelial carcinomas of the urinary bladder, the mean expression of MSH2 was lower in urothelial carcinomas than in urinary bladders,

and the mean expression of MSH6 was higher in urothelial carcinomas than in normal urinary bladders (Figure 20). Also, the mean ratio of MSH2 to MSH6 was lower in urothelial carcinomas than it was in normal urinary bladders (Figure 21). Using Mann-Whitney U tests, there were significant differences between urothelial carcinomas and normal urinary bladder samples in terms of %MSI ($p=0.003$), the expression of MSH2 ($p=0.001$), and the expression of MSH6 ($p=0.003$). However, there was no significant difference between urothelial carcinomas and normal urinary bladder samples in terms of the expression of MLH1 ($p=0.886$) or the expression of MSH3 ($p=0.470$). There were also no significant differences between infiltrating carcinomas and papillary carcinomas in terms of %MSI ($p=0.921$) or the expression of MLH1 ($p=0.116$), MSH2 ($p=0.562$), MSH3 ($p=0.204$) or MSH6 (0.751). Further, using Kreskas-Wallis tests and examining only urothelial carcinoma, there were no significant differences ($p>0.05$) between breed, sex, degree of cellular anaplasia, and degree of invasion into the urinary bladder wall, and expression of MLH1, MSH2, MSH3, or MSH6.

In regression analysis evaluating all cases including normal urinary bladders and urothelial carcinomas, there were no significant correlations between %MSI and expression of MLH1 ($p=0.408$), MSH2 ($p=0.263$), MSH3 ($p=0.799$), or MSH6 ($p=0.463$). Excluding normal urinary bladders, there remained no significant correlations between %MSI and expression of MLH1 ($p=0.343$), MSH2 ($p=0.518$), MSH3 ($p=0.629$), or MSH6 ($p=0.192$).

Survival analysis

Using Cox regression, there were no significant correlations in urothelial carcinoma between survival time and %MSI ($p=0.547$), MLH1 ($p=0.446$), MSH2 ($p=0.740$), MSH6

(p=0.262), age at diagnosis (p=0.290), breed (p=0.723), sex (p=0.485), treatment (p=0.0633), histologic classification of urothelial carcinomas as papillary or infiltrating (p=0.129), degree of anaplasia (p=0.054), degree of invasion into the bladder wall (p=0.185). Expression of MSH3, however, was associated with survival time (p=0.022).

Using Kaplan-Meier estimators and log rank tests, there was no significant correlation between survival time and MSI as categorized as stable, low, or high (p=0.283); breeds (p=0.704); sex (p=0.476); histologic classification as papillary or infiltrating (p=0.120); cellular anaplasia (p=0.132); degree of invasion into the bladder wall (p=0.307); or MSH3 expression when grouping cases by having > or < the mean MSH3 expression. There was, however, a significant correlation between survival time and treatment (p=0.017).

Discussion

These investigations demonstrated common, but not universal occurrence of MSI in canine urothelial carcinomas. Variation in frequency and magnitude of MSI in these cancers was correlated with genetic background in terms of breed and phylogenetic clade suggesting likely divergent pathways of urothelial carcinogenesis. There were significant differences in the expression of the MMR proteins, MSH2 and MSH6, between canine urothelial carcinomas and normal urinary bladder as evaluated by IHC and morphometric analysis using multispectral imaging. However, variance in MMR protein expression did not correlate with presence or degree of MSI, breed, or histomorphologic features of urothelial carcinomas. Neither MSI nor the expression of MMR proteins excluding MSH3 was associated with survival time.

Urinary carcinomas of the urinary bladder represent one of the many cancer types in humans in which a significant percentage of tumors have been reported to show defects in MMR. Development of such tumors has been associated with both hereditary and spontaneously developing MMR deficiency. While often occurring spontaneously, development of bladder cancers associated with defective MMR also has been well described as part of the Lynch syndrome. The Lynch syndrome is a hereditary predisposition for cancer development defined by somatic mutation in MMR genes.[12] A wide range of cancers has been described in patients with Lynch syndrome, the most extensively studied and widely reported of these is hereditary nonpolyposis colorectal carcinoma.[1-4, 15] A recent study of Lynch syndrome families from 4 nations including the US demonstrated that the urinary tract, including the kidney, renal pelvis, ureter, and bladder, had the highest organ system specific cancer risk after the colon and endometrium.[28]

The importance of MMR dysfunction and MSI in urothelial carcinomas of the upper urinary tract is well accepted in human medicine.[29-39] Studies of the MMR in urothelial carcinomas of the urinary bladder, however, have yielded variable and sometimes contradictory data. Cancers developing from urothelium of the upper urinary tract frequently have been associated with MMR defects and often exhibit MSI.[29-39] Although urinary carcinomas of the upper urinary tract represent the most common urinary tract cancers reported with Lynch syndrome, bladder cancers also occur in people harboring heritable MMR defects.[40-43] Lynch syndrome associated bladder cancer represents a very small proportion of bladder cancers; however, data from numerous studies over the last two decades indicate that a substantial subset of bladder cancers exhibit MSI suggesting that spontaneous development of MMR defects in urothelial carcinogenesis is common. [44, 45]

It has previously been suggested that due to the similarities of urothelial carcinomas of the urinary bladder in dogs and humans, that these canine cancers could be used as a research model.[20, 24] Similar to humans, urothelial carcinomas of the urinary bladder comprise a significant proportion of canine neoplasms. Urothelial carcinomas have a similar histomorphologic appearance in dogs and humans. [20, 24, 27] In fact, it has recently been suggested that canine urothelial carcinomas could be classified and graded according to the World Health Organization/International Society of Urologic Pathology consensus classification system, which was developed for use in human pathology.[27] Papillary and invasive carcinomas are prevalent in dogs and follow a similar clinical course to those reported in humans suggesting that the pathways involved in the development and progression of these tumors may be homologous.[20, 24] Further, particular breeds including

the Scottish terrier, West Highland white terrier, the Shetland Sheepdog, and Beagle, have significantly increased risk of developing urothelial carcinomas, making easier the study of the genetic basis of carcinogenesis in dogs.[20, 24, 25] One difference between human and canine urothelial carcinomas is that *in situ* carcinomas only rarely identified in dogs while they are by far the most commonly recognized form of urothelial carcinoma in humans.[24, 25, 46] This, however, may be due to the fact that bladder tumors are generally recognized in dogs only in late stages of disease when they are large enough to cause significant functional problems.

In the current study, there was a high incidence of MSI in canine urothelial carcinomas in comparison to other tumors with strong breed predispositions (gastric carcinomas and mammary tumors). Further, MSI in canine urothelial carcinomas was correlated with breed, with the phylogenetically related breeds Scottish terrier and West Highland white terrier often having high MSI.[26] Statistical correlations were particularly strong when comparing only the four breed groups with high relative risk for development of urothelial carcinomas. The relatively higher p-value obtained in permutation tests when breeds not predisposed for urothelial carcinomas was included in analysis as an additional category can be explained by the higher variability of MSI in tumors derived from this genetically heterogeneous group composed of 17 other purebreds and mixed breed dogs.

In humans, evidence suggests that tumors of different types with high MSI may differ in the type MS repeat affected. In human upper urinary tract urothelial carcinomas, MSI in mono- and dinucleotide repeat MS is common and usually associated with defective DNA mismatch repair (MMR).[47, 48] While the underlying mechanisms of development and significance are poorly understood, MSI in tetranucleotide repeats, termed EMAST

(elevated microsatellite alterations at select tetranucloetides), is common in human lower urinary tract urothelial carcinomas, while MSI in mono- or dinucleotide repeats is rare.[48, 49] Differences in the type of MS affected in specific tumors may indicate that while MSI occurs in many different tumor types, the mechanisms underlying MSI development within particular types of tumors varies. In the currently evaluated canine urothelial carcinoma set, MSI was observed in MS composed of both tertranucleotide and short mono- and dinucleotide repeats. While, there was no statistical associations between MSI in tetranucleotide repeat motifs and dog breed, there were associations between both magnitude and frequency of MSI in short (mono- and dinucleotide) repeats with the phylogenetically related breeds of West Highland white terrier and Scottish terrier being most prominently represented. This suggests that not only are these breeds predisposed for development of MSI, but that the mechanisms behind such development are similar.

We also hypothesized that the degree of MSI in individual canine urothelial carcinomas would be correlated with the expression of MMR proteins. Several studies have investigated expression of MMR proteins in bladder cancer using immunohistochemistry in humans.[44, 50-55] MMR IHC appears to correlate fairly well with MMR functional status in human colon cancers and similar findings are likely in other tumors.[56, 57] Multiple studies of MMR IHC in human bladder cancer report common loss or decreased expression of MMR proteins including MLH1, MSH2, MSH6, and MSH3 in subsets of tumors.[40, 42, 50-54, 58] The percentage of bladder cancers reported to exhibit MMR deficiency according to IHC evaluation of MMR proteins varies between studies, ranging from 0-69%.[40, 42, 50-54, 58] Such seemingly variable prevalence may be attributable to many factors including the particular

proteins evaluated and the histopathologic tumor subtypes included in the study, as well as technical differences in the performance and scoring of IHC.

In the canine urothelial carcinomas of the urinary bladder of the current study, lower expression of MSH2 and higher expression of MSH6 were in comparison to that of normal urothelium from unaffected urinary bladders. Such relatively low expression of MSH2 and high expression of MSH6 was often concurrent within individual urothelial carcinomas, and there were strong statistical differences between the ratios of MSH2 to MSH6 when comparing urothelial carcinomas to normal urinary bladders. The expression of MLH1 and MSH3 was not significantly different between canine urothelial carcinomas in comparison to that of normal urothelium from unaffected control bladders.

Interestingly, the expression of neither MSH2 nor MSH6 in canine urothelial carcinomas was associated with the frequency or degree of MSI. In fact, decreased MSH2 and increased MSH6 expression relative to that in normal urinary bladders were found more frequently in canine urothelial carcinomas than was high MSI. Given the lack of correlation between MMR protein expression and MSI, it is not surprising that no association was found between MMR protein expression and breed, which was strongly associated with MSI. The exact significance of this disjunction between variations in MMR protein expression and MSI is unclear. It is possible that IHC for MMR proteins is more sensitive in identifying variations in the MMR repair pathway, but that these variations are not functionally significant and therefore do not result in MSI. It is also possible that the relative small pool of MS evaluated in this study compared to the total number of MS in the genome was not completely reflective of MMR function. In human colon cancer, there has

been considerable controversy in deciding how best to evaluate tumors for defective MMR: microsatellite analysis or IHC[57, 59]. It seems that both methods have limitations.

With the rare exception of MSH3 survival correlating with survival time, there not appreciable correlations between MSI or MMR protein expression and survival time or histomorphologic features of canine urothelial carcinomas including classification as primary infiltrative or papillary, histologic grade, or depth of invasion into the urinary bladder wall. The significance of the correlation between MSH3 expression and survival time is unclear, especially given that there was no statistical difference in MSH3 expression between urothelium of normal bladders and urothelial carcinomas. Overall, these findings suggest that while the loss of MMR in certain bladder cancers is clear, the significance of such losses is less certain. In humans, an assortment of correlations between clinical and pathologic variables and MMR status has been reported; however, there is not universal agreement as to which variables correlate with MMR or the direction of the correlation (positive or negative). For instance, one study reports the loss of MLH1 and MSH2 is most common in high grade and invasive tumors[50], whereas another study associates MMR loss with noninvasive, well-differentiated tumors[53]. Further, differential expression of MMR proteins including MSH2, MSH3, and MLH1 has been associated with urothelial carcinoma grade and clinical outcome.[16, 48, 60] In seeming contradiction, several studies indicate that loss of MMR, even in invasive tumors may indicate a better prognosis similar to what has been reported in colon cancers.[50, 53, 54]

The current study was limited by the relative low number of cases and, in particular, the low number of cases of each breed and the low number of low grade urothelial carcinomas. High grade urothelial carcinomas are far more commonly diagnosed in dogs

than low grade tumors accounting for the low number of low grade tumors in the examined set. This may be due to an actual lower rate of occurrence in canine populations or diagnosis of urothelial neoplasms only late in the course of disease when larger, more aggressive tumors affect normal micturition or cause other clinical disease. Further, euthanasia is common in veterinary medicine and is especially common in animals with urothelial carcinomas due to the negative prognosis historically associated with such tumors, the effects on micturition, the common non-resectable nature and high number of cases with metastases of such tumors at the time of diagnosis, and client choice due to financial or homecare constraints. Treatment was also not standardized between animals. It is likely that variation in treatment affected survival time, especially given that treatment was one of the few variables examined in this study that correlated with survival time.

Despite inherent limitations, this study does suggest that canine urothelial carcinomas have potential as a model for further evaluation of the MMR system with respect to carcinogenesis. This work demonstrates that MSI can be observed in spontaneous canine tumors. Further, the frequent occurrence of MSI and the variation in expression of MMR proteins in canine urothelial carcinomas of the urinary bladder compared to canine epithelial tumors from other tissues suggests that MSI may contribute to urothelial carcinogenesis in the dog, and that there is likely an inheritable propensity for MMR dysfunction in specific breeds. The clinical implications of deficiencies in MMR in canine urothelial carcinomas are less clear as neither MSI nor MMR protein expression correlated with survival time or histomorphologic features often associated with prognosis.

Acknowledgements

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APPENDIX

Figure 16: *Unmixed composite images and unmixed pseudofluorecent images derived from multispectral imaging of MLH1, MSH2, MSH3, and MSH6 immunohistochemically labeled urothelium from a normal urinary bladder control and a grade II papillary urothelial carcinoma.* MLH1 and MSH6 IHC: Indirect biotin streptavidin and Fast Red chromogen, hematoxylin counterstain. MSH2 and MSH3 IHC: 3,3'-Diaminobenzidine (DAB) chromogen, hematoxylin counterstain.

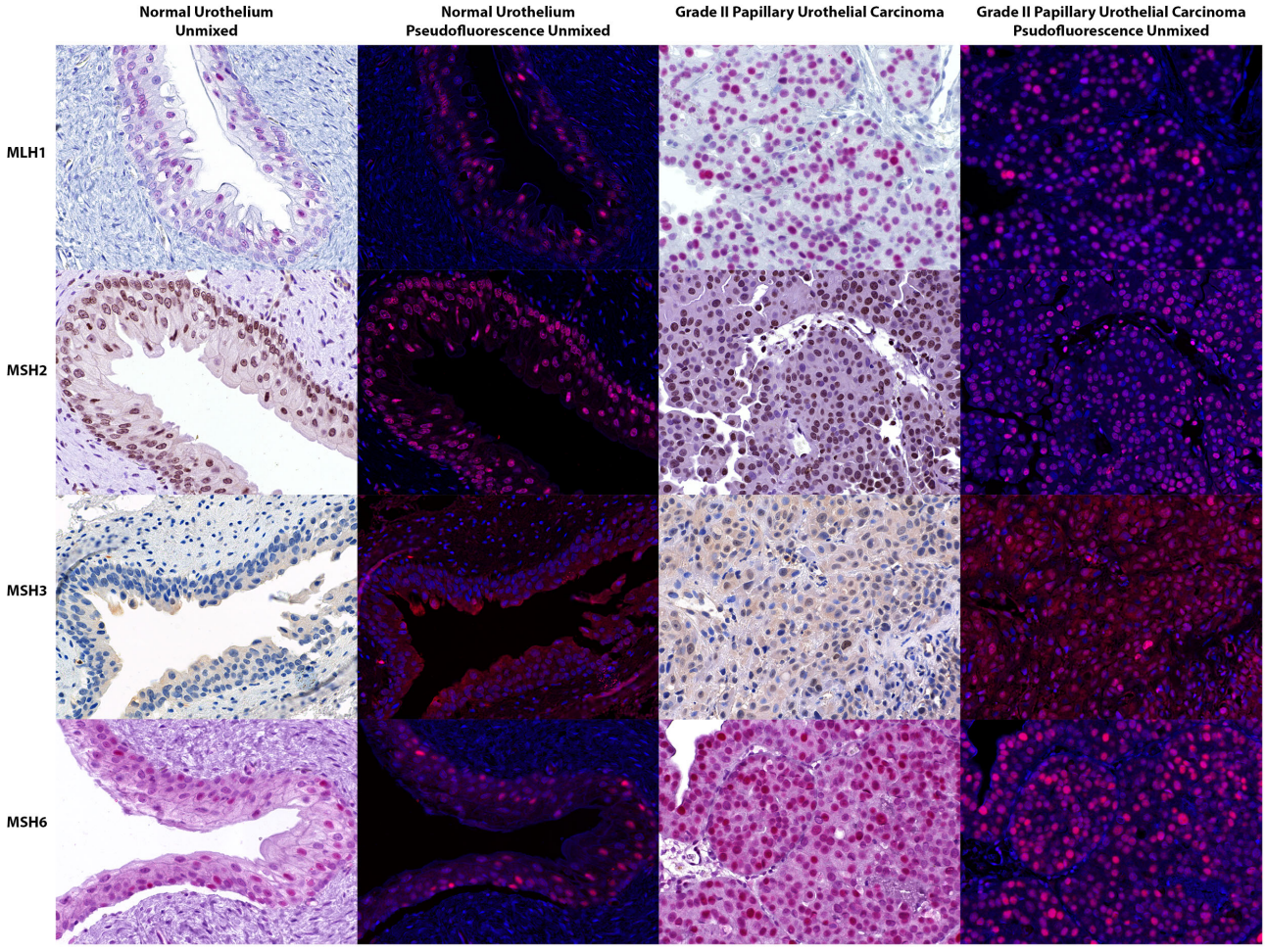

Figure 17: *Scatter plot depicting the frequency of microsatellite aberrations identified in canine gastric carcinoma tumor (N=15), mammary tumors (N=35), and urothelial carcinomas of the urinary bladder (N=46).* Each circle represents an individual tumor. The horizontal bar associated with each dataset indicates the mean %MSI for the group. Many urothelial carcinomas have high %MSI defined by 25% of evaluated MS having instability, while only few mammary tumors and no gastric carcinomas have high %MSI. In addition, the mean %MSI for urothelial carcinomas is higher than that of other evaluated tumors.

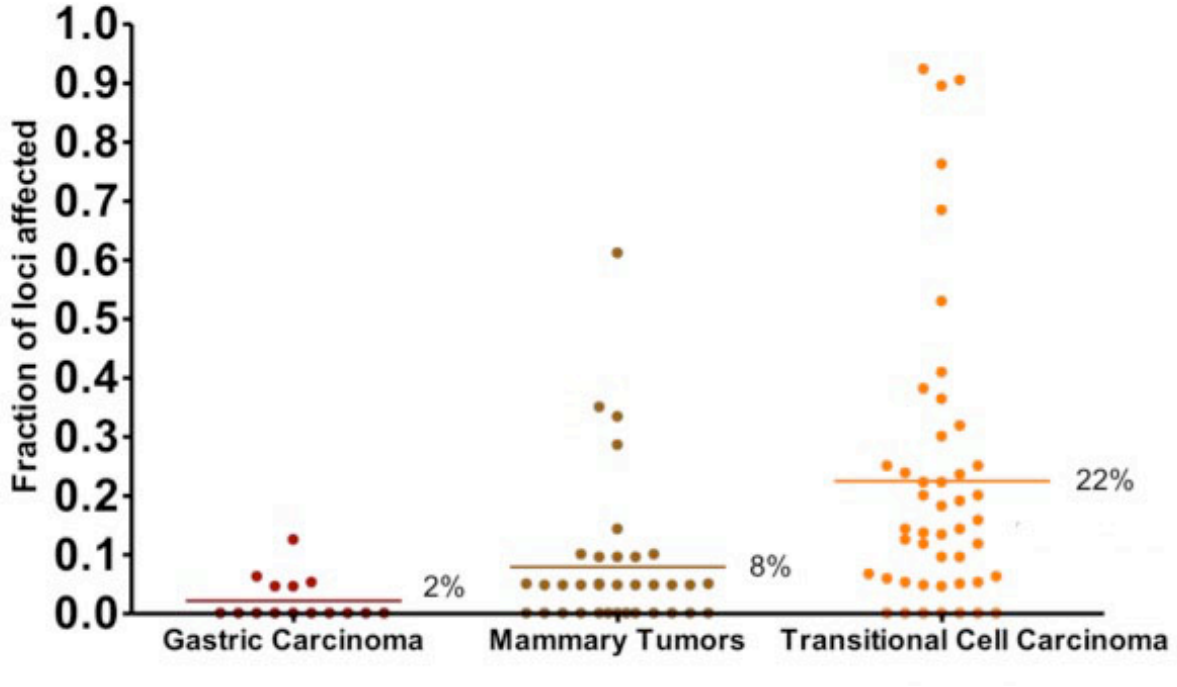

Figure 18: *Scatter plot depicting the frequency of microsatellite aberrations identified in canine urothelial carcinomas stratified by breed.* Each circle represents an individual urothelial carcinoma. The horizontal bar associated with each dataset indicates the mean %MSI for the group. The two dashed horizontal lines indicate the level for MSI-L and MSI-H classification. Variation in the fraction of MS with instability within individual breed groups is common; however, in comparison to other breeds, MSI-H is common in West Highland white terriers and Scottish terriers.

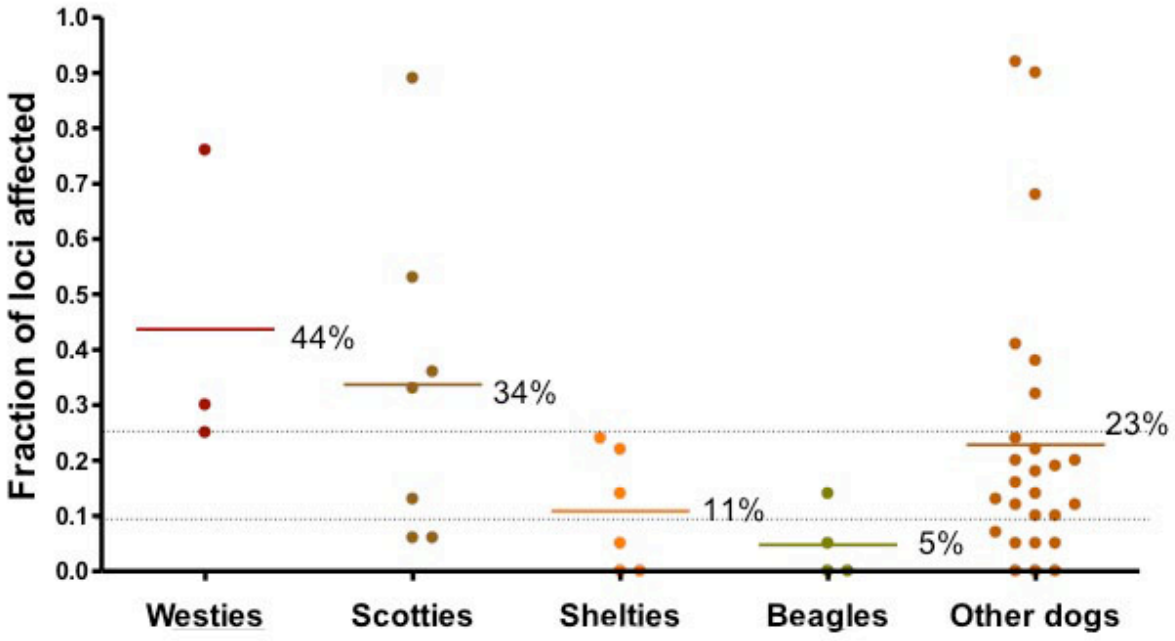

Figure 19: *Scatter plot depicting the frequency of microsatellite aberrations identified in canine urothelial carcinomas stratified by phylogenetic clade.* Each circle represents an individual urothelial carcinoma. The horizontal bar associated with each dataset indicates the mean %MSI for the group. The dashed horizontal lines indicate the level MSI-H classification. Variation in the fraction of MS with instability within individual phylogenetic groups is common; however, MSI-H is common phylogenetic clade 5, which includes West Highland white terriers and Scottish terriers.

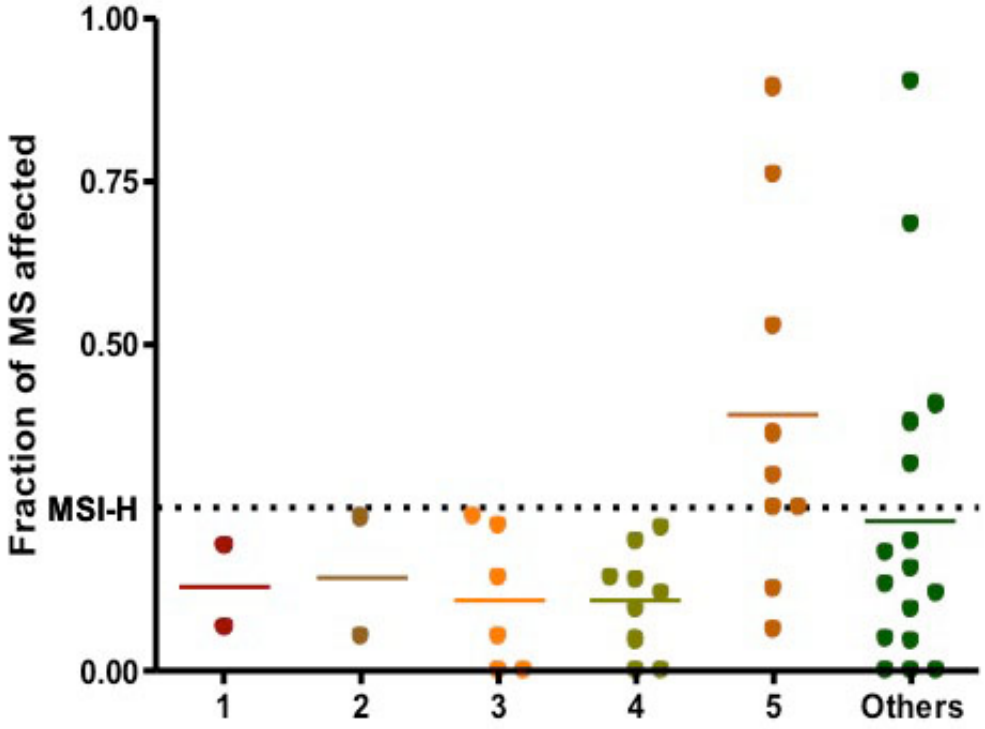

Figure 20: *Scatter plots depicting immunoreactivity of MSH2 and MSH6 in canine normal urinary bladder urothelium and urothelial carcinomas as determined by morphometric analysis of multispectral imaging.* Each circle represents an individual case. Horizontal bars associated with each dataset indicate the mean relative expression of MSH2 or MSH6 for each group. While there is variation in relative expression of MSH2 and MSH6 in both normal urothelium and urothelial carcinomas, the mean relative expression of MSH2 is lower in urothelial carcinomas, than in normal urothelium (A), and the mean relative expression of MSH6 is higher in urothelial carcinomas, than in normal urothelium (B).

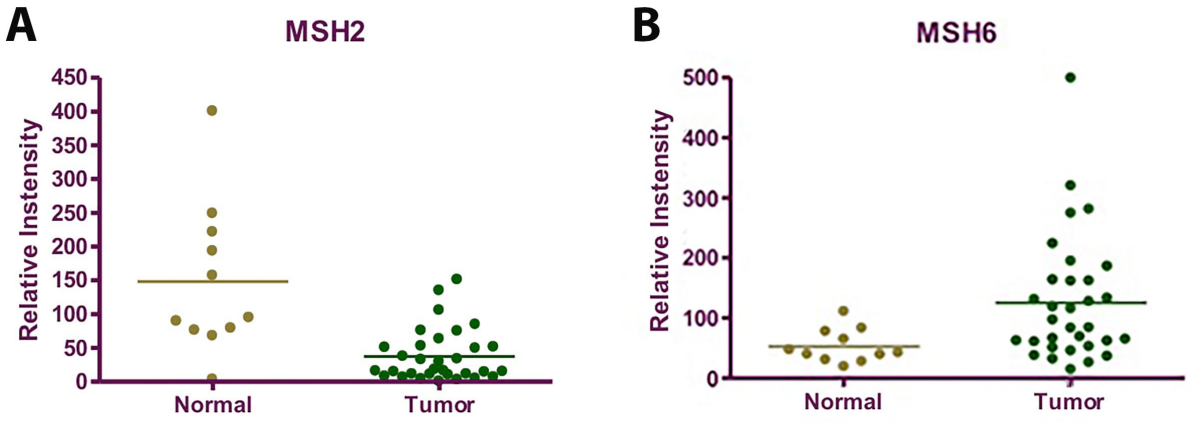

Figure 21: *Scatter plot depicting ratio of MSH2 and MSH6 immunoreactivity in individual cases of canine normal urinary bladder urothelium and urothelial carcinomas as determined by morphometric analysis of multispectral imaging.* Each circle represents an individual case. Horizontal bars associated with each dataset indicate the mean ratio of MSH2 and MSH6 for each group. While there is variation in the ration of MSH2 to MSH6 expression for individual cases in both normal urothelium and urothelial carcinomas, the mean ratio of MSH2 to MSH6 is lower in urothelial carcinomas, than in normal urothelium.

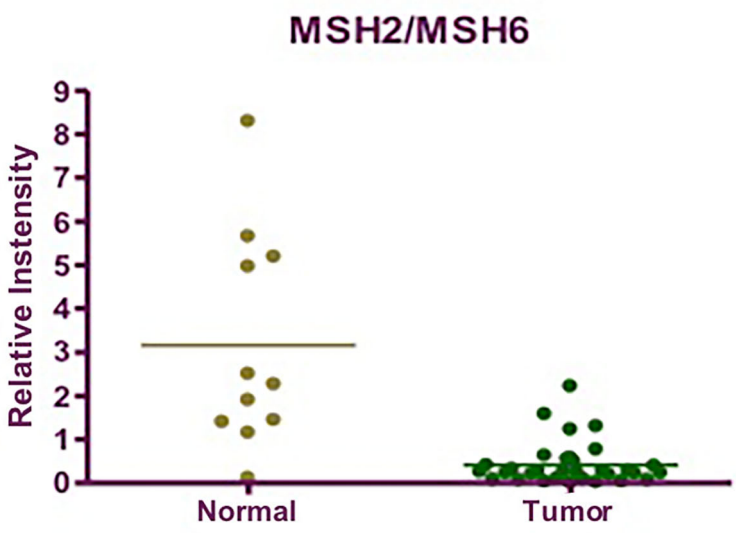

Table 10: *Distribution of microsatellite instability in canine epithelial tumors*

Tumor type	MSS	MSI-L	MSI-H
	No. tumors (%)	No. tumors (%)	No. Tumors (%)
GC	14 (93%)	1 (7%)	0 (0%)
MT	28 (80%)	3 (9%)	4 (11%)
TCC	16 (36%)	16 (36%)	13 (28%)

GC = Gastric carcinoma; MT = Mammary gland tumor; TCC = Transitional cell carcinoma of the bladder; MSS = microsatellite stable; MSI-L = at least 10% MSI; MSI-H = at least 25% MSI.

Table11: *Distribution of microsatellite instability in canine urothelial carcinomas of the urinary bladder classified by breed*

Breed	# of Tumors	# MSS	# MSI-L	# MSI-H	Mean %MSI
Westie	3	0	0	3	43.7% (± 28.2%)
Scottie	6	1	1	4	37.1% (± 30.7%)
Sheltie	6	3	3	0	10.9% (± 10.7%)
Beagle	4	3	1	0	4.8% (± 6.7%)
Other	26	7	13	6	22.8% (± 25.1%)

MSS = microsatellite stable; MSI-L = at least 10% MSI; MSI-H = at least 25% MSI.

Table 12: *Distribution of microsatellite instability in canine urothelial carcinomas of the urinary bladder by breed and repeat motif*

Breed	**Mean %MSI**	**Mean %MSI Di/Mono**	**Mean %MSI Tetra**
Westie	43.7% (± 28.2%)	52.8% (± 21.0%)	30.1% (± 41.8%)
Scottie	37.1% (± 30.7%)	32.2% (± 29.3%)	45.7% (± 36.1%)
Sheltie	10.9% (± 10.7%)	9.4% (± 8.9%)	13.0% (± 17.8%)
Beagle	4.8% (± 6.7%)	4.2%% (± 8.3%)	5.6% (± 6.4%)
Other	22.8% (± 25.1%)	21.4% (± 25.0%)	25.6% (± 26.4%)

% MSI = mean percentage of MS markers demonstrating instability for all tumors and all MS markers; Mean %MSI Di/Mono = Mean %MSI for di- or mononucleotide repeats only; Mean %MSI Tetra = Mean % MSI for tetranucleotide repeats only. Standard deviation is given in parentheses.

Table 13: *Distribution of %MSI and immunohistochemically evaluated MMR protein expression in canine normal urinary bladders and urothelial carcinomas of the urinary bladder*

	Mean	Median	Minimum	Maximum
%MSI				
All cases	14.4% (±3.2%)	9.5%	0%	89.0%
Normal urinary bladder	1.7% (±1.7%)	0%	0%	8.0%
All urothelial carcinomas	17.4% (±3.7%)	13.6%	0%	89.0%
Infiltrating carcinomas	27.2% (±16.0%)	11.8%	5.0%	89.0%
Papillary carcinomas	14.9% (±2.5%)	14.0%	0%	38.0%
MLH1 (postive pixels per nucleus)				
All cases	740.3 (±113.6)	618.5	7.1	2268.0
Normal urinary bladder	775.5 (±225.2)	755.5	26.6	1245.0
All urothelial carcinomas	695.4 (±111.0)	492.9	0.2	2268.0
Infiltrating carcinomas	571.1 (±229.2)	466.3	15.0	1353.9
Papillary carcinomas	797.4 (±158.0)	653.1	7.1	2268.0
MSH2 (postive pixels per nucleus)				
All cases	351.0 (±75.9)	209.7	1.7	1629.1
Normal urinary bladder	750.2 (±235.6)	768.2	189.2	1492.6
All urothelial carcinomas	328.8 (±67.6)	175.8	1.7	1629.1
Infiltrating carcinomas	164.8 (±52.1)	134.8	46.8	343.2
Papillary carcinomas	301.5 (±91.2)	157.0	1.7	1629.1
MSH3 (postive pixels per nucleus)				
All cases				
Normal urinary bladder	1410.0 (±197.9)	1291.9	684.0	2566.0
All urothelial carcinomas	1179.6 (±125.9)	1107.8	102.0	2662.0
Infiltrating carcinomas	915.8 (±248.7)	714.6	200.0	2133.0
Papillary carcinomas	1264.0 (±144.5)	1143.9	102.0	2662.0
MSH6 (postive pixels per nucleus)				
All cases	570.7 (±96.2)	399.3	27.8	2490.0
Normal urinary bladder	255.6 (±60.6)	256.0	112.4	399.3
All urothelial carcinomas	603.2 (±94.9)	417.1	1.9	2490.0
Infiltrating carcinomas	746.8 (±243.7)	1054.0	27.8	1225.8
Papillary carcinomas	619.8 (±131.4)	450.5	41.0	2490.0

% MSI = mean percentage of MS markers demonstrating instability for all tumors and all MS markers; Standard deviation for means is given in parentheses.

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CHAPTER 5

Evaluation of DNA mismatch repair in novel canine lower urinary tract urothelial carcinoma cell lines

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Abstract

The DNA mismatch repair system plays important roles in DNA post replication repair, damage recognition signaling, apoptosis, and meiotic recombination. Consequently, defects in MMR are associated with carcinogenesis in a variety of tissues and have been shown to affect response of cancers to chemotherapy. Recently, we have shown that many canine lower urinary tract urothelial carcinomas have deficiencies in MMR. In order to further investigate the role of MMR in carcinogenesis and the significance of MMR deficiency in the dog, 4 novel lower urinary tract urothelial carcinoma cell lines from 3 dogs established from primary cultured cells. Characterization of all cell lines including immunophenotyping for markers of urothelial differentiation was consistent with an urothelial origin. MMR gene expression in each cell line was evaluated using qPCR and protein expression was evaluated using Western blots. Further, MMR gene expression was evaluated in mice xenografts derived from the canine cell lines using qPCR on laser capture microdissection samples of tumor and MMR protein expression was evaluated using immunohistochemistry. One of the four cell lines, TYLER2, had decreased gene and protein expression of MSH2 and MSH6, which form portions of the MMR pathway. XTT survival assays were subsequently used to evaluate differences between the cell lines in sensitivity to a panel of chemotherapeutics. In comparison to other cell lines including one derived from the same primary tumor, TYLER2 had relative resistance to carboplatin and sensitivity to oxaliplatin, thiotepa, and methotrexate.

Introduction

Urothelial carcinomas of the urinary bladder comprise a significant proportion of canine neoplasms and have historically been associated with a poor prognosis. Because complete surgical resection is rarely achievable and 30-50% of these cancers metastasize, most animals die of their disease.[1, 2] Although frequently treated with chemotherapy, these cancers respond inconsistently; however, the choice of chemotherapeutic for urothelial carcinomas has traditionally been empirical rather than based on scientific evidence.[2, 3]

It is clear that the molecular constitution of cancer in one individual can differ considerably from that of cancers in others despite the fact that the cancers may have a similar morphology and anatomic localization. In other words, cancers even of the same histologic origin can be molecularly unique. Thus, in therapeutic terms, a standardized treatment prescription fails to address this diversity. In recent years, there has been considerable interest in determining ways to exploit the particular molecular constitution of a given tumor to improve therapeutic outcomes. One feature that differs between tumors that may be exploited through targeted therapy is DNA repair capability, including the functionality of their DNA mismatch repair system (MMR).

The MMR machinery participates in a variety of cellular processes. Most notably, this system is responsible for post replication repair of base-base mismatches and the resolution of insertion and deletion loops that can occur in repetitive regions of DNA.[4] The MMR system also participates in a variety of other processes including DNA damage recognition signaling, apoptosis, meiotic recombination, and other DNA repair pathways.[4] Given these varied roles played by MMR, it is not surprising that defects in MMR facilitate carcinogenesis. Similarly, it is not surprising that defects in MMR have significant effects on

response of cancers to therapy. The reported effects of MMR deficiency on chemotherapeutic response are complex. Depending on the mechanism of action for a given chemotherapeutic, MMR deficiency may confer either drug resistance or sensitivity.

Microsatellite Instability (MSI), which is defined as the accumulation of frame shift mutations in nucleotide repeat regions of DNA (called microsatellites) is considered a "signature" for MMR dysfunction.[4, 5] In previous investigations, we demonstrated that MSI is frequent in canine urothelial carcinomas of the urinary bladder and has strong associations with breed and phylogenetic clade (data presented in Chapter 3). In addition, we showed that expression levels of MSH2 and MSH6 as evaluated by immunohistochemistry differed in canine urothelial carcinoma from that observed in normal canine urotheluim (data presented in Chapter 3). Such frequent MSI and alterations MMR protein expression suggests MMR dysfunction is common in canine urothelial carcinomas and may be heritable. The exact cause and significance of MMR dysfunction in canine urothelial carcinomas remains unclear. However, based on reports of differential response of MMR proficient and MMR deficient cancers to treatment in humans, it is likely that MMR status could also affect treatment response in dogs with lower urinary tract urothelial carcinomas.

For the current study, we aimed to establish *in vitro* and xenograft models for further study of MMR dysfunction in canine lower urinary tract urothelial carcinomas. More specifically, we aimed to evaluate the expression of components of the MMR pathway at the gene and protein level in lower urinary tract urothelial carcinoma cell lines and xenografts, and to correlate differences in such expression to treatment response.

Materials and methods

All work done with canine tissues or with xenografts was preformed with approval from the Michigan State University Institutional Animal Care and Use Committee.

Characterization of canine lower urinary tract urothelial carcinoma cell lines

With owner consent for inclusion into the study, four canine cells lines (ANGUS, KINSEY, TYLER1 and TYLER2) were generated from primary cell culture of urothelial carcinomas of the lower urinary tract. ANGUS was derived from a papillary grade III urothelial carcinoma of the urinary bladder with invasion into the muscularis of a 10-year-old neutered male Scottish terrier, KINSEY was derived from an infiltrating urothelial carcinoma of the urinary bladder with invasion into the substantia propria of a 12-year-old spayed female Australian Shepherd, and TYLER cell lines were derived from a single papillary grade II urothelial carcinoma with invasion into the muscularis of the prostatic urethra of an 8-year-old neutered male Beagle. All animals were referred to the Michigan State University, Veterinary Medical Center, East Lansing, MI for treatment of primary bladder disease. Diagnoses of urothelial carcinomas were made based on results of histopathologic examination and evaluation of immunohistochemical markers at the Diagnostic Center for Population and Animal Health, Lansing, MI.

For ANGUS and TYLER cell lines, neoplastic cells were initially isolated from macerated fresh samples of each tumor taken from routine diagnostic biopsy. For KINSEY, cell lines were derived from cell pellets derived from centrifugation of 7mL of urine taken by cystocentesis. The resulting cell pellet was suspended in media and plated on cell culture plates. For all cell lines, neoplastic cells were grown on uncoated cell culture plates in 1:1 DMEM/F12 media (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal

bovine serum, l-glutamine, and penicillin/streptomycin in a 37°C humidified CO_2 incubator. For subculturing, cells were harvested from culture plates using 0.05% or 0.25% Trypsin-EDTA (Life Technologies).

Over multiple passages, two unique cellular morphologies were identified in the original TYLER cell line, which were separated by selective trypsinization over several passages. For selective trypsinization, mixed cell populations were incubated with 0.05% Trypsin-EDTA at 37°C for 1-3 minutes. Cells easily released from attachments on cell plates by such light trypsinization were removed by light rinsing of plates with culture media. Easily trypsinized cells were subsequently subcultured separately from more adherent cells resulting in establishment of two morphologically distinct cell lines, TYLER1 and TYLER2.

In addition subclones of TYLER1 and TYLER2 were established through serial dilution. Single colonies derived from dilution of cell suspensions plated on 96 well plates were identified at day 3 post plating. Subclones were established from these single cell colonies through subsequent expansion.

Development of xenografts

Xenografts were established in athymic nude mice by subcutaneous injection of 10^6 cells in 200ul of sterile media lacking FBS and antibiotics over the flank. Tumor volume was evaluated twice per week and animals were euthanized using CO_2 when tumor volume was >0.5cm^3 or when there grossly obvious ulceration of the skin over tumors. Fresh samples of xenograft tumors were flash frozen in liquid nitrogen and saved at -80°C. Additional fresh tissue samples were frozen over dry ice in optimum cutting temperature (OCT) media and samples were stored at -80°C. Remaining tumor samples were fixed for

24-48 hours in 10% buffered formalin and routinely processed for histopathologic examination.

Immunophenotyping of primary tumors, cell lines, and xenografts

For each of the four cell lines, cells were removed from 75% confluent cell culture plates with a cell scraper into culture media. After pelleting of cells under centrifugation, cells were suspended in 3ml of 10% buffered formalin. Following 6 hours of fixation, cells were pelleted under centrifugation, and the resulting cell pellet was routinely processed into paraffin blocks.

Markers of differentiation were evaluated in paraffin-embedded primary tumors, cell lines, and xenograft samples using immunohistochemistry for MNF116, E-cadherin, P-cadherin, N-cadherin, Cytokeratin 7, uroplakin III, Prostatic acid phosphatase (PAP), and vimentin. Previously described protocols were used for IHC, with the exception that for mouse derived primary antibodies, blocking of nonspecific binding to the mouse tissues was accomplished by preincubating primary antibody with secondary antibody and mouse serum prior to use (see chapters 1 and 2).

Evaluation of MMR protein expression by Western blots

Western blotting was used to evaluate expression of MLH1, MSH2, MSH3, and MSH6 in a cell lines along with a variety of normal canine tissues. Cell lines were harvested at 75% confluence using a cell scraper. Protein was subsequently isolated through incubation with RIPA buffer. Total protein was quantitated using Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA), a Victor X3 microplate reader (PerkinElmer, Shelton, CT USA) reading absorbance at 562nm, and comparison to a dilution series of bovine serum albumin. Proteins were separated using SDS-PAGE. For MLH1 and MSH2, proteins

from each sample were separated using 10% gels (BioRad, Hercules, CA, USA). For MSH3 and MSH6, proteins from each sample were separated using 7% gels (BioRad). Precision Plus Protein™, Dual Color (BioRad) was used for protein standards. Proteins were transferred to nitrocellulose by electroblotting. Following washes in TBST, blots were blocked using 3% powdered skim milk and incubated overnight with primary antibodies in 3% powdered skim milk as follows: 1:200 mouse monoclonal anti-MLH1 antibody (BD Biosciences, San Jose, CA, USA), 1:1500 rabbit polyclonal anti-MSH2 antibody (Santa Cruz Biotechnology, Dallas, TX, USA), 1:3000 rabbit polyclonal anti-MSH3 antibody (1:100, Abcam, Cambridge, MA, USA), 1:500 mouse monoclonal anti-MSH6 antibody (1:500, BD Biosciences). Following washes in TBST, blots were accordingly incubated for 2 hours with either 1:7500 goat anti-mouse IgG or 1:10,000 goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology) as appropriate. Blots were developed using a chemiluminescence detection system (Thermo Scientific) and using Amersham Hyperfilm™ MP autoradiography film (GE Healthcare, Little Chalfont, BM, UK). Blots were subsequently stripped for 1 hour using Western blot stripping buffer (Thermo Scientific). Following washes in TBST and blocking in 3% bovine serum albumin (BSA), blots were incubated overnight with 1:2000 goat anti-β-actin primary antibody in 3% BSA. Following washes in TBST, blots were incubated for 2 hours with donkey 1:7500 anti-goat secondary antibody in 3% BSA (Santa Cruz Biotechnology, Dallas, TX, USA), developed as described above.

qPCR for MMR genes

Cell lines were harvested at 75% confluence using .05% or .25% Trypsin-EDTA. Cell lysis, degradation of genomic DNA, and reverse transcription of RNA to cDNA was

accomplished using TaqMan® Gene Expression Cells-to-C_t kits (Life Technologies, Grand Island, NY, USA) using manufacturer's instructions and 10^4 cells.

For xenografts, laser capture microdissection (LCM) was used to isolate neoplastic canine urothelial carcinoma cells from mouse tissues. In TYLER1 and TYLER2 xenografts, epithelioid portions of tumors were harvested separately from portions with more discrete cell morphology. Briefly, serial sections of OCT embedded frozen xenografts blocks were made with a cryostat microtome using RNase-free techniques. Following brief fixation in methanol, staining with hematoxylin, and dehydration in alcohol, tissue sections were kept in xylene to maintain dehydration until LCM was performed. Laser capture microdissection (LCM) using an Arcturus PixCell IIe (Arcturus Engineering, Mountain View, CA, USA) with targeted regions of tumors being captured with CapSure® Macro LCM caps (Life Technologies). RNA was isolated from captured regions using an Arcturus Paradise Plus RNA Extraction and Isolation Kit (Life Technologies) following the manufacture's instructions. Reverse transcription was accomplished using High-Capacity cDNA Reverse Transcription Kits (Life Technologies) using manufacturer's instructions.

Quantitative real-time PCR was performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Grand Island, NY, USA) using commercially available TaqMan® gene expression assays for MLH1, MSH2, MSH3, MSH6, PMS2, β-actin, and HPRT1, and a custom designed TaqMan® gene expression assay for GAPDH (Applied Biosystems) (Table 14). Studies were performed with technical triplicates and were repeated twice with independent RNA isolation and generation of cDNA between studies. The baseline and threshold for detection was set by the StepOnePlus software, with threshold Ct being defined as the number of cycles at which the detection of fluorescent

signal exceeded the automatically set threshold. For cell lines, relative quantitation of target gene expression was evaluated using the ΔCt method using comparison of a compilation of control genes including GAPDH, β-actin, and HPRT1. For xenografts, only expression of MSH2 and MSH6 genes was evaluated, and analysis of relative expression of target genes was made to only GAPDH expression. Relative quantitation of gene expression between different cell lines or xenografts was performed using the delta-delta Ct (ΔΔCt) method.

Immunohistochemistry and morphometric analysis for MSH2 and MSH6 in xenografts

Immunohistochemistry (IHC) was preformed to evaluate MSH2 and MSH6 expression in xenografts using the antibodies described above for Western blots. Five μm sections of all formalin-fixed, paraffin-embedded tissues were processed for immunohistochemistry. For MSH2, deparaffinization, antigen retrieval, immunohistochemical staining and counterstaining was preformed on a Dako Link 48 Autostainer (Carpinteria, CA, USA) using a LSAB2 kit (Dako), which employs a 3,3' diaminobenzidine tetrahydrochloride (DAB) chromogen detection system. Retrieval for MSH2 was accomplished using heat induced epitope retrieval and incubation with citric buffer for 20 minutes. For MSH6, deparaffinization, antigen retrieval, immunohistochemical staining and counterstaining was preformed on a Benchmark XT™ autostainer (Ventana, Tucson, AZ, USA) using an Enhanced Alkaline Phosphatase Red Detection Kit (Ventana) that uses an indirect biotin streptavidin and Fast Red chromogen detection system. Blocking of nonspecific binding to the mouse anti-MSH6 antibody to mouse tissues was accomplished by preincubating primary antibody with secondary antibody and mouse serum prior to use. Retrieval for MSH6 was accomplished with 20

minutes of incubation with Cell Conditioning 1 solution (Ventana). Sections of normal urinary bladder were used as positive controls and run in parallel to cases for each of the IHC. For negative controls, homologous non-immune sera or buffer replaced primary antibodies.

With the exception of TYLER1 and TYLER2, seven randomly picked areas from each slide immunohistochemically labeled for the respective MMR proteins listed above were imaged using a CRi Nuance multispectral imaging system (Woburn, MA, USA). For TYLER1 and TYLER2, imaging was selectively performed to isolate regions with epithelial and discrete cell morphology. The resulting image cubes were converted to optical density units, then mathematically unmixed to separate chromogen labeling from counterstain using spectral libraries generated from imaging of only hematoxylin stained or immunohistochemically labeled and hematoxylin counterstained control specimens. Colocalized immunoreactivity was assessed as positive pixels per image using constant thresholds for detection and the multispectral imaging system software. The component images of the image cubes were then pseudocolored, converted to pseudofluorescent format, and unmixed for counting of nuclei. Such counting was done using Imagine morphometric analysis software developed at the Van Andel Research Institute (Grand Rapids, MI, USA) (Figures 22 and 23). Final results of morphometric analysis of immunoreactivity were reported as the mean number of positive pixels per nucleus for each evaluated sample.

Effect of chemotherapeutics on cell survival

Survival curves were generated for the each of the four cell lines in response to a panel of therapeutic agents using XTT assay kits (Trevigen, Gaithersburg, MD, USA) following the

manufacturer's instructions. Evaluated chemotherapeutics included cisplatin, carboplatin, oxaliplatin, methotrexate, thiotepa, lomustine (CCNU), paclitaxel, cytarabine, and gemcitabine. Briefly, $2x10^4$ cells of each cell line were incubated with a range of concentrations of each cytotoxic agent. Following incubation with XTT, a terazolium compound that is converted to a formazan dye within viable cells, absorbance was assessed using a Victor X3 microplate reader (PerkinElmer, Shelton, CT USA). Corrected absorbance was calculated by subtracting absorbance read at 630nm from absorbance read at 490nm. Surviving cell fraction was extrapolated from forecast models based on standards derived from incubating known numbers of concurrently plated cells from each cell line with XTT. Survival assays were performed with three technical repeats and repeated twice. Means of percent cell survival for each tested chemotherapeutic concentration were used to generate survival graphs.

Results

Characterization of canine lower urinary tract urothelial carcinoma cell lines and xenografts

Immunophenotyping of all primary tumors was consistent with urothelial origin. Specifically, neoplastic cells throughout all tumors diffusely had cytoplasmic immunoreactivity for MNF116, cytoplasmic and perimembranous immunoreactivity for cytokeratin 7, and perimembranous immunoreactivity for E-cadherin and P-cadherin (Figure 24). In addition, there was perimembranous immunoreactivity for uroplakin III in apical cell layers. There was no immunoreactivity for N-cadherin, vimentin, or PAP in any primary tumor.

After >20 passages ANGUS and KINSEY had a uniform epithelioid morphology. These cells were plump polygonal and grew as distinct, dense colonies (Figure 25). The original TYLER cell line after >10 passages was composed of a biphasic population of cells including plump polygonal epithelioid cells that grew in sheets and aggregates, and a population of more spindle to stellate cells. After separation of these distinct populations by selective trypsinization followed by >10 passages, the cell lines TYLER1 and TYLER2 were morphologically characterized. TYLER1 was composed of plump polygonal epithelioid cells that grew in discrete colonies. TYLER2 was composed of spindle to stellate cells that aggregated into sheets and bundles when near confluence.

All cell lines were confirmed as epithelial in origin by immunoreactivity to the pancytokeratin marker MNF116. Further, all cell lines expressed cytokeratin 7 which given the location and histomorphology of the primary urothelial carcinomas from which they were derived, is consistent with an urothelial origin. In addition, many cells from the TYLER1 and TYLER2 cell lines expressed prostatic acid phosphatase (PAP) (Figure 26). All

cell lines were immunoreactive for vimentin, and no cell line expressed uroplakin 3, a marker expressed late in urothelial differentiation and only by apical umbrella cells of the urothelium.

Characterization of xenografts

ANGUS failed to produce appreciable tumors in xenograft models. KINSEY, TYLER1, and TYLER2 grew well as xenografts forming rapidly growing masses. In xenografts, tumors derived from the KINSEY cell line were composed of neoplastic plump polygonal epithelial cells arranged in dense sheets and trabeculae. Three of 6 TYLER1 xenografts and 3 of 4 TYLER2 xenografts were composed of biphasic cell populations including multifocal regions of epithelioid differentiation and extensive regions of discrete round cells (Figure 27). Areas of epithelioid differentiation were composed of nests of polygonal neoplastic cells that often had central sharply defined, open or proteinaceous fluid filled cavitations. Discrete cells were round, loosely associated with one another, and arranged in dense sheets supported by scant fine fibrovascular stroma. The remaining 3 TYLER1 xenografts were composed of only epithelioid cells and the remaining TYLER2 cell line was only composed of discrete cells. Such biphasic cell populations were also seen in xenografts derived from subclones of TYLER1 and TYLER2 generated through serial dilution.

Using immunohistochemistry, neoplastic cells of KINSEY xenografts had immunoreactivity for the epithelial markers MNF116, E-cadherin, P-cadherin; immunoreactivity for cytokeratin 7 and uroplakin III, which are expressed by urotheluim; diffuse immunoreactivity for vimentin, an intermediate filament expressed in mesenchymal cells; and no immunoreactivity for PAP, a marker of prostatic epithelium (Figure 28). Epithelioid populations of TYLER1 and TYLER2 xenografts had expression of

MNF116, E-cadherin, P-cadherin, N-cadherin, cytokeratin 7, and uroplakin III (Figure 29). Discrete cell populations of TYLER1 and TYLER2 had no expression of MNF-116, E-cadherin, P-cadherin, cytokeratin 7, or uroplakin III. Both cell populations had strong expression of vimentin and few scattered individual cells of both populations had immunoreactivity for PAP.

Evaluation of MMR protein expression by Western blots

Western blotting yielded bands corresponding to expected molecular weights for all evaluated markers in all cell lines. The expression levels of MLH1 and MSH3 were relatively consistent across cell lines; however, the relative intensity of the MSH2 and MSH6 bands were lower in the TYLER2 cell line in comparison to that of other cell lines consistent with lesser expression of these proteins (Figure 30).

qPCR for MMR gene expression

With the exception of MSH6 in KINSEY in which there was moderate variance, there was only mild variance in relative gene expression between technical and biologic repeats. Examining expression of the MMR genes in cell lines relative to a compilation of the expression of control genes, GAPDH, β-actin, and HPRT1, TYLER2 had lower levels of expression of MSH2 and MSH6 than ANGUS, KINSEY, and TYLER (Figure 31). There were no obvious differences in expression of MLH1, MSH3, PMS2 or MLH1 between evaluated cell lines. There was significant variance in the relative expression of MSH2 and MSH6 relative to GAPDH for all evaluated xenografts; however, mean relative expression of MSH2 and MSH6 was higher in KINSEY than in either TYLER1 or TYLER2, and the there were no or only mild differences in mean relative expression of MSH2 and MSH6 between TYLER1 and TYLER2 (Figure 32). In evaluations of relative MSH2 gene expression in LCM

separated epithelioid and discrete cell populations of TYLER1 and TYLER2, there was differential expression of MSH2 between epithelial and discrete cell populations for both TYLER1 and TYLER2 (Figure 33). There was, however, no difference in MSH2 expression comparing the separated cell groups of TYLER1 to those of TYLER2.

Immunohistochemistry and morphometric analysis for MSH2 and MSH6 in xenografts

In general, the mean expression levels of MSH2 and MSH6 in terms of positive pixels per nucleus was lower in discrete cell populations than in epithelioid populations for both TYLER1 and TYLER2 (Figure 34; Tables 15 and 16). Further, the mean expression levels of MSH2 and MSH6, for both epithelioid and discrete cell populations were respectively higher in TYLER1 populations than in TYLER2 populations. The mean expression of MSH6 was higher in KINSEY in comparison to that of TYLER2 cell lines and discrete TYLER1 cell populations, but was not different from that of epithelioid portions of TYLER1. Expression of MSH2 in KINSEY was low relative to that observed in epithelioid populations. However, there was marked variation in quantitated immunoreactivity, and particularly large differences in immunoreactivity for MSH2 between the TYLER cell line subgroups.

Using one-way ANOVA tests, there were significant differences in the immunoreactivity for MSH6 between all cell groups ($p<0.001$) and when comparing only TYLER xenografts ($p=0.015$). There was, however, no significant difference between the immunoreactivity for MSH2 whether comparing all groups ($p=0.125$) or only TYLER xenografts ($p=0.230$).

Survival assays

Graphic results of XTT survival assays are presented in Figure 35. With regards to differences in chemotherapeutic sensitivity for TYLER1 and TYLER2, there were

appreciable differences in sensitivity to carboplatin, oxaliplatin, methotrexate, and thiotepa. TYLER2 was more sensitive to oxaliplatin, moderate concentrations of methotrexate, and lower concentrations of thiotepa than TYLER1, but less sensitive to carboplatin. ANGUS often was less sensitive to chemotherapeutics relative to other evaluated cell lines including for carboplatin, cisplatin, thiotepa, and gemcitabine. Sensitivity of KINSEY was similar to that of TYLER1 for carboplatin, TYLER1 and TYLER2 for cisplatin, ANUGS and TYLER1 for oxaliplatin, TYLER2 for methotrexate, and TYLER1 and TYLER2 for gemcitabine. For thiotepa, there were differences in sensitivity for all tested cell lines, especially at the lowest tested concentrations, with TYLER2 being most sensitive, followed by TYLER2, KINSEY and ANGUS. There was no appreciable difference in sensitivity of TYLER1, TYLER2, or KINSEY for gemcitabine. There were no or only mild differences in response of all evaluated cell lines to paclitaxel, cytarabine, lomustine.

Discussion

In humans, the potential application of MMR as a therapeutic target is highlighted by the differences observed in prognosis and the response to treatment between cancers of the same type that differ in MMR capacity. In terms of prognosis, MMR deficient cancers are generally associated with a more favorable clinical outcome than those that are MMR proficient.[6-10] In colorectal carcinomas, for example, MMR deficient tumors are associated with longer survival times and a decreased risk of metastasis compared to those that are proficient.[9] The reason for this difference in prognosis is likely multifactorial. Inherently, cancers with defects in MMR are genetically less stable that those with intact MMR leading to an increase in DNA lesions, which promotes cell cycle arrest and apoptosis signaling.[11] Also, there is evidence that MMR deficient cancer cells are often highly immunogenic. This is proposed to occur due to production of atypical proteins generated through frame shift mutations resulting in T cell mediated immune responses directed against the cancer cells.[12] Additionally, it has been shown that several genes associated with antitumor immune responses are over expressed in MMR deficient cancers and cell lines.[11]

Given the range of DNA repair and damage signaling pathways with which the MMR system is associated, it is not surprising that defects in MMR also have significant effects on response of cancers to therapy. The effects of MMR deficiency on chemotherapeutic response are complex. MMR deficiency is capable of conferring either drug resistance or sensitivity largely based on a given drug's mechanism of action.[8, 13-21] The differential effect of the MMR system relative to chemosensitivity likely in part reflects the ability of the MMR machinery to participate in a number of alternative DNA damage processing/signaling pathways. In the case of simple methylated bases in the DNA molecule, it has been

proposed that MMR may be involved in triggering apoptosis through either futile repair attempts or through conversion of the alkylated base to a lethal lesion such as a double strand break.[21] Thus, in this case, deficiency of MMR results in tolerance of alkylated bases and drug resistance. In contrast, an increased sensitivity to cytotoxins can be observed if defective MMR results in failure to repair certain types of DNA lesions. For example, certain interstrand cross-links are recognized by the MMR system and repair is thought to occur through MMR mediated homologous recombination.[13] Cells deficient in MMR do not effectively repair these cross-links and are sensitive to agents that induce such these lesions.

Of the 4 canine lower urinary tract urothelial carcinoma cell lines evaluated in the current study, TYLER2 was considered to be MMR deficient relative to the other cell lines in terms of MSH2 and MSH6 gene and protein expression. Such differential expression of factors in the MMR pathway in cell lines offers the opportunity to study the effect of differential expression of MMR on chemotherapeutic response in an *in vitro* canine model. This is particularly true as TYLER1 and TYLER2 provide the opportunity to evaluate naturally occurring differenced in MMR capacity while sharing a similar genetic background having been derived from the same primary tumor. The differential sensitivities observed in the MMR deficient TYLER2 to chemotherapeutics are consistent with reports in similar studies in humans. The fact that ANGUS was resistant to a number of chemotherapeutics relative to other cell lines is not surprising, as the primary tumor was aggressively treated with chemotherapy prior to establishment of the cell line.

Cisplatin, carboplatin, and oxaliplatin are platinum-containing chemotherapeutics that act by forming DNA adducts and interstrand crosslinks. Recognition of such DNA damage

promotes apoptosis through subsequent activation of apoptotic signaling pathways. In humans, resistance to cisplatin and carboplatin has been reported in MMR deficient cancers, while no such resistance has been reported to oxaliplatin.[8, 18, 22-26] While these drugs have similar mechanisms of action, it has been suggested oxaliplatin-related DNA adducts are not normally recognized by MMR and thus deficiency has little effect on effectiveness of oxaliplatin.[8, 26] Response of canine lower urinary tract urothelial carcinomas to carboplatin parallel these findings, where the MMR deficient cell line TYLER2 was relatively resistant; however, there was no difference in response to cisplatin, and TYLER2 was more sensitive to oxaliplatin.

Gemcitabine and cytarabine, which were evaluated in the current study, are pyrimidine nucleoside analogs. Radiosensitization with gemcitabine in the face of MMR deficiency has been suggested.[27, 28] Human carcinoma MLH1 and MSH2-deficient cell lines were found to be sensitively to cytarabine.[29] In contrast to these results, there was no apparent difference in the sensitivities of MMR proficient and deficient cell lines to gemcitabine or cytarabine.

In humans, MMR deficiency results in drug resistance to certain alkylating agents including the SN1 methylators, temozolomide and dacarbazine.[8, 13, 18, 19, 21] However, reports suggest that MMR deficient cells are highly sensitive to many of the interstrand cross-linking alkylators, including lomustine (CCNU) and mitomycin C.[13, 21] The role MMR directly played in sensitivity to alkylators in our canine urothelial carcinoma cell lines is unclear. While the MMR deficient TYLER2 cell was most sensitive to the alkylator, thiotepa, there was wide variation in response to treatment for all tested cell lines regardless of MMR status, and there were no apparent differences in response of the MMR deficient TYLER2 to lomustine.

Methotrexate is a folic acid inhibitor. There are contradictory reports regarding the effect of MMR proficiency on sensitivity to methotrexate. One study suggested an increased sensitivity to methotrexate in MMR deficient cell lines. In another study, comparison of the effect of methotrexate on a cell line with inactivation of MLH1 and decreased expression of PMS2 to a MMR proficient cell line found increased sensitivity in the MMR proficient cell line[30] In the canine urothelial carcinoma cell lines, the MMR deficient TYLER2 was more sensitive to methotrexate than the proficient TYLER1.

Paclitaxel (taxol) is an inhibitor of mitosis that acts by interfering with breakdown of microtubules. Previous studies have found no association between MMR proficiency and paclitaxel sensitivity, and no obvious differences were observed in cell lines of the current study.[24, 31]

The differential expression of MSH2 and MSH6 likely influenced the differential sensitivities of TYLER2 to select chemotherapeutics similar to that described in the human literature; however, differences in other carcinogenesis related pathways cannot be excluded as contributing to relative chemosensitivity or chemoresistance in the current study. While TYLER1 and TYLER2 were derived from the same tumor, produced similar tumors in xenografts, and maintained similar expression profiles of immunohistochemical markers, the exact similarity of the molecular constitution of these cell lines is unknown. This is particularly true given that there are marked differences in morphology of these cells in culture likely suggesting differences between the cell lines outside the MMR pathway. Further the growth of tumors in xenografts composed of biphasic cell populations suggests that each cell line may be composed of a mixed cell population, components of which may differ in MMR proficiency; however, some of the xenografts of TYLER1 and

TYLER2 cell line subclones also had epithelioid and discrete cell populations suggesting divergent differentiation within the mouse rather than injection of multiple cell types.

Overall, these studies suggest that there are similarities in dogs and humans regarding chemosensitivity of MMR proficient and deficient cells. In addition, the demonstrated variance in chemosensitivity suggests likely clinical implications for dogs affected with lower urinary tract urothelial carcinoma with regards to selection of treatment.

Acknowledgments

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APPENDIX

Figure 22. *Multispectral imaging of MSH2 immunohistochemistry in canine lower urinary tract urothelial carcinoma xenografts.* Immunoreactivity for MSH2 in unmixed images was detected by 3,3'-Diaminobenzidine (DAB) chromogen with hematoxylin counterstain. Colocalization of immunoreactivity (yellow) was determined using set thresholds defined from spectral libraries of controls. Morphometric analysis used to count nuclei in images converted to psedofluorescene.

Figure 22 (cont'd)

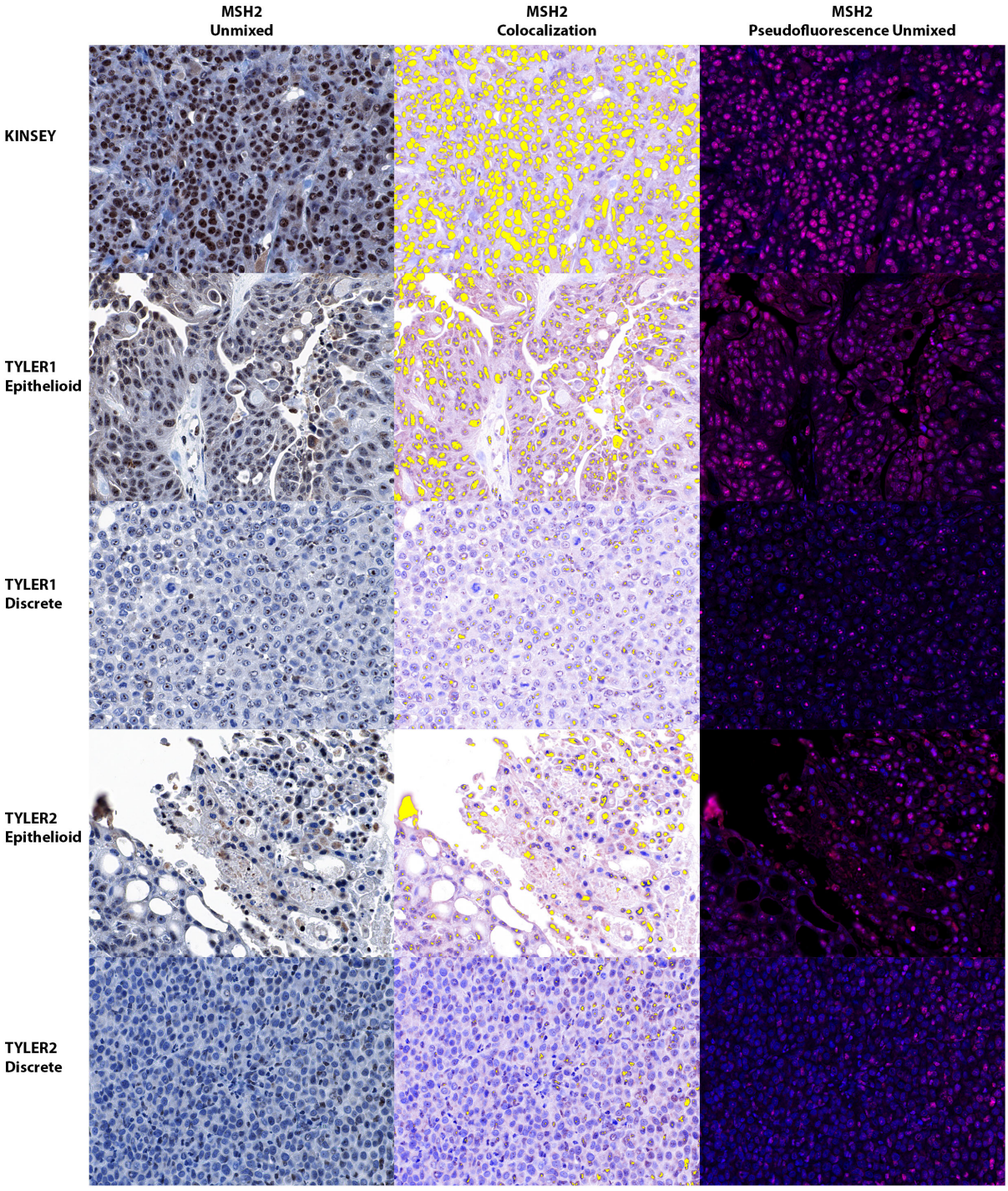

Figure 23: *Multispectral imaging of MSH6 immunohistochemistry in canine lower urinary tract urothelial carcinoma xenografts.* Immunoreactivity for MSH2 in unmixed images was detected by indirect biotin streptavidin and Fast Red chromogen, hematoxylin counterstain. Colocalization of immunoreactivity (yellow) was determined using set thresholds defined from spectral libraries of controls and morphometric analysis used to count nuclei in images converted to psedofluorescene.

Figure 23 (cont'd)

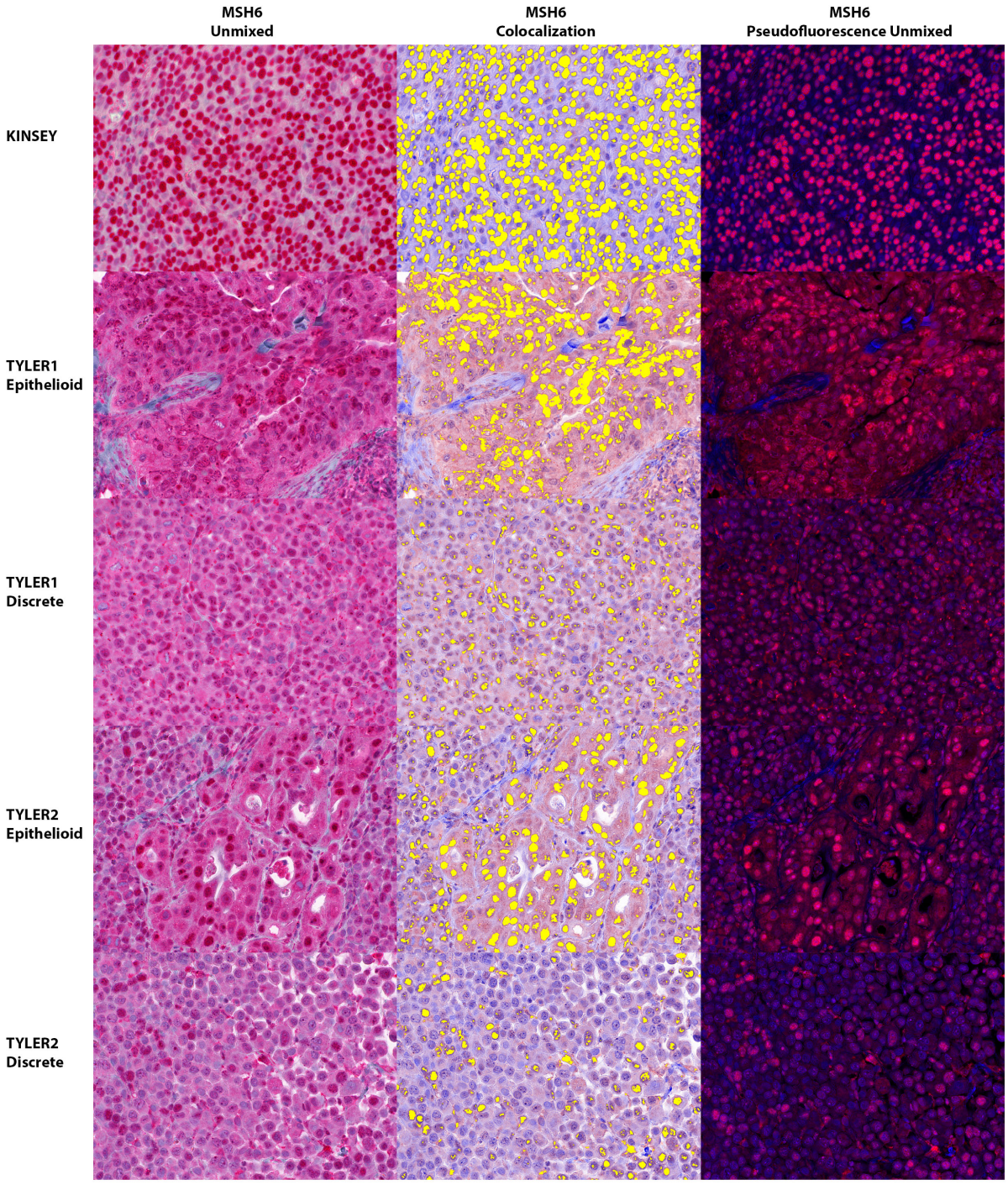

Figure 24: *Photomicrographs from the initial diagnostic biopsy and immunohistochemistry (IHC) on the papillary grade II urothelial from which TYLER cell lines were derived.* The histomorphology of the neoplasm and pattern or immunoreactivity for the tested IHC markers are consistent with a urothelial carcinoma. Neoplastic polygonal urothelial cells are arranged in dense nests that occasionally have open or necrotic centers, and that are supported by supported by scant fine fibrovascular stroma, hematoxylin and eosin (A). Neoplastic epithelial cells have strong cytoplasmic immunoreactivity for the pancytokeratin marker MNF116 (B) 3,3'-Diaminobenzidine (DAB) chromogen, hematoxylin counterstain. While stromal cells are immunoreactive, neoplastic urothelial cells are not labeled for vimentin (C), indirect biotin streptavidin and Fast Red chromogen, hematoxylin counterstain. There is strong perimembranous immunoreactivity for cytokeratin 7 throughout the neoplastic cells (D), strong apical expression Uroplakin 3 in cells at the center of nests (E), and no immunoreactivity for prostatic acid phosphatase (PAP), DAB chromogen, hematoxylin counterstain.

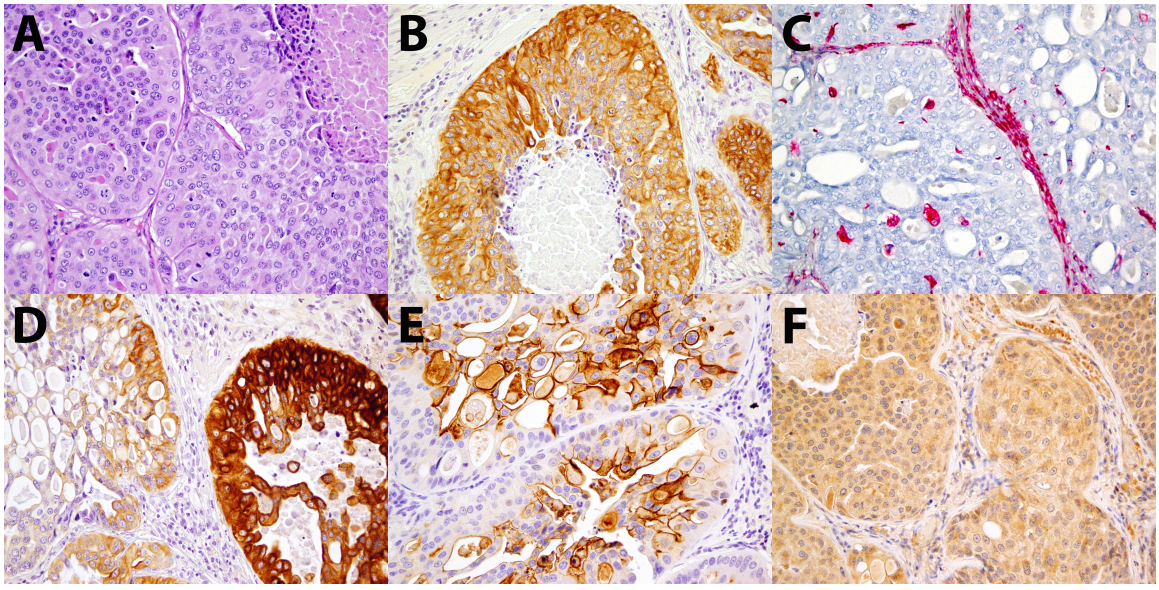

Figure 25: *Phase contrast photomicrographs of ANGUS, KINSEY, original TYLER cell lines.* ANGUS (A) and KINSEY (B) cell lines are composed of plump polygonal cells that grow in intimately associated colonies and sheets. Cells of ANGUS contain occasional refractile, sharply demarcated, vacuoles. The original TYLER cell line is composed of a biphasic population of plump polygonal cells that generally form dense, tightly aggregated colonies and sheets, and an intervening population of loosely arranged spindle to stellate cells (C).

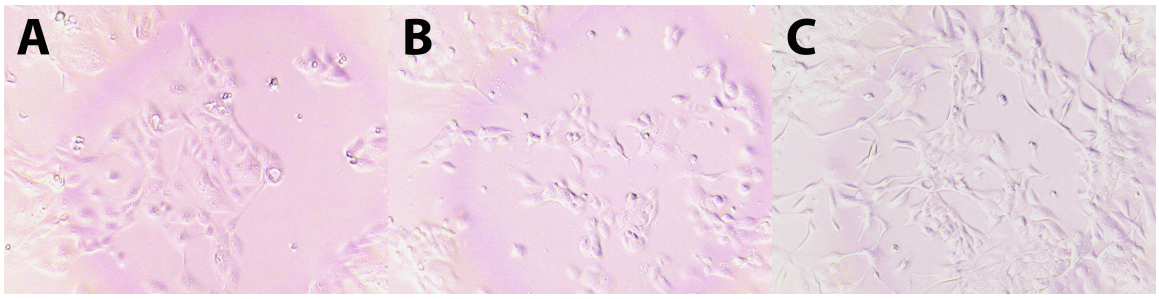

Figure 26: *Phase contrast photomicrographs of TYLER1 and TYLER2 cell lines and photomicrographs of IHC for differentiation markers in TYLER1 and TYLER2 cell lines.* In cell culture, TYLER1 is comprised of plump polygonal cells that generally form dense, tightly aggregated colonies and sheets. TYLER2 is composed of cells that have a spindle to stellate morphology and grow in loose, haphazardly arranged patterns. TYLER1 and TYLER2 have similar expression of immunohistochemical markers. In both cell lines, individual and scattered aggregates of cells have cytoplasmic immunoreactivity for cytokeratin 7 and prostatic acid phosphatase (PAP), 3,3'-Diaminobenzidine (DAB) chromogen, hematoxylin counterstain. Cells from both lines have strong cytoplasmic expression of vimentin, indirect biotin streptavidin and Fast Red chromogen, hematoxylin counterstain.

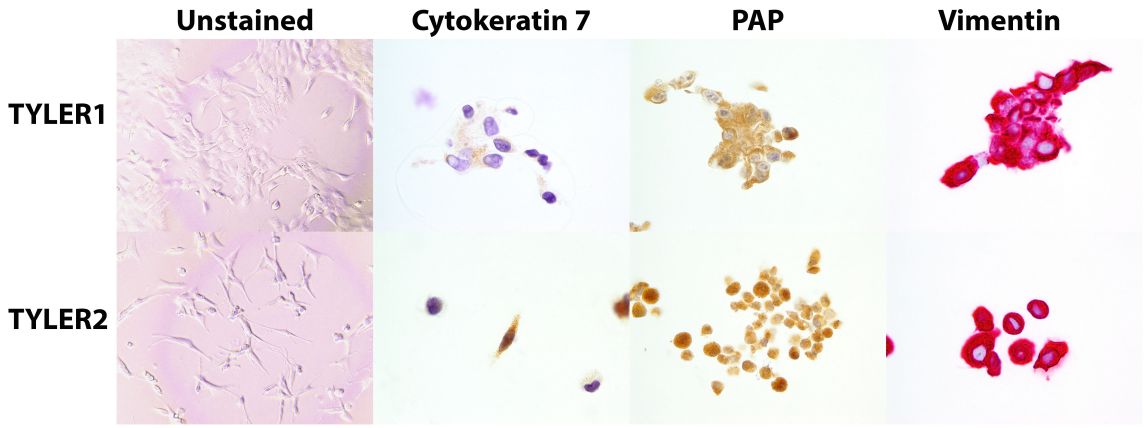

Figure 27: *Photomicrographs from a canine lower urinary tract urothelial carcinoma xenograft derived from the TYLER2 cell line showing two morphologically distinct cell populations, hematoxylin and eosin stain.* Neoplastic cell populations are biphasic with regions showing epithelioid or discrete cell morphology. In the lower magnification photomicrograph (A), epithelioid cells are arranged in nests and packets, which often have central cavitations that are open or that contain flocculent eosinophilic fluid and sloughed degenerate cells. Discrete cells are arranged in dense sheets supported by scant fine fibrovascular stroma. In the higher magnification photomicrograph, epithelioid cells are plump polygonal, and have distinct cell borders, moderate amounts of eosinophilic cytoplasm, and rare, sharply defined, open intracytoplasmic vacuoles. Discrete cells are densely packed, but loosely arranged, round, and have scant eosinophilic cytoplasm and variably distinct cell borders.

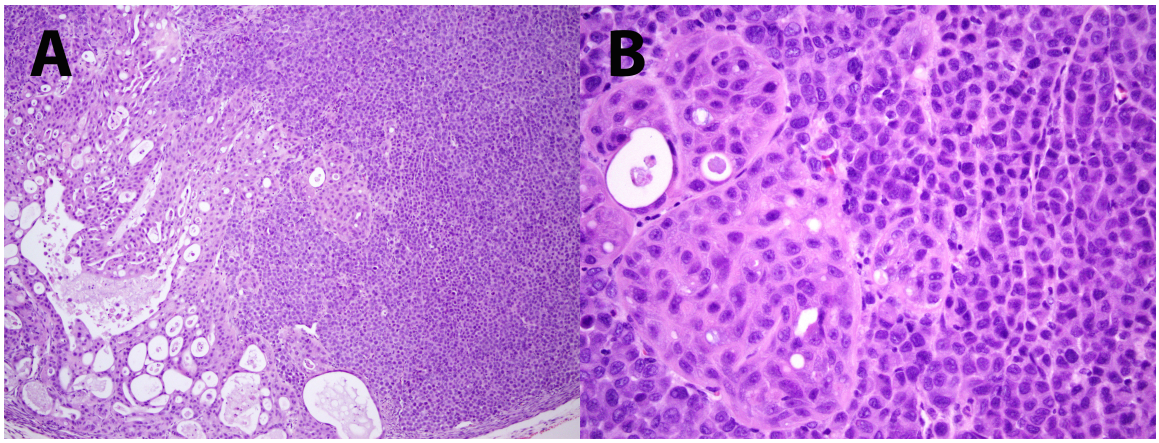

Figure 28: *Photomicrographs of IHC for differentiation markers in a canine lower urinary tract urothelial carcinoma xenograft derived from the KINSEY cell line.* Neoplastic epithelial cells have strong permembranous to cytoplasmic immunoreactivity E-cadherin (A), perimembranous immunoreactivity for P-cadherin (B), no labeling for N-cadherin (C) confirming an epithelial origin, 3,3'-Diaminobenzidine (DAB) chromogen, hematoxylin counterstain. There is strong perimembranous immunoreactivity for cytokeratin 7 throughout the neoplastic cells (D), no immunoreactivity for prostatic acid phosphatase (PAP) (E), which is consistent with an urothelial origin, DAB chromogen, hematoxylin counterstain. Similar to what was observed in plated cell lines, neoplastic cells have cytoplasmic expression of vimentin (F), indirect biotin streptavidin and Fast Red chromogen, hematoxylin counterstain.

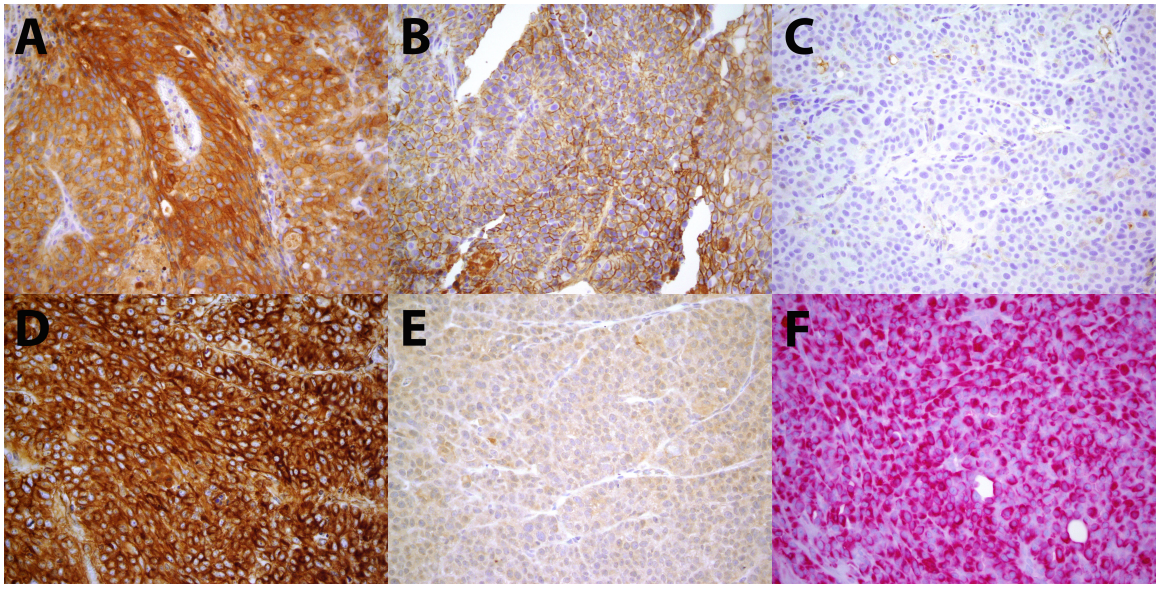

Figure 29: *Photomicrographs of IHC for differentiation markers in a canine lower urinary tract urothelial carcinoma xenograft derived from the TYLER2 cell line.* In regions of epithelioid differentiation, neoplastic epithelial cells have strong permembranous E-cadherin (A), P-cadherin (B), N-cadherin (C), and Uroplakin 3 (D) consistent with a urothelial origin, 3,3'-Diaminobenzidine (DAB) chromogen, hematoxylin counterstain. Discrete cell populations were not immunoreactive for E-cadherin (A), P-cadherin (B), N-cadherin (C), or Uroplakin 3 (D). There was rare cytoplasmic immunoractivity for prostatic acid phosphatase (E) in scattered individual cells in both epitheliod and discrete cell populations (E), suggesting some degree of prostatic epithelial differentiation, DAB chromogen, hematoxylin counterstain. Similar to what was observed in plated cell lines, epitheloid and discrete cells have cytoplasmic expression of vimentin (F), indirect biotin streptavidin and Fast Red chromogen, hematoxylin counterstain.

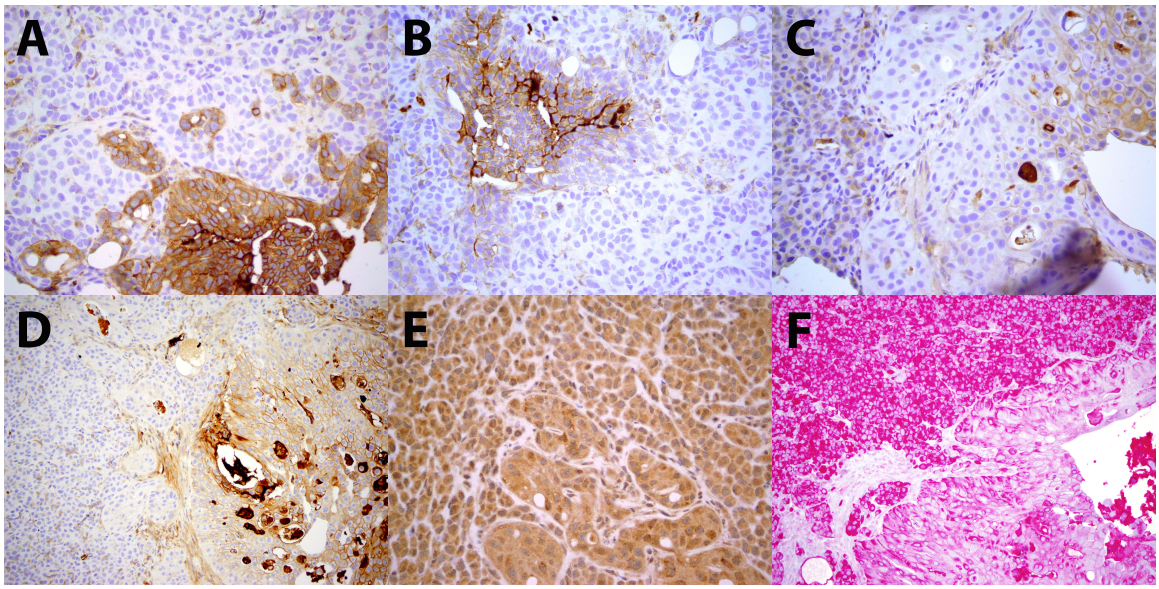

Figure 30: *Western blots show relative decreased expression of MSH2 and MSH6 in the TYLER2 cell line in comparison to that of ANGUS, KINSEY, and TYLER1.* Actin expression was evaluated to ensure equal protein loading.

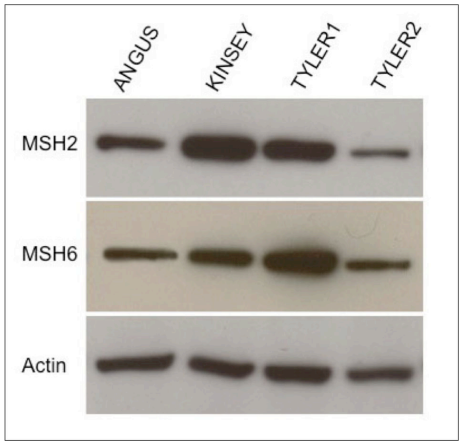

Figure 31: *Graph depicting results of qPCR for MLH1, MSH2, MSH3, MSH6, and PMS2 in the canine lower urinary tract urothelial carcinoma cell lines, ANGUS, KINSEY, TYLER1, and TYLER2 cell lines.* Columns represent relative quantification of each gene as determined by the ΔΔCt method with evaluation relative to a compilation of β-actin, GAPDH, and HPRT1 gene expression and relative to expression in TYLER2. Associated bars represent standard deviation. KINSEY, ANGUS, and TYLER1 respectively had greater than 10 fold, 5 fold, and 4 fold mean relative expression levels of MSH2 above that of TYLER2. KINSEY had greater than 6 fold mean relative expression levels and TYLER2 and ANGUS had greater than 1 fold mean relative expression of MSH6 above that of TYLER2. There were less than a fold differences in mean relative expression of MLH1, MSH3, and PMS2 between all cell lines.

Figure 31 (cont'd)

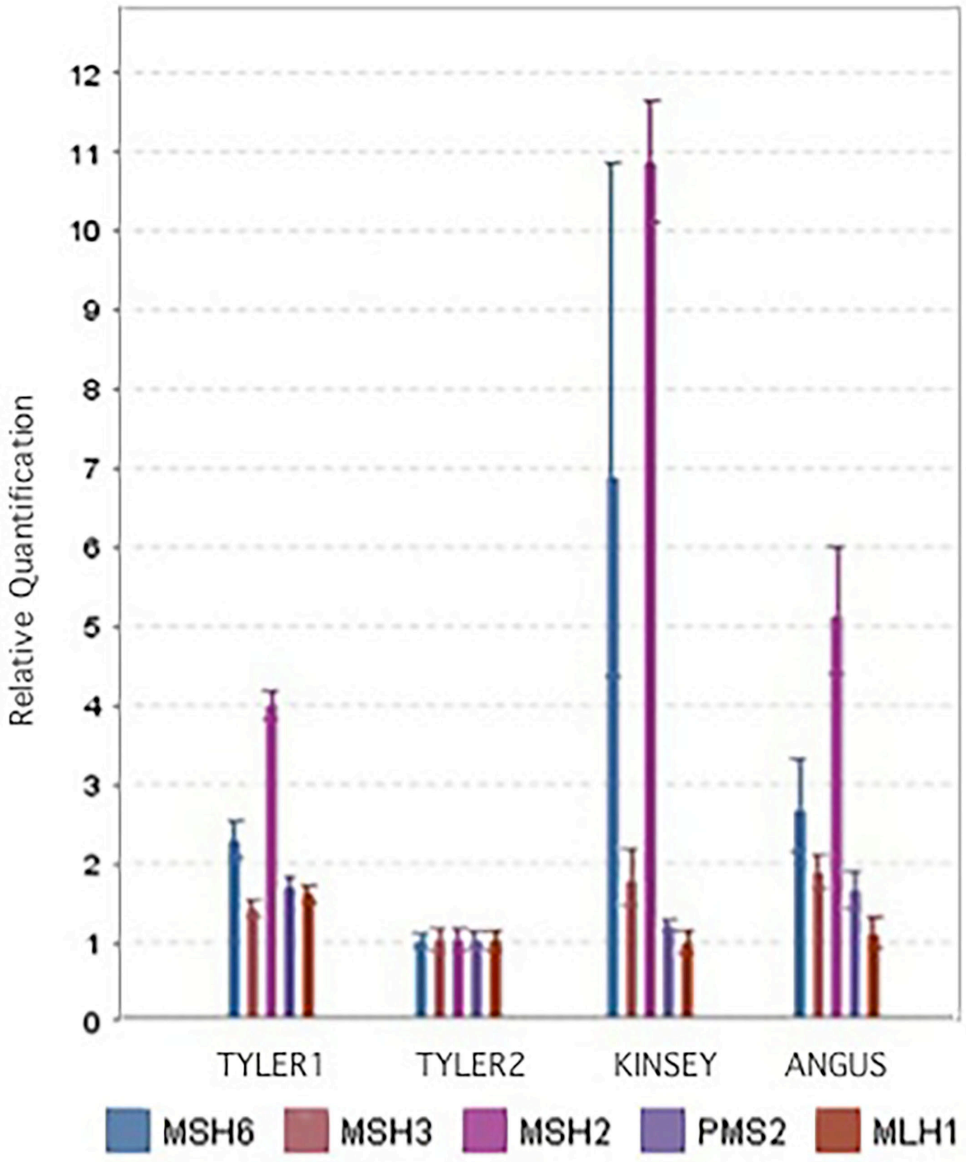

Figure 32: *Graph depicting results of qPCR for MSH2 and MSH6 in canine lower urinary tract urothelial carcinomas xenografts derived from TYLER1, and TYLER2 cell lines laser capture microdissection separated from mouse tissues.* Columns represent relative quantification of each gene as determined by the ΔΔCt method with evaluation relative to GAPDH gene expression and relative to expression in TYLER2. Associated bars represent standard deviation. Variance in the mean relative quantification of gene expression is depicted by wide standard deviations. The mean expression of MSH2 relative to GAPDH, was greater than 15 fold higher in Kinsey than in both TYLER1 and TYLER2. There was no appreciable difference in the mean expression of MSH2 between TYLER1 and TYLER2. The mean expression of MSH6 relative to GAPDH was greater than 25 fold higher in KINSEY than in TYLER2 and greater than 5 fold greater in KINSEY than in TYLER2. The mean relative expression of MSH6 in TYLER2 was greater than 4 fold higher than that of TYLER1.

Figure 32 (cont'd)

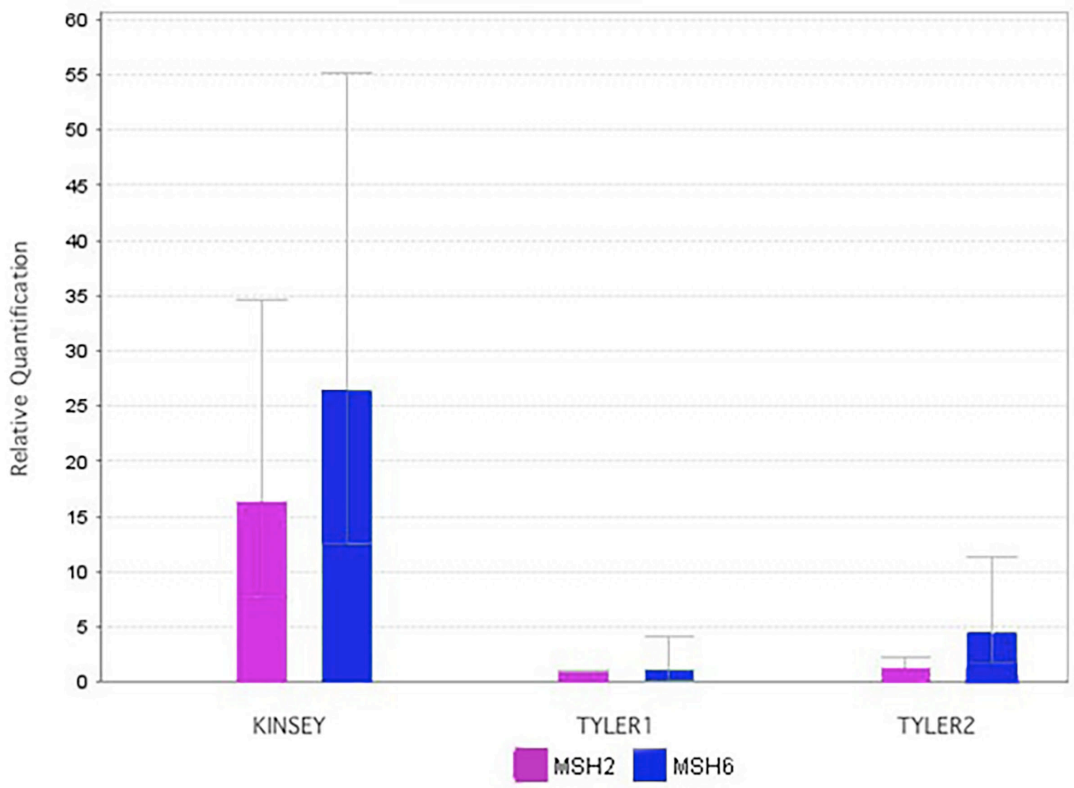

Figure 33: *Graph depicting results of qPCR for MSH2 in laser capture microdissection separated epitheliod and discrete cell populations of canine lower urinary tract urothelial carcinomas xenografts derived from TYLER1, and TYLER2 cell lines.* Associated bars represent standard deviation. Columns represent mean relative quantification of MSH2 expression as determined using the ΔΔCt method with evaluation relative to GAPDH gene expression and relative to expression in the TYLER1 discrete cell population. There was over a fold difference in the expression of MSH2 in TYLER1 epitheliod and TYLER2 epitheliod populations in comparison to that of the TYLER1 and TYLER2 discrete cell populations, respectively. There were only mild differences in relative MSH2 expression between the separately isolated epithelial and round cell populations when comparing TYLER1 and TYLER2.

Figure 33 (cont'd)

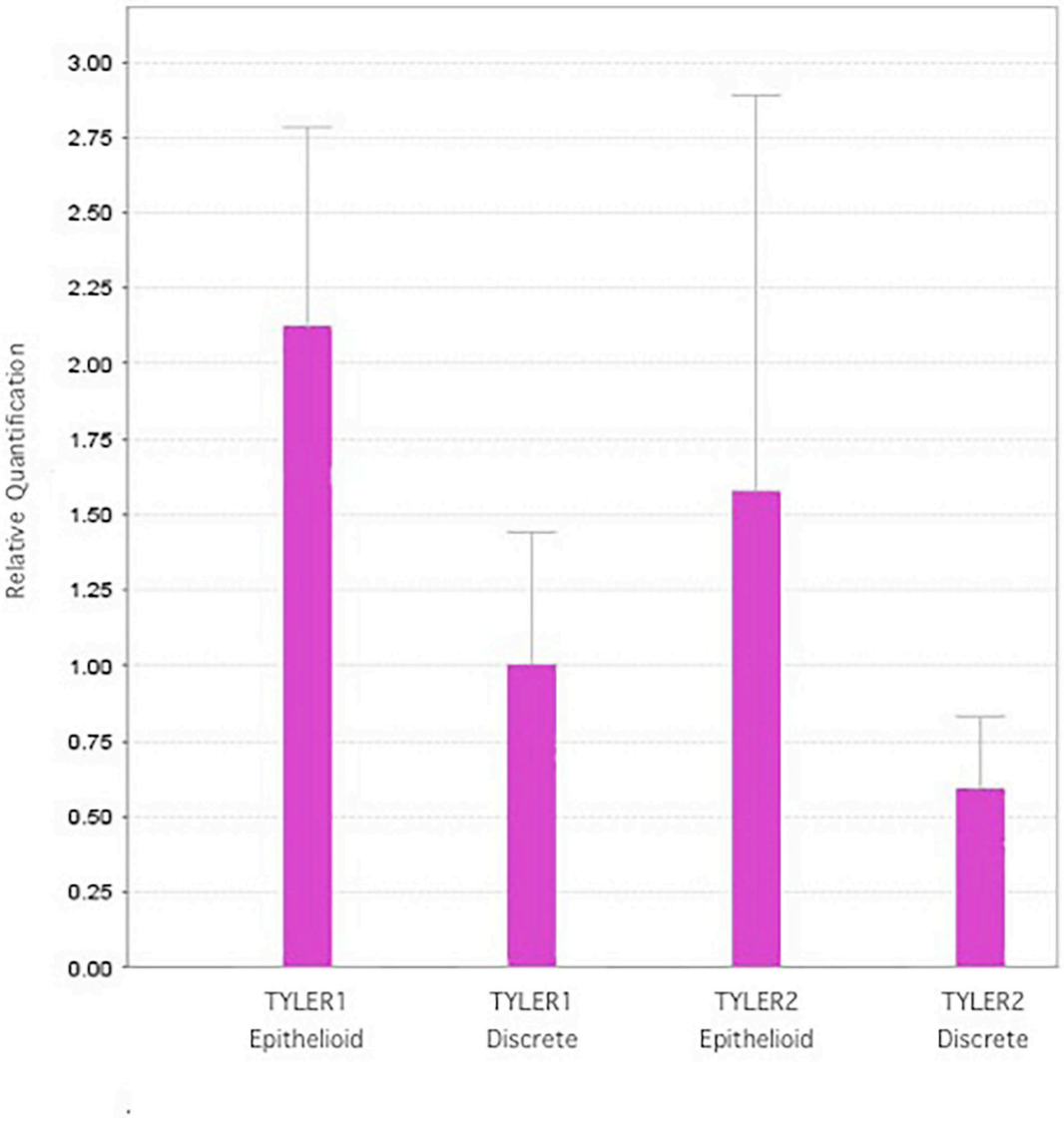

Figure 34: *Graph depicting results of morphometric analysis of multispectral imaging of immunoreactivity of MSH2 and MSH6 in canine lower urinary tract urothelial carcinomas xenografts derived from KINSEY, TYLER1, and TYLER2 cell lines.* For TYLER1 and TYLER2, areas with epithelioid and discrete morphology were imaged and analyzed separately. Quantitated immunoreactivity is reported as positive pixels per nucleus. Columns represent mean of expression of MSH2 and MSH6 between multiple xenografts with bars representing standard deviation (KINSEY: n=6; TYLER1 epithelioid: n-4; TYLER1 discrete: n=5; TYLER2 epithelioid: n=3; TYLER2 discrete: n=4). Marked variation in the expression of MSH2 and moderate variation in the expression of MSH6 within xenografts derived from the same cell line is evidenced by large standard deviations. The mean expression levels of MSH2 and MSH6 in terms were lower in discrete cell populations than in epitheliod populations for both TYLER1 and TYLER2. Further, the mean expression levels of MSH2 and MSH6, for both epithelioid and discrete cell populations were respectively higher in TYLER1 populations than in TYLER2 populations. The mean expression of MSH6 was higher in KINSEY in comparison to that of TYLER2 cell lines and discrete TYLER1 cell populations, but was not different from that of epithelioid portions of TYLER1. Expression of MSH2 in KINSEY was low relative to that observed in epithelioid populations.

Figure 34 (cont'd)

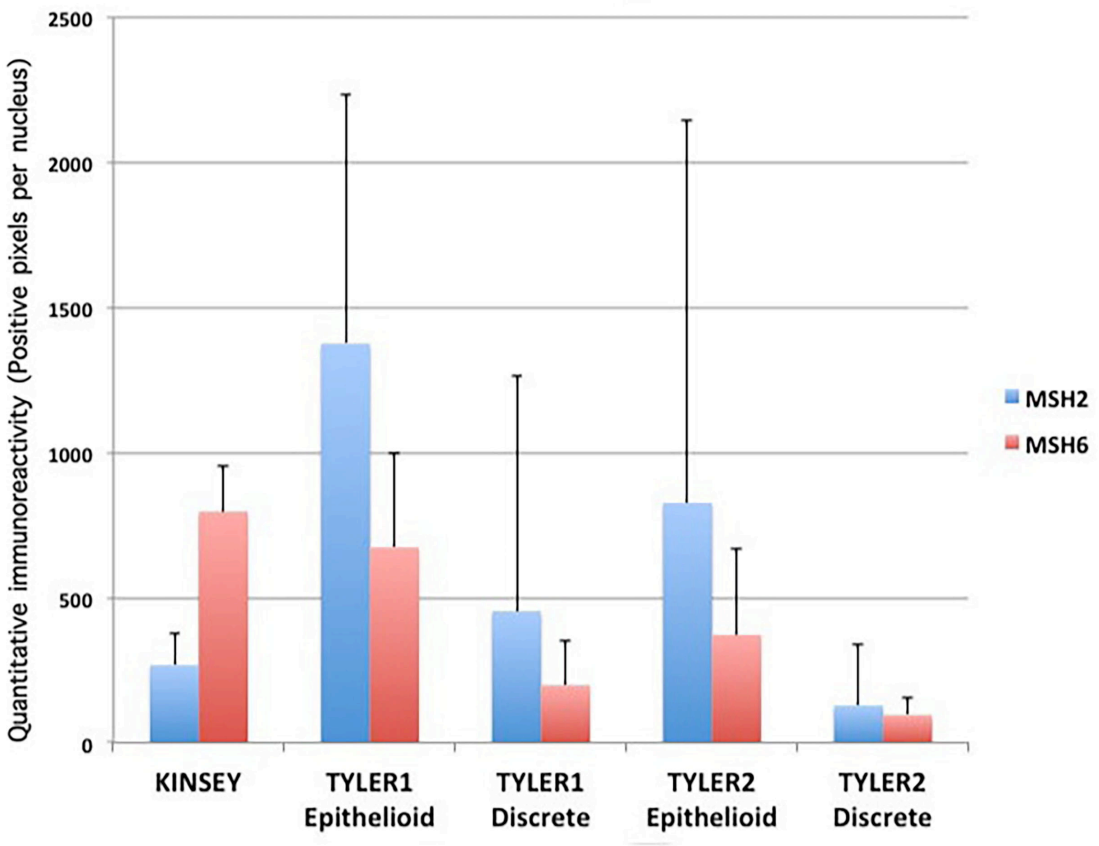

Figure 35: *Results of XTT survival assays comparing survival of canine urothelial carcinoma cell lines upon exposure to specific chemotherapeutics.* With regards to differences in chemotherapeutic sensitivity for TYLER1 and TYLER2, there were appreciable differences in sensitivity to carboplatin, oxaliplatin, methotrexate, and thiotepa. For carboplatin, there was low survival of TYLER1 relative to TYLER2 especially at higher tested concentrations; however, TYLER2 was more sensitive to oxaliplatin than TYLER1. TYLER1 was less sensitive than TYLER1 to moderate concentraions of methotrexate and lower concentrations of thiotepa. ANGUS often was less sensitive to chemotherapeutics relative to other evaluated cell lines including for carboplatin, cisplatin, thiotepa, and gemcitabine. Sensitivity of KINSEY was similar to that of TYLER1 for carboplatin, TYLER1 and TYLER2 for cisplatin, ANUGS and TYLER1 for oxaliplatin, TYLER2 for methotrexate, and TYLER1 and TYLER2 for gemcitabine. For thiotepa, there were differences in sensitivity for all tested cell lines, especially at the lowest tested concentrations, with TYLER2 being most sensitive, followed by TYLER2, KINSEY and ANGUS. There was no appreciable difference in sensitivity of TYLER1, TYLER2, or KINSEY for Gemcitabine, but ANGUS had relative resistance. There were no or only mild differences in response of all evaluated cell lines to taxol, cytarabine, and lomoustine.

Figure 35 (cont'd)

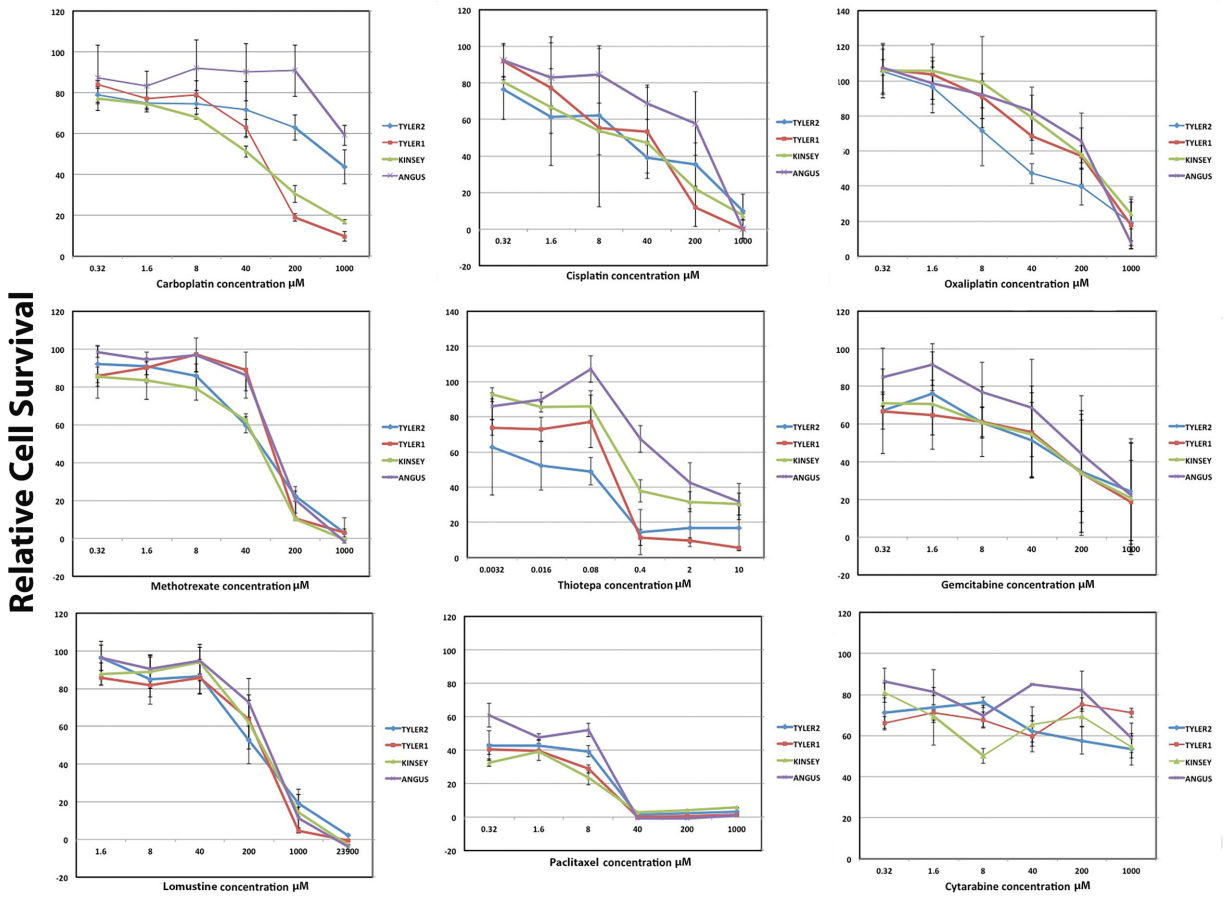

Table 14: *TaqMan® gene expression assays used for qPCR*

Gene	Amplicon length	Species	Number
MLH1	105	Dog	Cf02666410_m1
MSH2	83	Dog	Cf02626772_m1
MSH3	103	Dog	Cf02643735_m1
MSH6	77	Dog	Cf02641004_g1
PMS2	109	Dog	Cf02644577_m1
HPRT1	102	Dog	Cf02626256_m1
β-Actin	139	Human	Hs03023880_g1
Canine GAPDH custom designed TaqMan gene expression assay:			
Forward Primer: TCAACGGATTTGGCCGTATTGG Reverse Primer: TGAAGGGGTCATTGATGGCG FAM labeled probe: CAGGGTGCTTTTAACTCTGGCAAAGTGGA			

Table 15: *Distribution of MSH2 expression in canine urothelial carcinoma xenogafts as evaluated by morphometric analysis of immunohistochemistry*

Cell line xenograft	N	Mean MSH2 expression (± Standard Deviation)	Minimum	Maximum
KINSEY	6	267.1 (±111.9)	127.3	409.4
TYLER1 epithelioid	4	1275.7 (±855.3)	564.6	2544.7
TYLER1 discreet	5	453.4 (±808.3)	42.6	1896.3
TYLER2 epithelioid	3	826.3 (±1318.3)	24.2	2347.7
TYLER2 discreet	4	128.0 (±212.1)	9.1	445.7

Table 16: *Distribution of MSH6 expression in canine urothelial carcinoma xenogafts as evaluated by morphometric analysis of immunohistochemistry*

Cell line xenograft	N	Mean MSH6 expression (± Standard Deviation)	Minimum	Maximum
KINSEY	6	796.2 (± 158.5)	643.7	1128.5
TYLER1 epithelioid	4	672.8 (±325.9)	275.5	1035.7
TYLER1 discreet	5	197.9 (±153.9)	77	453.9
TYLER2 epithelioid	3	371.7 (±293.8)	54.3	634.1
TYLER2 discreet	4	95.2 (±59.7)	38.9	176.8

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CHAPTER 6

Conclusions

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Summary of findings

With regards to canine lower urinary tract urothelial carcinomas, these studies highlight the potential prognostic significance of multiple evaluated markers, emphasize potential targets for directed therapy, and highlight similarities between dogs and humans in multiple carcinogenesis pathways. However, these studies also highlight significant differences between urothelial carcinomas of the dog and human. As such, these findings contribute to the field of canine cancer prognostics and give hope to improved, targeted treatment for bladder cancer in dogs, but indicate that further study is needed. As an animal model, canine lower urinary tract urothelial carcinomas provide an interesting, although not perfect, parallel to such disease in humans.

Histologic classification and grading form cornerstones of routine tumor diagnostics and prognostication; however, the recently proposed classification and grading scheme for canine proliferative urothelial lesions based on the World Health Organization (WHO)/International Society of Urologic Pathology (ISUP) consensus system accepted in humans had not previously been evaluated with respect to prognostic relevance. This work shows that there are biologic differences between histologic classifications and grades as evidenced by differential expression of differentiation molecules and factors reflecting specific carcinogenesis pathways. Specifically, there were differences in expression of uroplakin III, cytokeratin 7, cyclooxygenase-2 (COX-2), P-cadherin, and β-catenin between classifications of proliferative urothelial lesions. Differentiation of urothelial tumors as papillary or infiltrating did correlate with survival time; however, degree of invasion and histologic grading based on degree of anaplasia did not.

Based on the common findings of invasion of carcinomas into the bladder wall and metastasis, it is likely that epithelial-to-mesenchymal transition occurs in canine urothelial carcinomas. Our studies suggest that the mechanisms underlying such transition in dogs differ from that reported in humans. In humans, correlations exist between the loss of expression of 15-hydroxyprostaglandin dehydrogenase (HPGD) and cadherin switching. This does not appear to be exactly the case in dogs as decreased expression of HPGD relative to that of normal urothelium was common, but changes in cadherin expression were not similar to that described in humans. There were differences in the expression of P-cadherin in canine urothelial carcinomas, with loss or aberrant localization of P-cadherin being most common in higher grade, invasive carcinomas and being associated with survival time. This contrasts with the increased P-cadherin expression described with epithelial-to-mesenchymal transition in humans. Further, while N-cadherin expression is reported with epithelial-to-mesenchymal transition in human urothelial carcinomas and was observed in human urothelial carcinoma cell lines lacking HPGD, no expression of N-cadherin was observed in any of the examined canine urothelial carcinomas. This suggests that while overall histomorphology and clinical progression of human and canine are similar, the mechanisms that govern invasion and metastasis at the molecular level are different.

Microsatellite instability (MSI) was found in a significant proportion of canine urothelial carcinomas and was associated with genetic background in terms of breed and phylogenetic clade of affected dogs. This finding alone makes evaluation of MSI and the possible heritable basis of MSI attractive for further study. High MSI, however, was not associated with clinical outcome in terms of survival time and was not specifically

correlated with subsequent evaluation of DNA mismatch repair (MMR) protein expression. Differences in MMR capacity were, however, associated with variable response to chemotherapeutic sensitivity in canine urothelial carcinoma cell lines. Specifically, the TYLER2 cell line had decreased expression of MSH2 and MSH6, resistance to carboplatin, and increased sensitivity to oxaliplatin, methotrexate, and thiotepa relative to TYLER1, which was derived from the same primary tumor and considered MMR proficient. Such differences in response to treatment *in vitro* suggest that there are likely similar differences between canine urothelial carcinomas *in vivo* and that MMR capacity should be taken into account with regards to choice of therapeutic.

Given the preponderance of MMR deficiencies in both hereditary and spontaneous human cancers and the clear differences reported in therapeutic response, there is clearly a need to optimize treatment plans and therapeutic options with respect to MMR capacity. Relevant and reproducible models for study of MMR and the effects of loss of MMR function on response to treatment are lacking. Based on our data, MMR is frequently, though not exclusively, defective in canine urothelial carcinomas. As such, urothelial carcinomas in dogs are likely to provide such a model for more generalized study of MMR.

Limitations of studies and unanswered questions

The major limitation of chapter 1 was the lack of clinical follow-up data. This was a retrospective study and cases evaluated were retrieved from archives of routine diagnostic specimens. As such, provided clinico-demographic information was of varying quality and completeness, and no information regarding treatment, clinical progression, or clinical outcome was available. Thus, while it was possible to compare and correlate expression of the evaluated immunohistochemical markers (uroplakin III, cytokeratin 7, COX-2 and caspase-3) to histomorphologic features that are often associated with prognosis in humans and in canine other cancers, the exact prognostic significance of differences in both histomorphologic features and evaluated immunohistochemical markers is unclear. COX-2 expression, at least, was further examined in chapter 2 and no correlations were found between expression and survival time; however, the grading of methods and means of evaluation of COX-2 expression was different between chapters 1 and 2.

In studies in which survival time was available (chapters 2 and 3), only urothelial carcinomas and normal urinary bladders were evaluated, and there were particularly low numbers of infiltrating and low grade papillary urothelial carcinomas. While differences were found in HPGD, P-cadherin, and β-catenin expression and in microsatellite instability between urothelial carcinomas and normal urothelium, it is unclear if such findings would be discriminatory between urothelial carcinomas and other proliferative urothelial lesions or inflammatory disease processes affecting the lower urinary tract. However, the lack of proliferative urothelial lesions besides urothelial carcinomas and the low number of infiltrating and low grade papillary carcinomas is largely reflective of the types of cases

that present to a veterinary referral clinic and the low rate of low grade or infiltrating carcinomas in the overall canine population.

Survival time from the date of diagnosis was the major indicator of prognosis evaluated in these studies. While overall survival time can be a relevant prognostic indicator, it can be skewed by many factors. In these studies, it is unclear how long tumors may have been present before diagnosis, treatment was not consistent, information regarding clinical progression was not available, and the extent of disease progression at the time of death was unclear. In contrast to humans, diagnosis of bladder cancer in dogs is often not made until late into disease progression when tumors cause mechanical problems or when systemic disease becomes apparent. Access to complete clinical follow-up was limited as most cases that had significant survival time were referred back to primary care veterinarians. Further, monitoring of disease progression in terms of changes in tumor size, invasion, or development of metastasis was inconsistent or incomplete, and results were often not available. The reason for death in most of these animals was reported to be related to progressive disease related to the urothelial carcinomas. In most cases, however, necropsies were not preformed to document extent of tumor growth or metastasis, and death was not by natural causes, but rather by elective euthanasia. The choice of euthanasia is a complicated issue with respect to statistical evaluation of survival in canine cancer. This is especially true given that there is significant variation in the point of disease progression at which the decision to euthanize is made. In lower urinary tract diseases, the choice for euthanasia may be related to local disease, and not necessarily metastasis or other systemic progression. Further, in veterinary medicine, home care considerations and financial impact of treatment also play large roles in the decision to euthanize. Euthanasia,

however, is an unavoidable confounding factor in analysis of cancer related statistics in veterinary medicine.

The exact significance of deficiency in mismatch repair as evaluated in chapter 3 remains unclear. While microsatellite instability was demonstrated in a number of canine urothelial carcinomas and was associated with breed, there was no clear association of MSI to MMR protein expression as evaluated by immunohistochemistry. The evaluation of MMR proficiency is complicated even in human medicine where it has been extensively used. Variable recommendations regarding evaluation immunohistochemcial of MMR protein expression and determination of MSI exist. The reagents used for evaluation in all of these studies were not specifically designed for use in dogs. This is particularly true of the antibodies used to assess protein expression by IHC. While we were able to show that the antibodies did label appropriately sized molecules in Western blot, aberrant labeling cannot be completely excluded as a confounder. The role of decreased MMR protein expression rather than total loss is unclear. Further, the morphometric methods of evaluating overall MMR protein expression differ from that described in most human studies.

In canine lower urinary tract urothelial carcinoma cell lines, the functional significance of decreased relative expression of MSH2 and MSH6 is unclear. Evaluation of the status of the MMR system of evaluated cell lines was largely inferred from evaluation of MMR gene and protein expression and comparison between cell lines. It is less clear to what degree the relative differences in MMR gene and protein expression had on the overall function of the MMR system. Outside of the studies reported in this dissertation, MSI was evaluated in ANGUS and the initial cultures of TYLER using the methods described in chapter 3. ANGUS

did not exhibit instability in any of the microsatellites evaluated. In contrast, there was variability in the length of select microsatellites comparing DNA of the TYLER cell line to that of DNA of blood samples collected from the dog from which TYLER was derived. This does suggest some functional deficiency microsatellite instability in TYLER. However, differences in TYLER1 and TYLER2 with respect MMR functional capacity have not been specifically assessed.

While the TYLER cell lines were derived from the same primary urothelial carcinoma, it is likely that these cell lines differ not only in MMR gene and protein expression. This is suggested by the marked phenotypic variation in cell culture and the production of biphasic cell populations in xenografts. These cell lines were derived from primary culture and as such are likely composed of a heterogeneous cell population. While such a heterogeneous cell population in cell culture may more closely resemble the natural heterogeneity of cancer *in vivo*, it complicates determination of the effects of specific differences in *in vitro* studies. In addition, if MMR deficiencies in TYLER2 are functional, an increased rate of mutation and an associated build up of secondary mutations over numerous passages in cell culture is likely. The reason for the marked variation in cell morphology between TYLER1 and TYLER2 is unknown, but is likely not determined only by differences in MMR. As such, it cannot be excluded that differences in sensitivity to chemotherapeutics observed between TYLER1 and TYLER2 were not related to specifically to differences in MMR proficiency. Further, even though differences in response to chemotherapeutics were observed in cell culture, this *in vitro* response may not be reflected *in vivo*.

Future directions

Larger and more in depth prospective studies are needed to more fully evaluate the prognostic significance of histologic classification and grading. Such studies should ideally include a larger representative group of low grade and infiltrating carcinomas. Such studies should also include careful monitoring to evaluate prognostic significance in terms of change in tumor size, change in stage, and time to metastasis. Based on the biologic differences observed in tumor classification and grade, it is likely that prognostic significance exists, but was not observed in the studied populations of this dissertation as only survival time from diagnosis was examined and there were low sample numbers, with low grade and infiltrating carcinomas being particularly underrepresented.

The mechanisms that underlie epithelial-to-mesenchymal transition in canine urothelial carcinomas remain unclear as does the significance of loss of HPGD. It is clear that epithelial-to-mesenchymal transition in dog urothelial carcinomas is different from that in humans. Comparative studies evaluating other features of epithelial-to-mesenchymal transition are needed to further characterize similarities and differences. Lack of HPGD expression was seen in all evaluated canine urothelial carcinoma cell lines, and while there was some degree of difference in P-cadherin expression, loss or up regulation was not often observed. Knock in studies for HPGD in cell lines along with evaluation of changes in cadherin expression and comparative invasion assays would serve to further determine the functional consequences of loss of HPGD.

It is unclear if MMR deficiencies play a role in development of other types of cancers in dogs. Based on the results of immunohistochemical MMR protein evaluation in bladder tumors, expression levels of MMR protein may not be well correlated with MSI given the

methods by which MSI was evaluated in these studies. It appears that lack of MSI, at least within the MS evaluated in our studies, does not preclude that differences or at least variation in MMR may be present. Given the fact that relative differences in expression of MMR protein expression as assessed by IHC and morphometric analysis of immunoreactivity did not correlate with MSI, it would be of interest to evaluate MMR protein expression in other tumors including gastric carcinomas and mammary tumors, for which it was shown that MSI is uncommon. It is likely that variance in MMR protein expression is common in canine cancer and possibly normal tissues.

The long-term goal of establishing differences in response to chemotherapeutics with respect to MMR status is to be able to make treatment selection for an individual patient based on MMR system evaluation. The results of chemotherapeutic sensitivity testing in canine urothelial carcinomas is promising, but the exact role of MMR proficiency and the exact implication these data have in animals *in vivo* remains unclear. In order to better isolate the effects that MMR proficiency have on treatment response *in vitro*, knockdown and knock-in studies would be required. TYLER1 and TYLER2 offer an excellent opportunity for such studies due to their common origin, but differential MMR capacity and demonstrated differences in chemosensitivity. In order to separate the effects of MMR deficiency form other potential sources of treatment response differences, MSH2 could be selectively knocked down in TYLER1 and other MMR proficient cell lines. Concurrent knock in studies could also be preformed in TYLER2 to increase the expression of MSH2 to the comparative levels of the other evaluated cell lines. Subsequent chemotherapeutic survival studies comparing manipulated cell lines to wild types would better distinguish differences in treatment response specifically associated with altered MMR expression.

Further, conditions in cell culture vary greatly from *in vivo* conditions as such response of cancerous cells to treatment may differ in each of these settings. Treatment dosages used in cell culture systems may not be achievable or may be ineffective in live animals. Drug concentrations that are applicable to cell cultures may cause toxicity in live animals or may not achieve sufficient penetrance of the target tissues. Xenografts of derived from cell lines could be evaluated in terms of *in vivo* response to treatment. The long-term goal for such studies would be to support clinical studies in naturally occurring bladder cancer dogs evaluating the response of tumors to standard chemotherapy with respect to the presence MSI and alterations in MMR protein expression as evaluated by IHC in routine diagnostic biopsies.

As a whole, the studies detailed in this dissertation suggest that many pathways are involved in carcinogenesis of canine urothelial tumors. These include prostaglandin regulation pathways, epithelial-to-mesenchymal transition, Wnt signaling, and DNA mismatch repair. However, it is unclear if and how the observed differences relate to one another or what other carcinogenesis related pathways may be altered in canine urothelial carcinomas. Carcinogenesis is a multifactorial process involving defects in multiple pathways related to DNA repair, the cell cycle, apoptosis, and a multitude of other processes. Once initiated, cancer progression involves changes in the expression of a plethora of genes due to a wide variety of mechanisms. Such individual changes and the sum of these changes act to determine clinical progression and response to treatment.

MMR deficiencies are associated with not only an increased rate of mutation, but with specific secondary mutations. In humans, whole genome gene expression profile of MMR deficient cancers are distinct from those of cancers with MMR proficiency. Large scale genomic analysis of MMR proficient and deficient canine urothelial carcinoma cell lines would allow for

evaluation of expression profiles of other DNA repair pathways, for comparison of differential expression of genes between MMR deficient and proficient cell lines, for comparison of changes in gene expression of both MMR proficient and deficient cell lines that occur in response to treatment, and for comparison of profiles in canine urothelial carcinomas to reported gene profiles in human cancers. Similar to what has been documented in human cancers with variable MMR capacity, differences in expression of genes related to apoptosis, antitumoral immunity, and MMR would be expected. With evaluation of profiles with response to treatment, cell lines with varying MMR capacity will undoubtedly have significant differences in expression of multiple genes as MMR capacity is associated with varying sensitivity to cytotoxic agents. Such differential gene profiles would serve to highlight additional potential targets for therapeutic intervention. Comparisons of gene profiles in dogs to those of humans would also further the supposition that dogs are accurate models of urothelial carcinogenesis and MMR deficiency for human cancer.