EVALUATION OF SURVIVIN, AN INHIBITOR OF APOPTOSIS, IN CANINE URINARY BLADDER TISSUES

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Master of Science

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

EVALUATION OF SURVIVIN, AN INHIBITOR OF APOPTOSIS, IN CANINE URINARY BLADDER TISSUES

presented by Wendi Velando Rankin,

a candidate for the degree of master of science,

and hereby certify that, in their opinion, it is worthy of acceptance.

Dr. Carolyn Henry
Dr. Jonathan Green
Dr. James Turk
Dr. Jeffrey Tyler

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EVALUATION OF SURVIVIN, AN INHIBITOR OF APOPTOSIS, IN CANINE URINARY BLADDER TISSUES

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ABSTRACT

Introduction: Survivin, an inhibitor of apoptosis, is overexpressed in human urinary bladder transitional cell carcinoma (TCC). The objectives of this study were to evaluate expression of survivin in canine TCC, cystitis, and normal urinary bladder, and correlate expression with cell proliferation index. The hypothesis was that survivin is overexpressed in canine urinary bladder TCC.

Materials and Methods: Immunohistochemistry (IHC) with an anti-survivin antibody was performed on archival canine TCC, cystitis, and normal urinary bladder tissues. Reverse-transcriptase polymerase chain reaction (PCR) was performed on fresh-frozen tissues (when available). Ki-67, a marker for cell proliferation, was also evaluated by IHC.

Results: Nuclear survivin was present in 27/41 TCC, 12/24 cystitis, and 0/46 normal bladders. Differences between TCC versus normal and cystitis versus normal were significant. Cytoplasmic survivin was present in 7/41 TCC, 2/24 cystitis and 17/46 normal tissues; differences between normal and cystitis were significant. Six of 6 TCC samples, 4/7 cystitis, and 11/22 normal bladder tissues were positive for mRNA, but levels were not significantly different. Tissues with nuclear survivin had a significantly higher Ki-67 score than those without.

Conclusions: As in human tissues, survivin is present in canine TCC. While the presence of survivin in cystitis and normal bladder demonstrates that this is unlikely to be a biomarker for cancer, nuclear survivin is present in TCC and cystitis tissues, but not normal bladder. Nuclear survivin may to play a role in cell proliferation and therefore, may be a target for therapy and prognostic tool for canine bladder tumors.

CHAPTER 1

Introduction: Literature Review and Goals

INTRODUCTION

Apoptosis—or programmed cell death—plays a critical role in tissue homeostasis and remodeling as well as organism development.[1] The two families of proteins that regulate apoptosis are the BCL2 family[2], which affects release of cytochrome c from the mitochondria, and the inhibitors of apoptosis (IAP) family, which inhibit caspases that participate in the apoptotic cascade.[3]

The inhibitors of apoptosis share structural characteristics that include one to three copies of a 70-amino acid zinc-finger fold, called the baculovirus repeat (BIR).[3] Survivin, in addition to being the smallest IAP, is the most recently discovered protein in its family.[4] Survivin is a 142 amino acid, 16.5 kDa protein that, unlike other IAPs, only contains a single BIR domain and also has a long C-terminal alpha-helix coiled region and a dimeric arrangement.[5] The BIR domain participates in anti-apoptosis and the coiled region in tubulin binding in regulation of cell division.[6]

In addition to the full-length (or wild-type) survivin, alternative splicing of the human survivin gene gives rise to four additional isoforms of the protein: survivin-2B, survivin-Δex3[7] survivin-3B[8] and survivin-2α.[9] These isoforms are thought to have varying abilities to regulate apoptosis; survivin-2B has reduced apoptotic capabilities, whereas survivin-Δex3 retains its anti-apoptotic capabilities.[6] In addition, the splice variants have different subcellular localization: survivin (wild type) and survivin-2B localize to the cytoplasm, where as survivin-Δex3 is usually in the nucleus.[10]

Although survivin is an inhibitor of apoptosis, it is regarded as a bi-functional protein since it also regulates cell proliferation. Survivin expression is cell-cycle dependent, as it is expressed in the G_2/M phase and localizes to the mitotic spindle

apparatus to regulate cell proliferation.[4] The absence of survivin leads to defects in cell division such as failure of cytokinesis, multipolar mitotic spindles and polypoid or multinucleated cells.[6] In embryogenesis, survivin contributes to cell proliferation, angiogenesis, cardiogenesis, and neural tube closure.[11,12] It is essential to development of the fetus, as embryos deficient in survivin die at 4-5 days.[13]

The specific mechanism for inhibition of apoptosis by survivin is controversial.[6,14,15] The BIR domain in inhibitors of apoptosis allows binding to and inhibition of caspases; however, it is unknown whether survivin is a direct or indirect inhibitor of caspases[6] or if it inhibits apoptosis by antagonizing cell-death signals in the mitochondria.[15]

Survivin is a unique IAP because it is expressed in embryonic and fetal tissues, but not most normal, differentiated adult tissues.[4,16] Interestingly, survivin is also overexpressed in various human cancers, including sarcomas, many carcinomas and hematopoietic tumors.[4,17-25]

Because of its expression in malignancies, but not normal differentiated tissue, survivin is being evaluated as a prognostic tool, diagnostic marker, and a therapeutic target in a variety of tumors. Expression of the survivin protein is highly correlated with aggressive disease in human patients with neuroblastoma,[26] soft-tissue sarcoma,[27] diffuse large B-cell lymphomas,[23] oral and cutaneous squamous cell carcinoma,[17] colorectal cancer,[28,29] non-small-cell lung cancer,[19,30] breast cancer[31] and urinary bladder cancer.[21,32]

Survivin in human urinary bladder transitional cell carcinoma (TCC) has shown great promise as a novel diagnostic tool, prognostic tool and therapeutic target. Urinary survivin has been demonstrated as a non-invasive specific marker for human patients

with recurrent or new urinary bladder TCC in several studies.[21,32-36] In addition, survivin expression in tumor tissues correlates with higher stage, recurrent disease and shorter disease-free interval in TCC patients.[22,36-41] Survivin may also be a novel therapeutic target for bladder TCC, as recent studies have shown that down-regulation can inhibit cell growth or increase chemosensitivity.[42-44]

Canine urinary bladder TCC is a naturally occurring tumor that serves as a good model for the human disease.[45] Histopathologic characteristics, biological behavior, molecular features, response to medical therapy, and prognosis are all features that are similar between human and canine TCC. However, while human patients with TCC may be cured with treatment due to the non-invasive nature of their disease at initial diagnosis, canine TCC is often detected at advanced stages and median survival times for canine patients are less than 1 year.[46] Because of this overall poor prognosis, additional methods for early diagnosis to improve therapy and prognosis are necessary for dogs with bladder cancer.

With the publication of studies in human malignancies that demonstrate a role for survivin in tumor growth, there is hope that this novel protein may improve our understanding of tumor progression and therapy in veterinary patients. However, studies of survivin in the dog are lacking, given that the messenger RNA (mRNA) was only recently identified and sequenced from canine testis tissue.[47] In that study, survivin mRNA was identified in a variety of canine tumors, but no associations could be made between survivin mRNA levels and tumor types. More recently, the protein has been identified in canine mast cell tumors and oral squamous cell carcinoma.[48,49] There are no reports of survivin evaluation in canine urinary bladder tissue.

OBJECTIVES

Because survivin has not yet been evaluated in canine urinary bladder tumors, the first objective of this thesis was to identify survivin in bladder TCC. This was performed with an anti-survivin rabbit polyclonal antibody (Novus Biologicals, Inc.) previously used to identify survivin in human and canine tissues.[39,48,50] In addition, we wanted to identify survivin messenger RNA to confirm that the message for the survivin protein was present. The identification of survivin would allow us to proceed with the next several steps in order to determine if survivin has a role in tumor growth.

The second objective of the series of studies was to evaluate survivin expression in canine TCC and compare its expression to that of normal urinary bladder tissue. The protein expression patterns and the presence or absence of survivin in these tissues would help determine if survivin would be useful as a novel biomarker for TCC. Once we established that survivin mRNA was present, we evaluated mRNA levels. This would help us determine if higher levels were found in tumor tissue compared to normal bladder tissue. Results of these projects are presented in Chapters 2 and 4.

Although inflammation of the urinary bladder is a risk factor for bladder tumors in humans,[51-53] this is not an established risk factor for TCC in dogs. Additionally, cystitis can create false-positive results with the Veterinary-Bladder Tumor Antigen Test (a commercially available screening test for canine TCC).[54] Our third objective, therefore, was twofold: we wished to evaluate survivin in canine cystitis tissues to determine if survivin could be useful to differentiate between TCC and cystitis and to determine if survivin could link cystitis to bladder tumor development.

Knowing that survivin has a bi-funcitonal role in cells—inhibition of apoptosis and regulation of cell proliferation—we were lead to our final objective. The final objective, presented in Chapter 5, was to evaluate survivin expression and correlate expression patterns with cell proliferation (measured by Ki-67) to determine the role of nuclear and cytoplasmic survivin in cell proliferation. Ki-67 is a nuclear protein expressed during all phases of the cell division cycle, but not present in resting cells;[55] therefore, it can be used as a measure of the proliferative activity of a cell population. This final section of the study helps determine the role of survivin in canine urinary bladder cell proliferation and tumorigenesis.

All of the aforementioned objectives were designed to test the overall hypothesis of this thesis – survivin is overexpressed in canine urinary bladder TCC; therefore, it regulates cell proliferation and tumor growth. The work presented here are the initial steps in determining if survivin has a role in predicting prognosis or as a target for therapy against canine bladder tumors.

CHAPTER 2

Identification of Survivin, an Inhibitor of Apoptosis, in Canine Urinary Bladder Transitional Cell Carcinoma

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INTRODUCTION

Survivin is a member of the inhibitors of apoptosis (IAP) family which is normally present during fetal development. Survivin plays a role in mitosis[12] and inhibition of apoptosis. The protein is present in fetal tissues[16] and contributes to cell proliferation, angiogenesis, cardiogenesis, and neural tube closure;[11] however, survivin is typically absent in normal, differentiated adult human tissues.[4]

This protein is of particular interest in oncology, as survivin is overexpressed in many human malignancies including lung, pancreatic, colon, breast and prostate cancers, and lymphoma.[4] In addition, survivin expression has been correlated with increased aggressiveness of human neuroblastoma,[56] soft-tissue sarcoma,[31] diffuse large B-cell lymphoma,[23] oral and cutaneous squamous cell carcinoma,[17] colorectal cancer,[28,29] non-small cell lung cancer,[19] and breast cancer.[31] Survivin expression has also been associated with risk of recurrence in human urinary bladder tumors.[22]

Methods to detect survivin include immunohistochemistry (IHC), enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR). An anti-survivin rabbit polyclonal antibody (NOVUS Biologicals, Inc., Littleton, CO) has been used for IHC staining of canine tumor tissue (mast cell tumors and oral squamous cell carcinomas).[48] Studies of survivin in canine tissues are rare, as survivin mRNA was only recently identified and sequenced.[47] There are no reports of survivin evaluation in canine urinary bladder tissues.

Survivin is being investigated as a diagnostic and prognostic tool, as well as a target for therapy in human urinary bladder cancer. Detection in the urine has proven to be a sensitive marker of bladder cancer in people[21,32] and higher levels of protein

expression are associated with an increased risk of bladder cancer and high grade tumors.[32] Similarly, survivin expression in tissues is more common in higher-grade tumors[22] and in patients with shorter disease-free intervals.[22,37,57] Higher levels of survivin mRNA in bladder transitional cell carcinoma (TCC) tissue are also associated with higher histologic grade.[34,38] In bladder cancer cell lines, down-regulation of survivin mRNA leads to decreased cell survival and increased apoptosis,[42,43,58,59] indicating that survivin may be a target for therapy in human TCC.

Naturally-occurring canine TCC shows striking similarity to human invasive urinary bladder cancer.[45] Histopathologic characteristics, biological behavior, molecular features, response to medical therapy, and prognostic factors are all features of urinary bladder TCC that are similar between people and dogs. Our group has reported preliminary evidence that survivin is present in canine TCC, and that it may have prognostic significance.[49] In a retrospective study of survivin expression in canine TCC tissues in which clinical outcome data were available, dogs with bladder tumors that had positive nuclear survivin immunoreactivity (n=5) had a median survival time of 81 days, compared to 226.5 days for dogs with negative nuclear immunoreactivity (n=10). There was not a significant difference between groups (P=0.1). Although it was a retrospective pilot study, the results provided initial evidence that further evaluation of survivin in canine bladder tumors is warranted.

Results of the recent studies of survivin in human urinary bladder cancer give us hope for using survivin as a prognostic tool or therapeutic target in canine patients. Dogs with advanced bladder cancer have an overall grave prognosis with standard therapy, with median survival times that do not surpass 1 year.[46] Therefore, there is considerable

enthusiasm to determine if survivin is useful as a target for therapy. Since reports of survivin in canine bladder tissues are lacking, the first step in this line of research is to determine the extent of survivin expression in canine TCC and to determine if survivin is preferentially expressed in cancer tissues compared to normal tissues.

The objective of this study was to evaluate the expression of survivin in normal canine urinary bladder tissue and canine urinary bladder TCC. Our hypothesis was that the expression of survivin detected in the nucleus would be much greater in TCC tissues compared to normal bladder tissues and therefore, nuclear survivin may have a role in tumor progression.

MATERIALS AND METHODS

Tissue samples for Immunohistochemistry

Archival formalin-fixed, paraffin-embedded urinary bladder TCC samples were obtained from the Veterinary Medical Diagnostic Laboratory (VMDL), Columbia, MO, School of Veterinary Medicine, University of California, Davis, CA and the School of Veterinary Medicine, Purdue University, West Lafayette, IN. Normal bladder tissue samples were obtained from cadavers presented to the VMDL for routine necropsy and from terminal surgery dogs in veterinary student laboratories; these tissues were formalin-fixed (for 24-48 hours) and paraffin-embedded. One pathologist (James Turk [JRT]) reviewed all slides to confirm the diagnosis of TCC and to ensure that inflammatory and/or neoplastic changes were not present in normal tissue samples.

As in the initial pilot study, fetal bovine renal tissue was used as a positive control, as extrapolated from the methods of Ambrosini, et al.[4] Negative controls included bladder tissue treated with rabbit IgG instead of anti-survivin antibody.

Immunohistochemistry Methods

A rabbit polyclonal anti-survivin antibody available from NOVUS Biologicals, Inc. (Littleton, CO) was used for formalin-fixed samples. Tissue sections were cut at 4 μm and deparaffinized. Slides were steamed in citrate buffer (DAKO, Fort Collins, CO) at pH 6.0 for 20 minutes and cooled for 20 minutes. Slides were then treated with 3% H_2O_2 for 15 minutes and protein blocked (DAKO) for 5 minutes. A 1:400 dilution of the primary antibody for survivin was used for 30 minutes at room temperature. Negative controls were treated with IgG rather than anti-survivin antibody. Envision + (DAKO) was used for the detection system and the chromagen used was nova red (Vector Laboratories, Burlingame, CA) with Mayer's hematoxylin counterstaining.

Immunoreactivity was evaluated by one pathologist (Susan Turnquist [SET]); samples that had insufficient epithelium for evaluation were excluded. As described in a human study using this antibody,[28] immunoreactivity was determined to be positive ($\geq$5% of cells with immunoreactivity) or negative (<5% of cells with immunoreactivity). Samples were classified as positive for cytoplasmic survivin ($\geq$5% cells with cytoplasmic immunoreactivity), nuclear survivin ($\geq$5% of cells with nuclear immunoreactivity) or no reactivity.

Tissue Samples for Polymerase Chain Reaction

Tumor tissue specimens were collected during sterile surgical procedures from patients undergoing cystoscopy or cystotomy. Samples of normal bladder mucosa were

collected from terminal surgery patients in veterinary student laboratories with approval from the Animal Care and Use Committee (University of Missouri-Columbia). As a positive control for RNA tissues,[47] normal testes were collected at surgery from adult patients (≥2 years old) undergoing routine castration. All tissue specimens were snap-frozen immediately after collection in liquid nitrogen and stored at -80° C. Tissues collected for PCR analysis were evaluated by one pathologist (JRT) to confirm the diagnosis of tumor and ensure that normal bladder and normal testis was present.

RNA Isolation and first-strand synthesis

Total RNA from testes, tumor tissue and normal urinary bladder tissue was extracted from 30 mg of frozen sections using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The concentration of RNA was determined using the NanoDrop® ND-1000 (NanoDrop® Technologies, Wilmington, DE). Total RNA (100 ng) was used for first-strand synthesis using SuperScript™ III First-Strand (Invitrogen, Carlsbad, CA) with random hexamers according to the manufacturer's instructions.

Traditional qualitative PCR

Reverse-transcriptase PCR was performed using 1 μl of complementary DNA (cDNA) from the first-strand reaction. The primers for survivin were developed using Vector NTI Software (Invitrogen, Carlsbad, CA) from the canine survivin sequence (GenBank accession number AB180206). The primers for survivin were as follows: forward primer 5' CAC CGC GTC TCT ACG TTC AAG AAC TG 3'; reverse primer 5' CCG TTC TCC TTT CCT AAG GCA CAG TG 3'. β-actin was used as a housekeeping gene and primers used were as follows: forward primer 5' ATG GTG GGA ATG GGT

CAG AAG GAC 3' and reverse primer 5' TAC ATG GCT GGG GTG TTG AAG GTC. The first-strand product was amplified using EconoTaq™ DNA polymerase (Lucigen, Middleton, WI) on an ABI Prism® Thermacycler (Applied Biosystems, Foster City, CA) over 32 PCR cycles (30 sec at 94° C, 30 sec at 58° C, 30 sec at 72° C). Only samples with a positive β-actin PCR product were included in the final analysis.

Sequencing

To confirm that the PCR product was survivin, the survivin PCR product was extracted from one of the tumor tissues with a QIAquick gel extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The PCR product was ligated into a pCR 2.1 vector (TOPO) and transformed into One Shot DH5α-TI® (Invitrogen, Carlsbad, CA) competent *E. coli* cells. Cells were incubated at 4° C for 15 minutes and heat-shocked for 30 seconds at 42° C. 250 μl of SOC medium was added and cells were incubated at 37° C for 1 hr. 20 μl of cell solution was spread on pre-warmed ampicillin plates. A bacterial colony was collected with a sterile toothpick and placed in 50 ml LB medium overnight at 250 rpm at 37 C. The medium was spun at 3,000 rpm for 10 minutes at 4° C. To the pellet, 9 ml of GTE was added with 50 mg of lysozyme and placed at 4° C for 10 minutes. A 0.2 N NaOH/1% SDS solution was added and 8 ml of 5 M KOAC added, then placed on ice for 10 minutes. Tubes were spun at 3,000 rpm for 20 minutes at 4° C. Supernatant was mixed with isopropanol and centrifuged at 3,000 for 15 minutes at room temperature. The pellet was washed with 70% ethanol and resuspended in 300 μl of TE and 5 ul RNase added. DNA sequencing was performed with an Applied Biosystems 3730 DNA Analyzer (Foster City, CA) using Applied Biosystems Prism BigDye Terminator cycle sequencing chemistry.

STATISTICAL ANALYSIS

Immunohistochemistry

Samples were classified as positive or negative for survivin immunoreactivity and grouped into those with nuclear survivin expression and those with cytoplasmic survivin expression. The proportions of positive tests were compared among groups (TCC and normal) by use of the Chi squared test. In those instances in which the observed frequency for any permutation of the group and test result was less than 5 observations, the Fisher's exact test was used. Differences were considered statistically significant if $P<0.05$.

PCR

Tissue samples were classified as either positive or negative for survivin with traditional qualitative PCR. Differences between TCC and normal were evaluated using Fisher's exact test and considered significant if $P<0.05$.

RESULTS

Immunohistochemistry

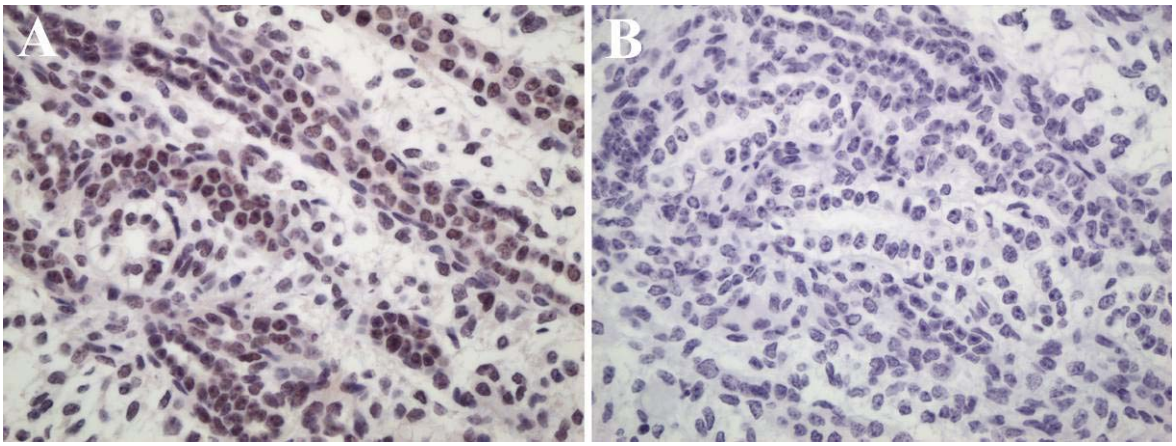
Samples from 41 dogs with TCC were included in the immunohistochemical evaluation. Archival samples were obtained from July, 1991 to May, 2006. Twenty-one were from the University of California, Davis, 13 from the University of Missouri-Columbia and 7 were from Purdue University. Patient characteristics were unknown for two of the TCC samples. Of the 39 cases for which patient information was available, the median age was 11.3 years (range 5 to 16 years). Twenty-four (58.5%) samples were

collected at the time of cystotomy, either via surgical biopsy or partial cystectomy. Other means of sample collection included cystoscopy (7/41 [17.1%]), necropsy (6/41 [14.6%]) and diagnostic catheterization (1/41 [2.4%]). Methods of collection were unknown in 3/41 (7.3%). For the cases with recorded patient information, 24/39 (61.5%) dogs were female spayed with 12/39 (30.8%) castrated males, 2/39 (5.1%) intact males and 1/39 (2.6%) intact female. There were 9/41(22%) mixed-breed dogs, 4/41 (9.8%) beagles, and 4/41 (9.8%) Scottish terriers. Breed was unknown in 5 dogs, but other breeds included Labrador retrievers (2/41), Jack Russell terrier (2/41), West Highland white terriers (2/41) and one of each for the following breeds: border collie, Basenji, Bernese mountain dog, Cairn terrier, Cocker spaniel, English springer spaniel, collie, golden retriever, Lhasa apso, miniature schnauzer, maltese, standard poodle and pointer.

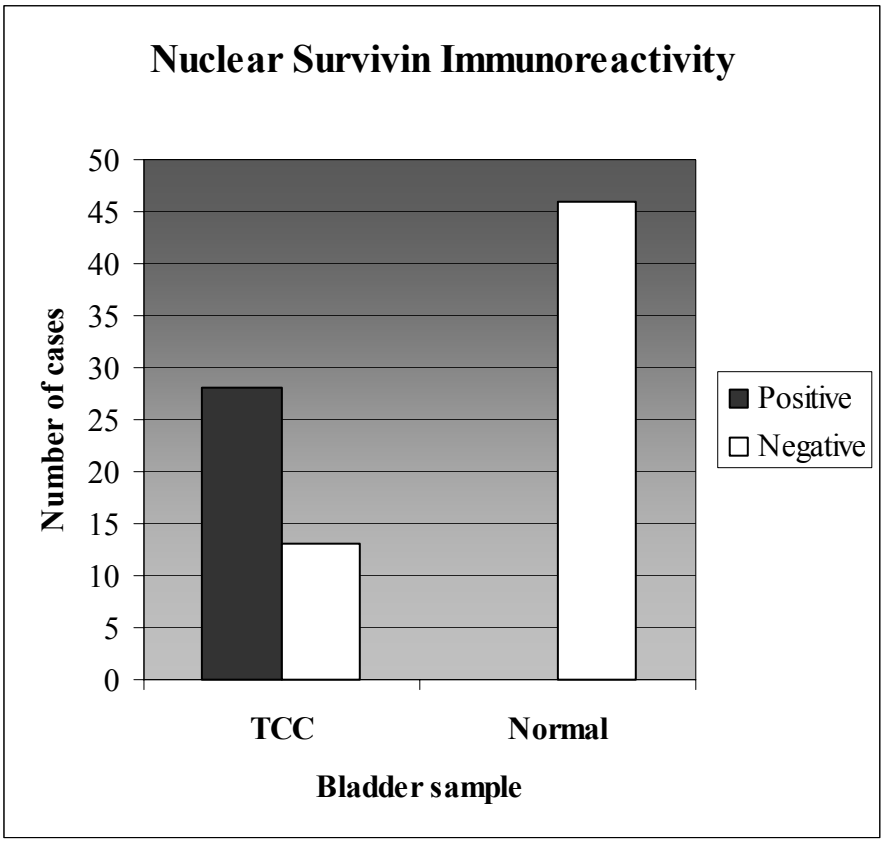
A total of 46 normal urinary bladder samples were evaluated with IHC at the University of Missouri-Columbia from December 2004 to June 2006. Four of the samples were obtained from dogs being euthanatized for diseases unrelated to the urinary tract (atrial fibrillation [n=1], metastatic osteosarcoma [n=2], and multiple myeloma with dirofilariasis [n=1]); these four dogs had a median age of 10.9 years (range 7.1 to 12 years). The remaining 42 samples were obtained from terminal surgery dogs from a veterinary student laboratory at the time of surgery, just prior to euthanasia. The exact signalments of the terminal surgery dogs were unknown, although they appeared to be young adults.

Immunoreactivity to survivin was detected in the nuclei of proximal tubule cells from fetal bovine kidneys (Figure 2-1), and slides of these tissues were used as a comparison for tumor tissues. Nuclear survivin immunoreactivity was evaluated in the

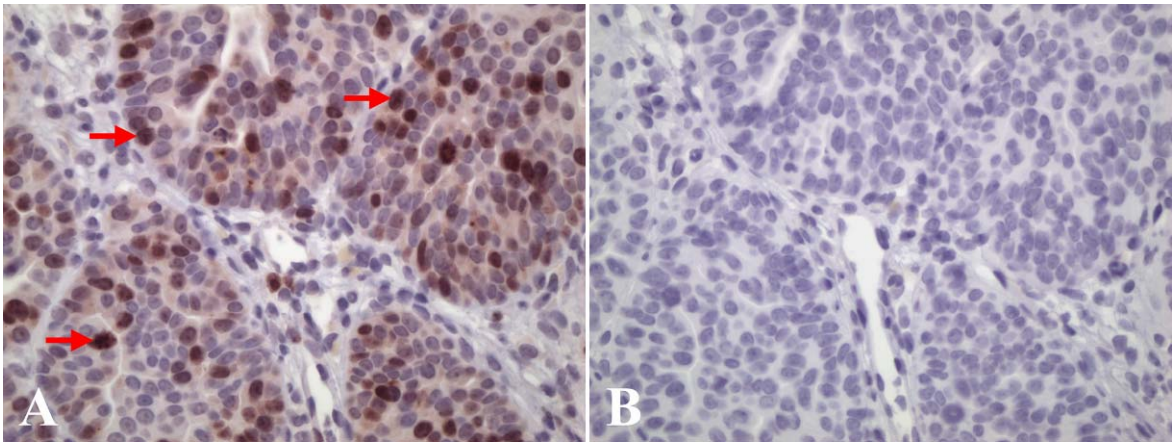
two groups (Figure 2-2). Of the 41 TCC samples, 28 (68.3%) were positive for nuclear survivin (Figure 2-3) and 13 (31.7%) were negative. None of the normal urinary bladder tissues had nuclear immunoreactivity. These proportions differed significantly (P<0.001).



- **Figure 2-1**: Survivin immunohistochemistry for fetal bovine renal tissue (400X). Renal tubular cells were positive for nuclear survivin (A) and negative control incubated without anti-survivin antibody is shown for the same tissue (B).

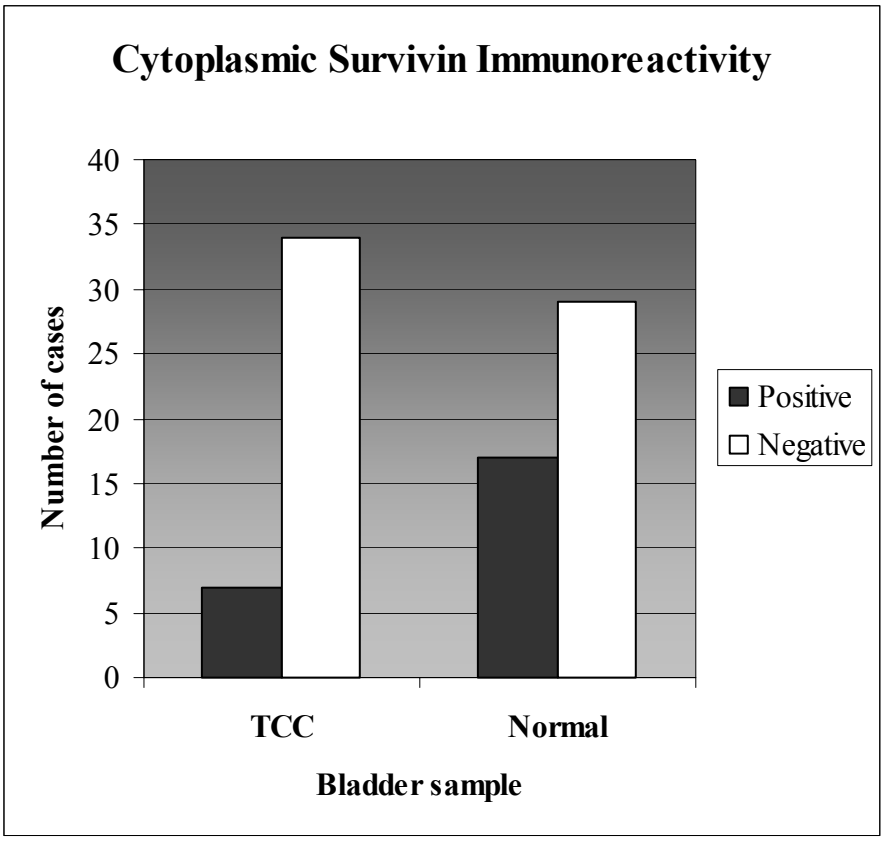


- **Figure 2-2.** Nuclear survivin immunoreactivity in transitional cell carcinoma (TCC) and normal samples. Differences between TCC and normal tissues were significant ($P<0.001$).

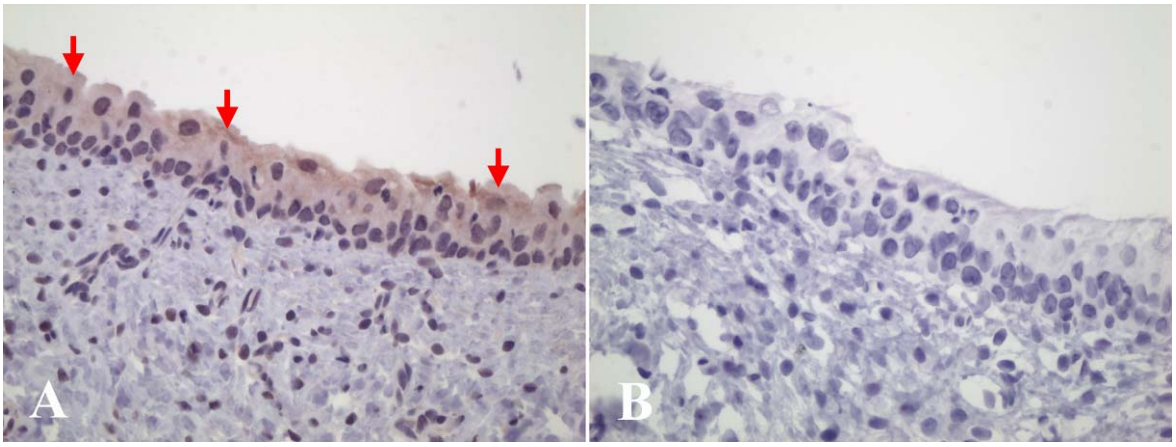


- **Figure 2-3**. Survivin immunohistochemistry for urinary bladder transitional cell carcinoma tissue (400X). Tissue incubated with anti-survivin antibody demonstrates positive nuclear immunoreactivity (A). Arrows point to positive nuclei. The negative control was incubated without anti-survivin antibody (B).

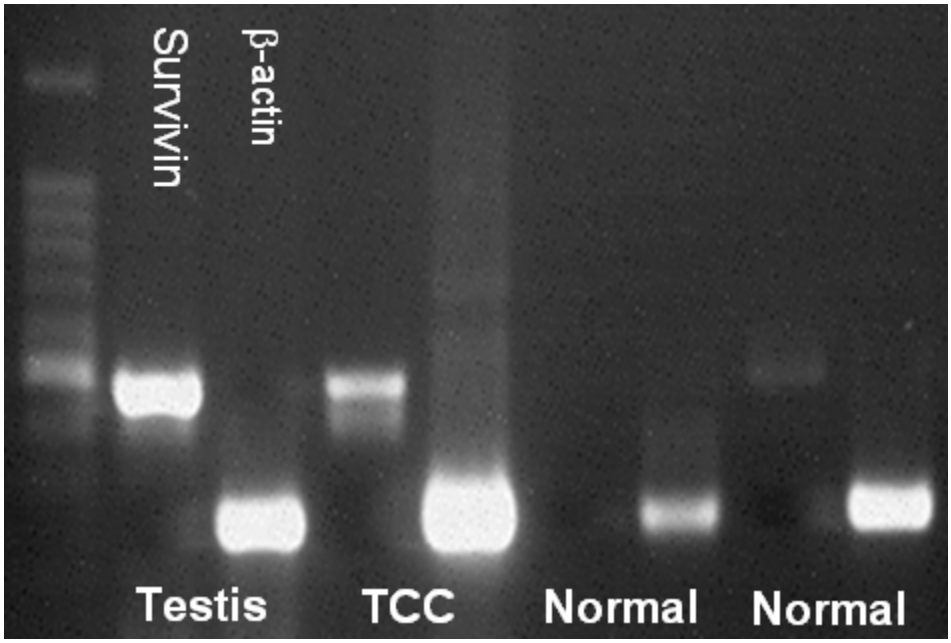
Cytoplasmic survivin immunoreactivity was variable in the groups, but more normal tissues had survivin immunoreactivity in the cytoplasm than did TCC tissues (Figure 2-4). Seven tumor tissues (17.1%) showed positivity and 34 (82.9%) were negative. Seventeen of 46 (37%) normal samples had positive cytoplasmic immunoreactivity (Figure 2-5) and 29 (63%) had negative immunoreactivity in the cytoplasm. These proportions did not differ significantly (P=0.07).



- **Figure 2-4**. Cytoplasmic survivin immunoreactivity in transitional cell carcinoma (TCC) and normal urinary bladder tissues. Differences between TCC and normal samples were not significant (P=0.07).



- **Figure 2-5.** Survivin immunohistochemistry for normal urinary bladder urothelium (400X). Arrows point to positive cytoplasmic survivin immunoreactivity (A). The nuclei in this sample are negative for survivin. Negative control is shown (B).



- **Figure 2-6.** Gel electrophoresis of survivin PCR products. The first lane is a 100 base pair (bp) ladder. Note that the testis (positive control) and TCC samples are positive for both b-actin (269 bp) and survivin (496 bp). The first normal sample is positive for b-actin but negative for survivin. The second normal tissue is positive for b-actin but has a weak band for survivin.

PCR

A total of six tumor samples were included for traditional PCR analysis. All of these patients had histopathological confirmation of tumor tissue; only two had enough formalin-fixed tissue for survivin IHC evaluation. All six TCC samples were positive for a 496 base pair (bp) survivin product, as were all five of the positive controls (testes). To confirm that the PCR product was survivin, one of the 496 bp products from a TCC sample was cloned and sequenced and the sequence was identical to that of a previously published sequence from canine testis.[47] Both tumor samples for which IHC was performed were positive for nuclear survivin with IHC. Of the 22 normal bladder tissues,

11 (50%) were positive for survivin mRNA, some of which showed weak survivin bands (Figure 2-6). Eighteen of the 22 normal samples had sufficient tissue for IHC and none of these samples had cytoplasmic or nuclear survivin immunoreactivity. Fisher's Exact test showed that the difference between TCC and normal tissues for traditional PCR was not significant (P=0.06) (Table 2-1).

Tissue	Survivin +	Survivin -	Total
Testis	**5**	**0**	**5**
TCC	**6**	**0**	**6**
Normal	**11**	**11**	**22**

- **Table 2-1**. Survivin mRNA expression with reverse-transcriptase PCR. All of the positive controls (testis tissues) were positive for survivin, as were TCC tissues. Half of the normal samples were positive for survivin. The difference between the TCC samples and normal samples was not significant (P=0.06).

DISCUSSION

The objective of this study was to evaluate the expression of survivin in normal canine urinary bladder tissue and bladder TCC tissue. Our results indicate that survivin is expressed in both TCC and normal urinary bladder tissue, making it unlikely that survivin will be a useful as a diagnostic test for TCC. However, the differences in expression of survivin support our hypothesis that TCC tissues have a greater expression of nuclear survivin compared to normal urinary bladder. While further studies are necessary to

determine the role of nuclear survivin in tumors, it may have potential as a target for TCC therapy.

Survivin is not typically present in normal, terminally differentiated adult tissues in humans,[4] but this is not supported by all studies of survivin in human[60-67] and canine tissue.[47,48] The fact that survivin is expressed during the G_2/M phase of the cell cycle[68] suggests that it has a role in cell division and proliferation. Several studies also show that tumor tissues positive for survivin are more likely positive for cell proliferation markers.[25,69-72] The presence of survivin in normal epithelial tissue of the bladder in this study is not surprising, since epithelial tissue has the ability to self-renew and proliferate rapidly. In addition, although survivin is present in some normal tissues, the expression is generally lower than in cancer tissues.[73] In this study, our objective was to determine if survivin was present in bladder tissues, and future studies are planned to determine if survivin mRNA levels are lower in normal canine urinary bladder tissue compared to TCC.

While survivin was identified in the cytoplasm of normal canine urothelial cells (Figure 2-5), none of the normal samples exhibited nuclear survivin. This is similar to a study using human tissues, where none of the healthy bladder tissues exhibited nuclear immunoreactivity and 26 of 45 (57.7%) TCC samples were positive for nuclear survivin ($P<0.001$).[74] As with this study, cytoplasmic survivin immunoreactivity in that report did not differ significantly between normal bladder and TCC. It is possible that nuclear survivin, because it is present in TCC tissues but not in normal tissues, plays a role in cell proliferation by inhibiting apoptosis, but survivin may not be as active in the cytoplasm.

Nuclear survivin is a poor prognostic indicator in human esophageal squamous cell carcinoma,[50] cholangiocarcinoma[75] and non-small-cell lung cancer[76] compared to cytoplasmic survivin. Results have been inconsistent, as some reports indicate a longer disease-free interval associated with nuclear survivin expression.[74,77] In this study, no associations can be made between survivin localization and prognosis since treatments varied between patients. Prospective studies are warranted to determine if nuclear survivin has a role as a prognostic tool. In addition, because none of the normal samples exhibited nuclear survivin, *in vivo* studies are warranted to determine if targeting nuclear survivin may inhibit tumor progression.

The subcellular localization may indicate the presence of survivin splice variants. Alternative splicing of the human survivin RNA can lead to different messenger RNA (mRNA) which give rise to five different isoforms of the protein: survivin, survivin-2B, survivin-Δex3[7] survivin-3B[8] and survivin-2α.[9] In addition to having different anti-apoptotic properties (survivin-Δex3 has anti-apoptotic properties, survivin-2B has lost its apoptotic capability[7] and survivin-Δex3 is inversely correlated to apoptotic index[78]), the different isoforms of survivin proteins have different subcellular localization. Survivin and survivin-2B are found mainly in the cytoplasm, where as survivin-Δex3 is predominantly nuclear.[10] Survivin splice variants have not been reported in canine tissues but have been found in human TCC.[79] The splice variant properties reported from human survivin and their subcellular localization suggest that further evaluation of survivin isoforms and their differential localization in canine tissue is warranted.

All six TCC tissues were positive for survivin mRNA with PCR and half of the normal bladder tissues were positive (Table 2-1), but the differences were not significant

(P=0.06), likely due to a small number of samples. The positive PCR in 2/6 tumor samples with nuclear survivin on IHC confirmed the presence of mRNA for survivin protein synthesis. Since survivin mRNA was present normal samples that did not have positive IHC, it is possible that in these normal tissues, mRNA levels were low enough that survivin was not translated into a detectable amount of protein. This may indicate that survivin mRNA is a suitable target for therapy as previously demonstrated in TCC cell cultures.[42-44]

The ages of dogs in the control population and of the archival samples may have affected IHC results. While the age of the dogs with normal bladders was unknown, they appeared to be young adults; therefore age is unlikely to play a role in the different survivin expression between normal bladder and TCC. Additionally, older archival TCC samples may have different IHC characteristics than those collected more recently. Despite the differences in sample age and control population age, results indicate that there is different survivin expression between normal tissues and TCC and this provides evidence that future studies are necessary.

In this study, we demonstrate that survivin is present in canine urinary bladder tumor tissue and in some normal urinary bladder samples. The finding of survivin in canine TCC provides additional evidence that canine urinary bladder cancer can serve as a model for the human disease. Future studies are warranted to determine the role of nuclear survivin in tumorigenesis and cell proliferation, and we plan further investigation of survivin as a prognostic tool and therapeutic target.

CHAPTER 3

Evaluation of Survivin in Canine Urinary Bladder Cystitis Tissues

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INTRODUCTION

Survivin is an inhibitor of apoptosis that is expressed in fetal tissues[16] and regulates mitosis and cell proliferation.[12] The protein is overexpressed in human cancers such as various carcinomas,[4,19,22,28,32,80,81] hematopoietic tumors[4,23,24] and sarcomas.[25] Canine survivin has been recently identified in testis tissue[47] and is found in several canine tumors, including mast cell tumors,[48] oral squamous cell carcinoma[48] and urinary bladder transitional cell carcinoma (TCC).[49]

The authors previously evaluated survivin in canine urinary bladder TCC and normal urinary bladder tissue.[49] In that study, 28 of 41 (68%) of the tumor samples and none of the normal bladder tissues had nuclear survivin; the differences were significant ($P<0.001$). Of the normal bladders, 17 of 46 (37%) had positive cytoplasmic survivin and only 7 of 41 (17%) tumor tissues were positive; however, differences were not significant. The results of this initial study showed that nuclear survivin was present in TCC tissues, but not normal urinary bladder.

The role of nuclear versus cytoplasmic survivin in canine tissues is unknown. Studies in human cancers have demonstrated that nuclear survivin may be associated with a poor prognosis in some types of carcinomas.[50,75,76,82] Nuclear survivin is thought to be involved in promotion of cell proliferation, whereas cytoplasmic survivin may participate in cell survival but not proliferation.[77] In addition, survivin splice variants in human tissues have different roles in apoptosis and localize to different parts of the cell.[10] The splice variants survivin (wild type) and survivin-2B localize to the cytoplasm, where as survivin-Δex3 is usually in the nucleus.[10] While survivin splice variants have not been reported in canine tissues, the different localization of survivin in the cell and the

different splice variants suggest that survivin isoforms and cell localization play a role in regulation of cell proliferation and apoptosis in healthy as well as tumor tissue.

Because defective apoptosis can lead to persistence of mutated neoplastic cells,[83] it is possible that survivin may have a role in pre-neoplastic lesions or early carcinogenesis. In human tissues, survivin has been found in hyperplastic and dysplastic lesions of the prostate,[84] skin,[85] colon[18,86] lung,[87,88] cervix,[89] oral cavity[90] and endometrium.[91] In some studies, there was increased survivin expression in samples with higher dysplasia or neoplasia compared to the corresponding tissues with just hyperplasia or low-grade dysplasia.[18,88] This suggests that survivin may participate in cellular transformation to neoplastic lesions.

Not only are hyperplastic tissues associated with cancerous lesions, but there is an increasing amount of research linking inflammation and cancer. Reports in human tissues suggest that inflammation can lead to various cancers, including cancer of the breast, liver, endometrium and colon.[92-96] Cytokines and other cell signals expressed during inflammation can promote carcinogenesis,[97] but recent reports have also demonstrated that survivin is upregulated with inflammatory cytokines.[62,98] Additionally, therapies aimed at suppressing inflammatory cytokines also suppress survivin expression.[99,100] These studies suggest a link between survivin, inflammation and carcinogenesis.

Although urinary bladder cystitis has not been clearly documented as a risk factor for canine urinary bladder TCC, chronic irritation and infection are risk factors for bladder tumors in humans.[51-53,101] Since canine TCC shares many similar features with human TCC,[45] we wanted to determine if survivin expression in cystitis tissues could demonstrate a role of cystitis in urinary bladder tumorigenesis.

The objective of this study was to evaluate the expression of survivin in canine urinary bladder cystitis tissues and compare its expression to that of normal urinary bladder and TCC tissues that were previously evaluated and reported by the authors.[49] Because previous human studies have demonstrated a role for survivin in inflammation and hyperplasia, our hypothesis was that nuclear survivin is present in canine cystitis tissues and, therefore, nuclear survivin may play a role in canine urinary bladder cell proliferation and tumor growth.

MATERIALS AND METHODS

Immunohistochemistry

Archival formalin-fixed (24-48 hours), paraffin-embedded urinary bladder cystitis samples were obtained from the Veterinary Medical Diagnostic Laboratory (VMDL), Columbia, MO, College of Veterinary Medicine. Criteria used to confirm cystitis were the presence of inflammatory leukocytes in the bladder sections examined. One pathologist (JRT) reviewed all slides to confirm the diagnosis of cystitis.

Formalin-fixed, paraffin-embedded normal canine urinary bladder (n=46) and TCC tissues (n=41) that were previously evaluated for survivin immunoreactivity[49] were used to compare survivin expression with the cystitis tissues in the current study. Tumor and normal tissues had been previously reviewed by one pathologist (JRT) to confirm the diagnosis of TCC and ensure that no neoplastic or inflammatory changes were present in the normal urinary bladder tissues.

As used in the previous study,[49] bovine fetal kidney was used as a positive control as extrapolated from the methods of Ambrosini, et al.[4] Negative controls included tissue

treated with rabbit IgG instead of anti-survivin antibody. A rabbit polyclonal anti-survivin antibody available from NOVUS Biologicals, Inc (Littleton, CO) was used for samples, with tissues processed and stained according to methods previously described.[49] One pathologist (SET) reviewed tissues to evaluate survivin immunoreactivity in the cystitis tissues. As with the previously evaluated samples (TCC and normal bladder), immunoreactivity was positive (≥5% of cells with immunoreactivity) or negative (<5% of cells with immunoreactivity). Samples were classified as positive for cytoplasmic survivin (≥5% cells with cytoplasmic immunoreactivity), nuclear survivin (≥5% of cells with nuclear immunoreactivity) or no reactivity.

Polymerase chain reaction

When available, fresh cystitis tissue specimens were collected during sterile surgical procedures from patients undergoing cystoscopy or cystotomy and snap-frozen immediately after collection in liquid nitrogen and stored at -80° C. Fresh TCC samples from the previous study were collected and stored in the same manner. Fresh normal urinary bladders from the previous report were collected from terminal surgery patients with approval from the Animal Care and Use Committee (University of Missouri-Columbia). RNA isolation, complementary DNA synthesis and traditional qualitative reverse-transcriptase polymerase chain reaction (PCR) were performed according to the methods previously described.[49] Fresh-frozen canine testis was used as a positive control[47] and deionized water without complementary DNA was used as a negative control in PCR reactions as well as normal samples from the previous study[49] known to be negative for survivin mRNA.. β-actin was used as a housekeeping gene for each sample and samples with positive PCR bands for β-actin were included in the final

analysis. The survivin primers used were developed from the canine survivin sequence (GenBank accession number AB180206).

STATISTICAL ANALYSIS

Immunohistochemistry

Samples were classified as positive or negative for survivin immunoreactivity and grouped into those with nuclear, cytoplasmic, or no survivin expression. The proportions of positive tests were compared among groups (TCC, cystitis and normal) with the Chi squared test. When an observed frequency of the group and test result was less than 5 observations, the Fisher's exact test was used. Differences were considered statistically significant if $P<0.05$.

PCR

Tissue samples were classified as either positive or negative for survivin with traditional qualitative PCR. Differences between cystitis, TCC and normal were evaluated using Fisher's exact test and considered significant if $P<0.05$.

RESULTS

Patient characteristics

Twenty-four cystitis samples collected from September 2004 to October 2006 were included for immunohistochemical evaluation. Samples were collected by cystotomy in 17/24 (70.8%), necropsy in 5/24 (20.8%), and methods were unknown in 2/24 (8.3%). Of the cystotomy patients, 5/17 (29.4%) had cystotomies due to cystic calculi, 2/17 (11.8%) had cystotomies for a thickened bladder wall or bladder wall mass

and 8/17 (47.1%) had cystotomies for unknown reasons. The remaining 2 patients had cystotomies for a ruptured bladder (one secondary to trauma and one secondary to cystic calculus obstruction). Of the 5 patients with cystitis that had bladder samples obtained on necropsy, two were euthanized due to spinal tumors causing urinary incontinence and others were euthanized for conditions unrelated to the urogenital system (1 for being hit by a car, 1 for a mediastinal mass, and 1 for suspected immune-mediated hemolytic anemia). There were 10/24 (41.7%) spayed females, 9/24 (37.5%) castrated males, 4/24 (16.7%) intact males, and gender was unknown in 1/24. Of the breeds represented in this group, 5/24 were mixed-breed dogs, 2/24 were Labrador retrievers, 2/24 were Golden retrievers, 2/24 were boxers, 2/24 were pugs, and one of each of the following breeds: Bichon Frise, Dalmation, Doberman pinscher, German shorthaired pointer, Lhasa Apso, Maltese, miniature pinscher, miniature schnauzer, Schnauzer, Yorkshire terrier and standard poodle. The median age of the cystitis patients was 6.4 years (range 1.7 to 12.8 years).

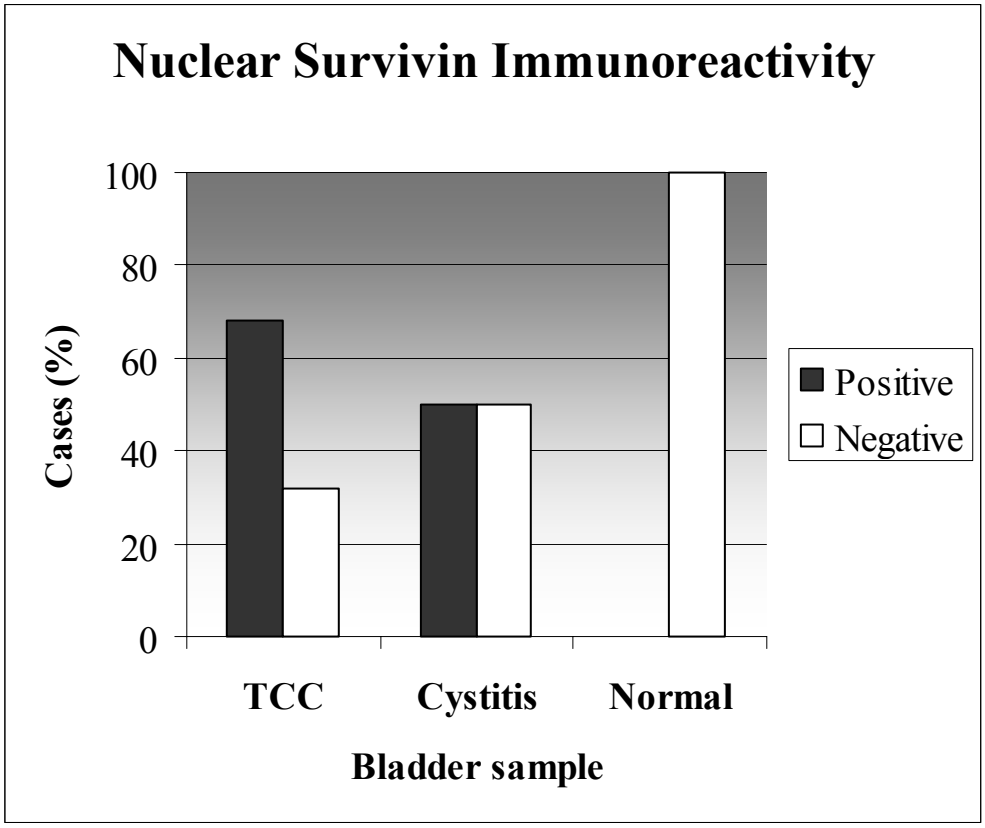
Patient characteristics for the TCC and normal urinary bladder samples are reported in the previous study.[49] Briefly, archival samples from 41 TCC patients were evaluated from the University of California, Davis (n=21), University of Missouri-Columbia (n=13) and Purdue University (n=7). The median age for patients where signalment information was available (n=39) was 11.3 years (range 5 to 16 years). Forty-six normal urinary bladder samples were evaluated in the previous study; 42 of those were from terminal surgery dogs (ages unknown) and 4 were from dogs being euthanatized for diseases unrelated to the urinary tract.

Immunohistochemistry

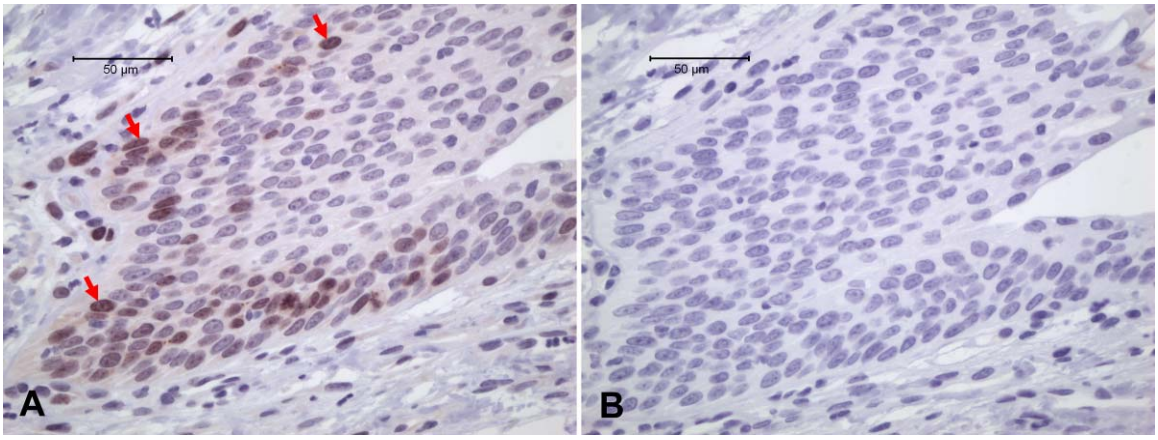
Fetal bovine renal tubules were positive for survivin immunoreactivity, as shown in the previous report;[49] these positive controls were used as a comparison for cystitis samples and the TCC and normal tissues from the previous study. Nuclear survivin immunoreactivity was evaluated in the cystitis tissues and compared with previously reported results from TCC and normal bladder (Figure 3-1). Half (12/24) of the cystitis samples showed positive immunoreactivity for nuclear survivin (Figure 3-2) whereas 12/24 were negative. Chi-squared analysis among the groups showed that differences were statistically significant with $P<0.001$. The differences between normal and TCC samples were statistically significant ($P<0.001$) as reported in the previous study.[49] Differences between normal and cystitis samples using Fischer's exact test were significant ($P<0.001$). We were not able to detect a difference between TCC and cystitis nuclear immunoreactivity ($P=0.23$), but the power of this comparison was low at 0.17.

For cytoplasmic survivin immunoreactivity, the cystitis samples exhibited 2/24 (8%) with positive cytoplasmic survivin and 22/24 (92%) with negative immunoreactivity. When cystitis samples were compared to TCC and normal bladder tissues from the previous report, the cytoplasmic survivin immunoreactivity was variable in the three groups (Figure 3-3). Overall Chi-squared analysis showed that differences among the groups were statistically significant ($P=0.008$). As previously reported,[49] the difference between normal and TCC cytoplasmic survivin was not significant ($P=0.07$); however, the power of this test was 0.43. The difference between normal and cystitis cytoplasmic immunoreactivity was significant ($P=0.02$). No difference was found

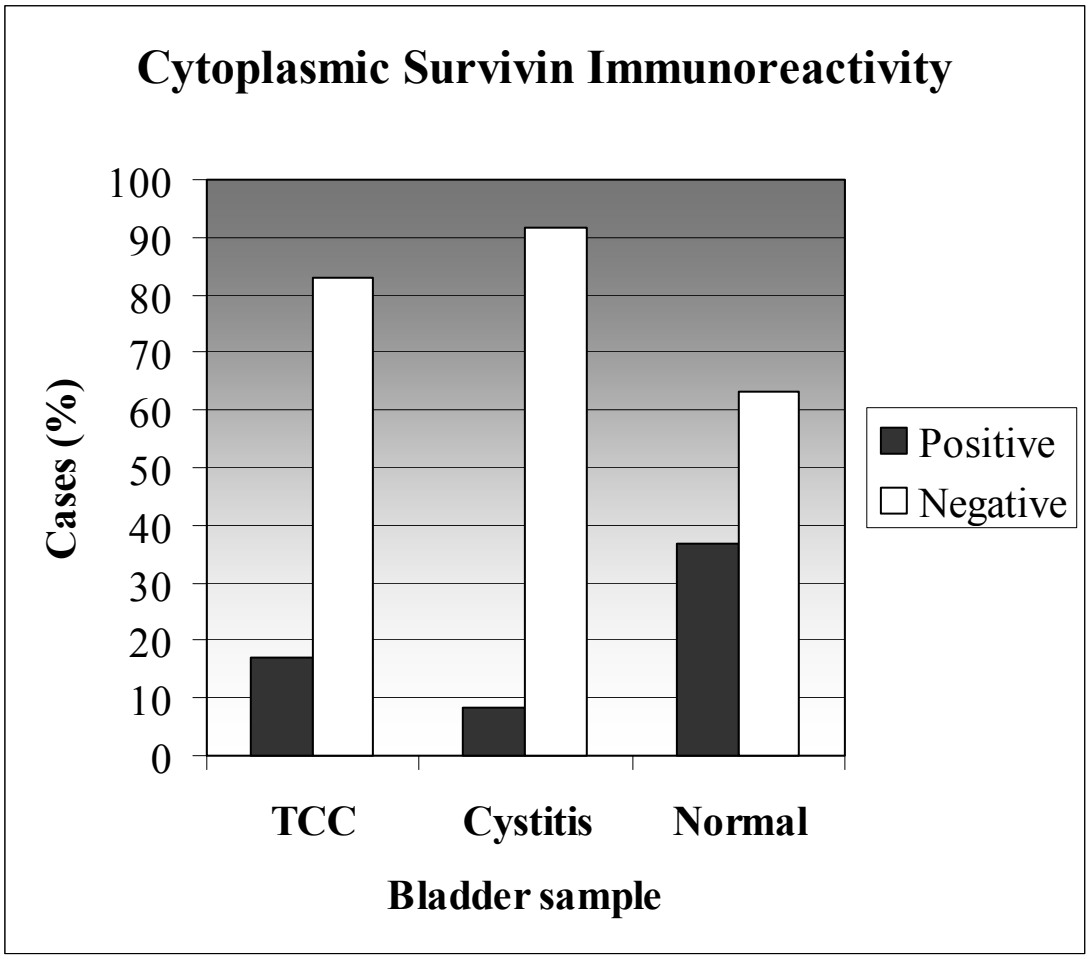
between TCC and cystitis cytoplasmic immunoreactivity (P=0.7), but the power of this comparison was 0.06.



- **Figure 3-1**. Nuclear survivin immunoreactivity in TCC, cystitis and normal urinary bladder. Differences between normal and TCC and normal and cystitis were significant (both P<0.001), but not between TCC and cystitis (P=0.23).



- **Figure 3-2**. A. Cystitis sample positive for nuclear survivin at 400X. Arrows point to positive nuclei. B. Negative control incubated with rabbit IgG instead of anti-survivin antibody.



- **Figure 3-3**. Cytoplasmic survivin immunoreactivity in transitional cell carcinoma (TCC), cystitis and normal urinary bladder. Differences between normal and cystitis were significant (P=0.02), but not between TCC and cystitis (P=0.7) or TCC and normal (P=0.07).

PCR

Seven fresh cystitis tissues were available for PCR analysis. Of these 7 samples, 3 also had enough formalin-fixed tissue for survivin immunohistochemistry. Four of the 7 tissues were positive for survivin mRNA (Table 3-1). Of the 3 samples that had both IHC and PCR, 2/3 were positive for nuclear survivin protein and survivin mRNA. The

remaining sample was negative for both survivin protein and survivin mRNA. When comparing survivin mRNA expression in cystitis tissues to TCC and normal tissues, the differences between the tissues (TCC versus cystitis and normal versus cystitis) were not significant (P=0.19 and P=1.0, respectively); however, the power of the tests were 0.007 and 0.032, respectively.

Tissue	Survivin +	Survivin -	Total
Testis	5	0	5
TCC	6	0	6
Cystitis	4	3	7
Normal	11	11	22

- **Table 3-1**: Survivin mRNA expression in testis, transitional cell carcinoma (TCC), cystitis, and normal urinary bladder tissues. Differences between groups were not significant (TCC versus normal, P=0.06; TCC versus cystitis, P=0.19; normal versus cystitis, P=1.0).

DISCUSSION

The objective of this study was to evaluate survivin expression in canine urinary bladder cystitis and compare expression to that of TCC and normal bladder. These results support our hypothesis and indicate that nuclear survivin is present in canine cystitis tissues. The study demonstrates that survivin is not likely to be a specific diagnostic marker for canine TCC, but we show that nuclear survivin is present in both TCC and

cystitis tissues. Such observations warrant further investigation to better define a role for survivin in cell proliferation and regulation of apoptosis.

It is possible that nuclear survivin may have a role in hyperplastic and neoplastic cell proliferation. None of the normal samples exhibited nuclear survivin immunoreactivity whereas 50% of the cystitis and 68% of the TCC samples had nuclear survivin (Figure 3-1). This is supported by a study utilizing human TCC samples, where TCC tissues with greater nuclear survivin had a significantly higher cell proliferation (assessed by Ki-67 scores) than patients with low nuclear survivin scores.[57] In addition, survivin is higher in human hyperplastic prostatic tissue,[84] hyperproliferative skin lesions,[85] hyperplastic colonic mucosa[86] and hyperplastic endometrium[91] compared to their normal tissue counterparts. In several studies, the expression of survivin was higher in malignancies compared to the hyperplastic or inflammatory counterparts.[85,86,91,102] These studies may suggest a role for survivin in cell proliferation and tumor progression, either by regulation of cell proliferation or by indirect inhibition of apoptosis.

The localization of survivin in the cell in cystitis and TCC tissues compared to normal tissues may reflect different functions based on its location. Previous studies in human tissues have suggested that nuclear survivin generally promoted cell proliferation, while cytoplasmic survivin was involved in cell survival and maintenance.[30,77] In addition, because survivin localizes to the mitotic spindle in the G_2/M phase,[68] nuclear survivin is thought to regulate the cell cycle and cell proliferation, whereas cytoplasmic survivin is proposed to regulate apoptosis.[103] Therefore, it is possible that the cytoplasmic survivin in canine bladder tissues may function to maintain cell survival in normal

bladder urothelium, whereas the nuclear survivin in hyperplastic bladder and TCC facilitates cell proliferation.

Another explanation for the subcellular localization could arise due to splice variants. A few survivin splice variants that encode for slightly different proteins with different cellular localization and functions have been described in humans. Survivin-Δex-3 localizes to the nucleus and has anti-apoptotic properties, whereas survivin-2B localizes to the cytoplasm and has reduced anti-apoptotic properties compared to Δex-3.[7] Additionally, survivin Δex-3 is associated with a higher rate of lung tumor recurrence compared to survivin-2B in humans.[30] The presence of these survivin splice variants in human tissues may explain the different expression patterns in canine bladder tissues; however, further evaluation is necessary to determine if distinct survivin isoforms are associated with the different subcellular localization patterns in the dog.

The presence of nuclear survivin in cystitis and TCC tissues may support the theory that hyperplasia or inflammation of the bladder can lead to malignant transformation in dogs. Previous reports have suggested a role of inflammation in the development of human bladder tumors.[51-53,101] To our knowledge, survivin has not been evaluated in human cystitis tissues, but it has been demonstrated in several pre-neoplastic lesions in human tissues such as breast adenoma,[104] oral epithelial dysplasia,[90] actinic keratosis,[105] colorectal adenomas[18] and cervical intraepithelial lesions.[89] Survivin is also upregulated in human keratinocytes and mouse skin exposed to ultraviolet-B radiation, a known risk factor for skin cancer.[106] In addition, one study demonstrated that survivin was present in only 33% of precancerous oral lesions in humans that did not have malignant transformation, but was present in 94% of oral lesions that progressed to

squamous cell carcinoma.[90] When comparing normal, inflammatory, and malignant pleural lesions in human tissues, survivin expression increases with the severity of disease,[102] and inflammatory cytokines such as granulocyte-macrophage colony stimulating factor increases survivin expression in human tissues.[62,98] These findings suggest that survivin may be a mechanism for development of tumors in hyperplastic or inflamed tissues. Future studies are necessary to determine if cystitis may be a precancerous lesion in dogs and whether survivin can be a target for anti-tumor therapy.

The nuclear and cytoplasmic survivin immunoreactivity between TCC and cystitis samples were not statistically significant. This may be due to a small number of samples (particularly in the cystitis group) considering the powers of the tests were low. In addition, TCC samples may induce secondary cystitis, and therefore survivin positivity may have been due to concurrent inflammation in tumor samples. In this study, it was not possible to evaluate all samples for inflammation (many tumor samples were too small to evaluate the degree of inflammation). In addition, because this was a retrospective analysis, it is difficult to determine survivin expression in progression of disease from cystitis to TCC development. However, future studies are planned to evaluate survivin expression levels in bladder tissues to determine if cystitis samples may have lower levels than TCC tissues.

The presence of survivin messenger RNA (mRNA) in 4/7 of the cystitis tissues in this study and the tissues from the previous study (all 6 of the TCC tissues and 11/22 normal bladder) (Table 3-1) confirmed the message for the survivin protein. While no differences were detected among the samples, the powers of the tests were low due to the

small sample size. Future studies evaluating larger numbers of samples are necessary to determine if the presence or absence of survivin mRNA is different between the groups.

Of the three cystitis and 2 tumor samples for which there was also IHC, the presence of survivin mRNA correlated with positivity for nuclear survivin on IHC. The one cystitis tissue that had no survivin mRNA had no survivin immunoreactivity on IHC. With normal bladder, however, samples positive for survivin mRNA had neither cytoplasmic nor nuclear survivin. The mRNA levels in the cystitis and tumor tissues may have been high enough to be translated into detectable protein; however, normal samples may not have had high enough levels of mRNA for translation or high enough levels of detectable protein. Because the objective of this study was to identify survivin expression in cystitis tissues, we did not perform quantitative analysis of mRNA. Future studies evaluating mRNA levels in the different tissues are planned to determine if mRNA levels correlate with the disease states.

The results of our study show that survivin is present in canine urinary bladder cystitis tissues. We found that nuclear survivin is present in cystitis and TCC tissues, but not in normal bladder tissues. With the rapidly expanding research linking inflammation and carcinogenesis, it is possible that survivin may be another mechanism—in addition to or independent of cytokines—for bladder cancer development. Future studies are planned to determine if cell proliferation is higher in tissues with nuclear survivin compared to cytoplasmic survivin. If so, nuclear survivin may be an early marker for bladder tumors or potential therapeutic target of chronic cystitis and canine urinary bladder tumors.

CHAPTER 4

Survivin Messenger RNA levels in Urinary Bladder Tissues

INTRODUCTION

The preceding two chapters reported that survivin protein and mRNA are present in canine urinary bladder tissues. We demonstrated that—due to its presence in normal, cystitis and TCC tissues—survivin would not be a reliable biomarker for canine bladder tumors. The next reasonable step, therefore, would be to determine if the level of survivin would correlate with different disease states of the urinary bladder.

Previous studies show that survivin is overexpressed in human malignancies, including bladder cancer.[4,17,19,22,23,26,28,29,31] Early reports stated that survivin is not in most normal adult tissues,[4] but some tissues such as endothelial and hematopoietic cells do express survivin.[107] That survivin is present in these tissues suggests that it may have a role in normal as well as neoplastic tissue.

In canine tissues, survivin mRNA was detectable in many normal, differentiated tissues, but overexpressed in three different types of tumors in a previous study;[47] however, urinary bladder tissues were not evaluated in that study. In addition, survivin mRNA was detected in 11 of 22 normal bladder specimens, but these 11 did not have evidence of survivin protein on immunohistochemistry (see Chapter 2). These studies suggest that while survivin mRNA is present in normal tissues, the levels may not be high enough to be translated in to protein.

Levels of survivin mRNA have been evaluated in human urinary bladder TCC. In the urine, higher levels of survivin are found in TCC patients than in healthy controls.[33,34,108] In urinary bladder tissues, the levels of survivin mRNA correlates with invasiveness, grade, stage, and increased risk of recurrence in human TCC.[34,38,40] Cystitis

tissues were not evaluated in those studies, but the results suggest that survivin levels may be useful to predict prognosis in patients with TCC.

The objective of this study was to evaluate survivin mRNA levels in canine TCC, cystitis, and normal urinary bladder tissues. Based on previous studies in humans, our hypothesis was that TCC samples would have higher levels of survivin mRNA compared to cystitis and normal samples and therefore, higher survivin mRNA correlates with more aggressive disease of the urinary bladder.

MATERIALS AND METHODS

Fresh-frozen tissue samples were obtained and complementary DNA made according to the methods described in Chapters 2 and 3. Fresh-frozen canine testes were obtained from adult dogs (>2 years old) undergoing routine castration.

Complementary DNA (50 ng) from the first-strand reaction was used for quantitative PCR of survivin mRNA using the SYBR Green kit (QIAGEN, Hilden, Germany). Primers and probe for the survivin amplification are as follows: forward primer 5' TTC TGC TTC AAG GAG CTG GAA 3'; reverse primer 5' CAC AAC CAG ATG AAT GTT TTT TAT GC 3'. β-actin control was used to normalize expression levels. The primers used for β-actin control were as follows: forward primer 5' CAG GAT GCA GAA GGA AAT 3'; reverse primer 5' GCT GAT CCA CAT CTG CTG GAA 3'. Reactions were performed in a Model 7500 Applied Biosystems Real-Time PCR System. Reaction conditions were 50° C for 20 minutes, 95° C for 15 minutes followed by 40 cycles of the amplification step (95° C for 30 sec, 58° C for 30 sec, and 72° C for 45 sec). Expression of survivin was calculated by normalizing the expression

level to that of β-actin in each sample with the formula $2^{-(Ct\ survivin - Ct\ \beta\text{-}actin)}$.[109] Relative expression was determined after normalizing survivin expression and comparing it to that of normal tissue as per the manufacturer's instructions (ABI Prism Sequence Detection System, Applied Biosystems).

For statistical analysis, the ratios of survivin to β-actin were calculated for each sample and differences between groups (normal, TCC and testis) were evaluated using one way analysis of variance.

RESULTS

Normal bladder tissue (n=9), cystitis tissues (n=4), TCC tissue (n=5) and testes (n=6) were evaluated with quantitative real-time PCR. The ratios of survivin:β-actin were compared between the groups for statistical analysis. Expression levels relative to normal bladder are shown in Table 4-1. There were significant differences between survivin level in testis compared to other groups ($P<0.001$); however, no significant differences were noted between TCC, cystitis, and normal expression levels. Quantitative analysis showed that survivin expression relative to that of normal bladder tissue was greatest in testis (123 times that of normal), but TCC tissue and normal tissue expression levels were similar with overlapping ranges (Table 4-1).

Tissue	Expression relative to normal	Range
Testis (n=6)	123.95	5.82 to 2638.23
TCC (n=5)	0.63	0.19 to 2.05
Cystitis (n=4)	0.16	0.06 to 0.4
Normal (n=9)	1.000	0.31 to 3.27

- **Table 4-1**. Survivin mRNA expression levels of urinary bladder tissues relative to normal urinary bladder samples. Testis tissues (n=5) had the highest expression of survivin relative to normal. When comparing survivin:b-actin ratios for statistical analysis, differences between testis and the 3 other tissues were significant (both $P<0.001$) but between TCC versus cystitis, TCC versus normal, and normal versus cystitis results were not significant ($P=0.39$, $P=0.85$, $P=0.08$, respectively).

DISCUSSION

Quantitative analysis of survivin mRNA in urinary bladder tissues showed no significant differences in TCC, cystitis and healthy mucosa. Based on these results, our hypothesis was rejected and we cannot say that tumor tissue has higher expression levels of survivin mRNA.

The authors know of only one previous study that evaluated survivin mRNA with quantitative PCR in canine tissues.[47] Among normal tissues, the testis showed highest expression levels of survivin mRNA, but levels were detectable in lung, heart, kidney, liver, spleen and gastrointestinal tract tissues. Urinary bladder was not evaluated. Eighteen malignant and 9 benign tumors of various tissues were evaluated for survivin mRNA and many of the expression levels for tumors were within the range of normal

tissue levels. While the three highest levels of survivin mRNA were in malignant tumors (mammary gland, synovioma and melanoma), no conclusions can be made regarding the significance of expression levels compared to normal tissues in that study. With the paucity of information regarding survivin levels in normal and malignant canine tumors, future studies are necessary that evaluate one particular tumor type and its corresponding normal tissue to determine if mRNA levels correlate with diseased tissues.

It is not clear why survivin would be detected in normal dog tissue, but not human tissue. In contrast to the study by Uchide et al.,[47] in adult human tissues, survivin mRNA has not been detected in normal, differentiated adult tissue such as the lung, heart, liver, spleen and kidney.[4] In addition, there was no detectable survivin mRNA in normal human bladder mucosa in one study.[34] One possible explanation is that survivin functions differently to control cell proliferation and apoptosis in the dog compared to humans. Another explanation may be that canine tissues have different pathways for degradation or translation of survivin in that mRNA levels do not necessarily correspond directly to protein levels of survivin.

In this study, no significant differences were noted between tumor, cystitis, and normal tissues. However, in human tissues survivin mRNA levels were associated with higher pathologic stage and grade of bladder tumors, but there was no detectable survivin mRNA in normal bladder mucosa.[34] From our understanding of survivin and its regulation of cell proliferation and apoptosis, it is reasonable to conclude that survivin levels correlate with more aggressive disease. While this is not the case for our current study, the number of samples in this study may not be high enough to note significant differences. In addition, as discussed in the previous paragraph, survivin mRNA in canine

tissues may have a different role than in the human and that it is even detectable in normal tissues suggests that it may have a different function in dog tissue. Thus, findings of survivin mRNA in human tissues may not directly translate into dog studies.

The results of this study demonstrate that canine testis has high levels of survivin mRNA and therefore it can be used as a positive control for mRNA studies. Although we did not find significant differences between TCC, cystitis and normal urinary bladder tissues, future studies are necessary to evaluate the role of survivin mRNA in the pathogenesis of cancer and cell proliferation. It is possible that mRNA levels in the dog do not correlate to diseased states and therefore, perhaps evaluation of the survivin protein is a more reliable way to assess survivin's role in canine malignancy.

CHAPTER 5

Correlation of Survivin and Ki-67 Immunoreactivity in Canine Urinary Bladder Tissues

INTRODUCTION

The preceding chapters reported that survivin is present in the nuclei of some tumor and cystitis specimens, but not normal urinary bladder tissue. We also found that the presence of survivin mRNA in normal tissues does not correlate with survivin protein expression, and that the levels of mRNA may not correlate with disease status in the samples evaluated. The next step in evaluating the role of survivin in urinary bladder tissues would be to correlate Ki-67 immunoreactivity with survivin expression.

As previously discussed, survivin has a dual role in tissues—not only does it regulate apoptosis, but it participates in regulation of cell proliferation. Several studies in human hepatocellular carcinoma have demonstrated that survivin function may depend on its subcellular localization. Nuclear survivin is found in malignant cells, but only cytoplasmic staining (and not nuclear survivin) is found in nonmalignant hepatocytes.[110] Nuclear survivin in these cells control cell proliferation, whereas cytoplasmic survivin inhibits caspase-3-dependent apoptosis.[111] This suggests that nuclear survivin plays a role in malignant cell proliferation, whereas cytoplasmic survivin is involved in normal cell survival.

Ki-67 is a protein in the nucleus that is expressed during all active parts of the cell cycle, including G_1, S, G_2, and mitosis, but not in resting cells (G_0 phase).[112] Because of its expression in many parts of the active cell cycle, Ki-67 labeling index (the percentage of positively-staining cells) is a sensitive method to assess proliferation in tumors. Ki-67 has also been utilized in various malignancies as a marker of cell proliferation, and is of prognostic value in many tumors, including urinary bladder carcinomas.[113-117] Because of

its association with cell proliferation and its value as a prognostic tool in urinary bladder TCC in humans, Ki-67 can be a useful marker for cell proliferation in canine TCC.

Ki-67 and survivin have been evaluated concurrently in several different tumor types. Survivin expression has been positively correlated to Ki-67 labeling index in human colonic adenomas,[18] mantle cell lymphoma,[118] hepatocellular carcinoma,[103] ependymomas[71] and urinary bladder transitional cell carcinomas (TCC).[57,117] These studies suggest that survivin has a role in tumor cell proliferation. More specifically, nuclear survivin is positively correlated with cell proliferation in human urinary bladder tumors.[57,117]

The correlation between survivin and tumor cell proliferation in canine tissues is unclear. In canine mast cell tumors, there was no positive association between survivin expression and Ki-67 index.[48] There are no reports of survivin and correlation with cell proliferation in canine urinary bladder tissue. The objective of this study was to determine if there is a relationship between survivin subcellular expression and cell proliferation (as assessed by Ki-67 labeling index) in canine urinary bladder tissues. Our aim was to test the hypothesis that nuclear survivin, but not cytoplasmic survivin, correlates with Ki-67 immunoreactivity.

MATERIALS AND METHODS

Formalin-fixed, paraffin-embedded (FFPE) canine urinary bladder TCC, cystitis, and normal urinary bladder tissues that were used in the previous studies (Chapters 2 and 3) were used when available. One pathologist (JRT) confirmed the diagnoses, and tissues

were incubated with anti-Ki-67 antibody if they also had survivin immunohistochemistry from the previous studies (Chapters 2 and 3).

FFPE tissues were cut at 4 μm, placed on positively-charged slides, microwaved and left on a 43° C slide warmer overnight. They were hydrated and steamed at 95° C for 20 minutes in Citrate Buffer (Dakocytomation, Carpenteria, CA) pH 6.0, cooled at room temperature for 20 minutes, rinsed, then placed in Tris buffer for at least 5 minutes before staining. Subsequent staining was done on the Dakocytomation Autostainer. Slides are treated with 3% H_20_2 for 15 minutes, washed in buffer, treated with Protein Block (Dakocytomation, Carpenteria, CA) for 5 minutes and drained. They were then incubated in mouse anti-Ki-67 antibody (clone 7B11) (Zymed, South San Francisco, CA) at a 1:200 concentration for 60 minutes. Negatives are treated with mouse IgG (Sigma, St. Louis, MO) at a 1:1000 dilution for 60 minutes. Secondary and tertiary reagents used were LSAB2 (Dakocytomation, Carpenteria, CA) for 20 minutes each with a tris buffer rinse. The chromogen used was DAB (Dakocytomation, Carpenteria, CA.) for 5 minutes. Slides were then counterstained in Mayer's Hematoxylin (Newcomer's Supply, Appleton, WI.) for 1 minute, dehydrated and coverslipped. Mouse gastrointestinal tract epithelium was used as a positive control.

Ki-67 score was evaluated by one author (Wendi Rankin [WVR]). As extrapolated from a previous study,[57] ten fields (400X) were evaluated and the number of positive nuclei were counted and divided by the total number of epithelial or tumor cells in each field. The score was expressed as a percentage and the 10 fields were averaged for each sample. Samples were categorized into cytoplasmic survivin, nuclear survivin or no immunoreactivity as reported from the previous studies (Chapters 2 and 3). The

average Ki-67 labeling index was calculated for each group (TCC with nuclear survivin, TCC without nuclear survivin, TCC with cytoplasmic survivin, TCC without cytoplasmic survivin, cystitis with nuclear survivin, cystitis without nuclear survivin, cystitis with cytoplasmic survivin, cystitis with cytoplasmic survivin, normal bladder with cytoplasmic survivin, normal bladder without cytoplasmic survivin). Groups were compared with one way analysis of variance. A $P<0.05$ was considered statistically significant.

RESULTS

Of the samples that had previous immunohistochemistry (IHC) for survivin, 19 TCC samples, 42 normal bladders, and 23 cystitis tissues had enough remaining tissue to clearly evaluate Ki-67 IHC. For TCC samples, those with cytoplasmic survivin immunoreactivity (n=7) and those without cytoplasmic survivin (n=12) had no statistically significant difference in Ki-67 scores (27.17% versus 22.95%, respectively, P=0.5) (Table 5-1). Normal urinary bladder samples had a Ki-67 score of 2.74% for samples with cytoplasmic survivin (n=16) and 2.19% for samples without cytoplasmic survivin (n=26); differences were not significant (P=0.4) (Table 5-1). Since there was only one cystitis sample that exhibited cytoplasmic survivin immunoreactivity, correlation between cytoplasmic survivin immunoreactivity and Ki-67 score was not evaluated.

TCC samples without nuclear survivin (n=8) had a Ki-67 score of 17.99% compared to a 31.17% Ki-67 score for those samples with nuclear survivin (n=11) (Table 2) and the difference was significant (P=0.02). The Ki-67 immunoreactivity patterns in

TCC tissues were heterogenous (Figure 5-1). Urinary bladder cystitis tissues with negative nuclear survivin (n=11) had a Ki-67 score of 7.06% compared to those with positive nuclear survivin (n=12) having a Ki-67 score of 44.63%; the difference was statistically significant (P<0.001). Ki-67 immunoreactivity in cystitis tissues is shown in Figure 5-2.

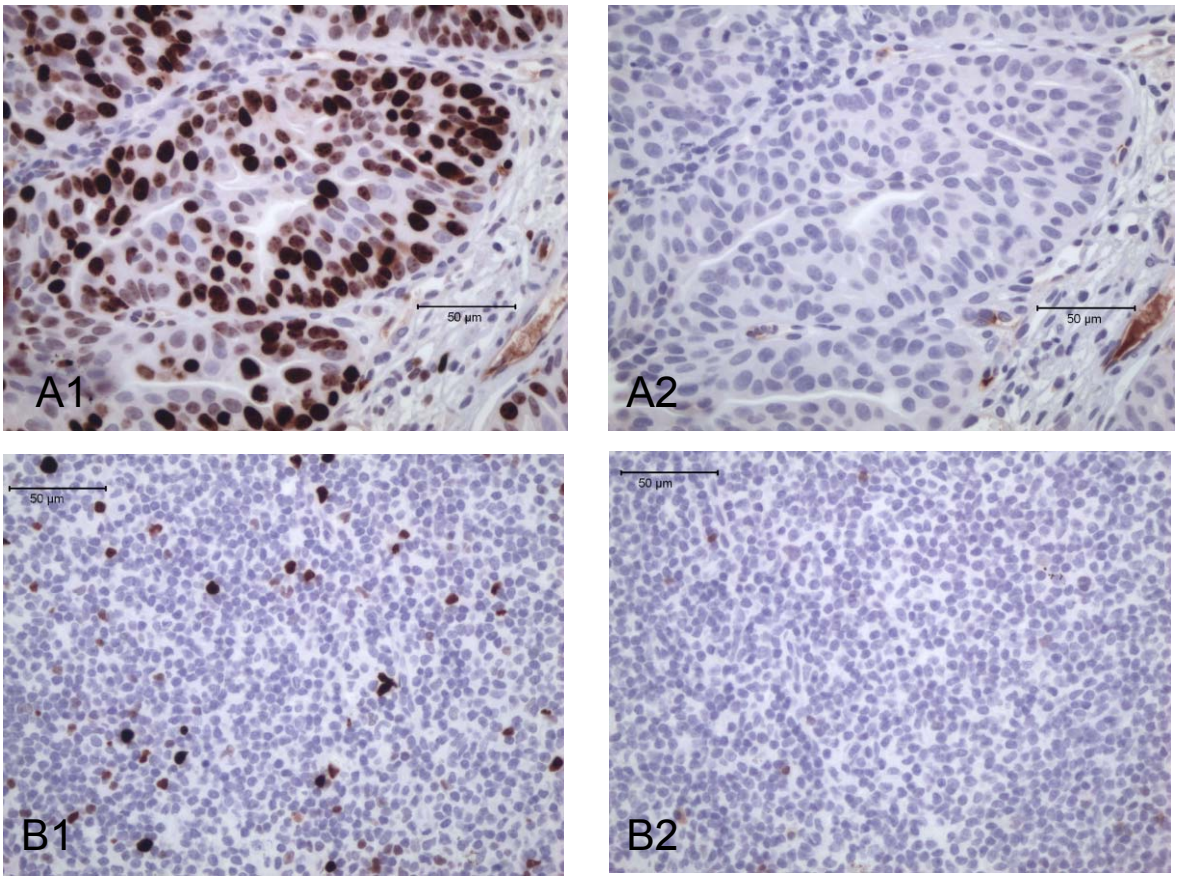
Sample	**Cytoplasmic survivin**	**Ki-67 score (%, average +/- standard deviation)**	**P-value**
TCC	Negative (n=12)	27.17 +/- 9.22	P = 0.5
	Positive (n=7)	22.95 +/- 17.19	
Normal	Negative (n=26)	2.74 +/- 2.3	P = 0.4
	Positive (n=16)	2.19 +/- 1.76	

- **Table 5-1**. Cytoplasmic survivin and correlation with Ki-67 score in TCC tissues and normal urinary bladder.

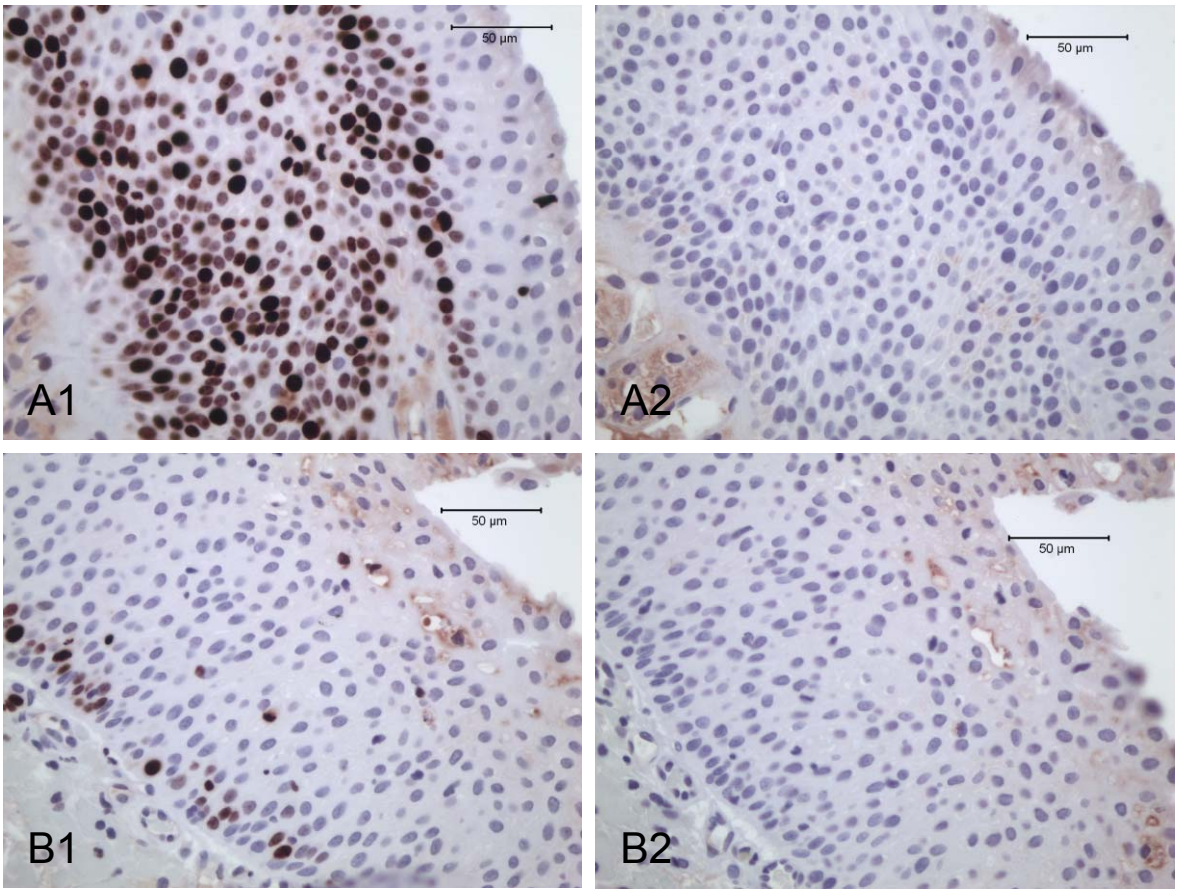
Sample	**Nuclear survivin**	**Ki-67 score (%, average +/- standard deviation)**	**P-value**
TCC	Negative (n=8)	17.99 +/- 8.48	P = 0.02
	Positive (n=11)	31.17 +/- 12.15	
Cystitis	Negative (n=11)	7.06 +/- 10.94	P < 0.001
	Positive (n=12)	44.63 +/- 16.29	

- **Table 5-2**. Nuclear survivin and correlation with Ki-67 score in TCC and cystitis

tissues.



- **Figure 5-1**. Ki-67 immunoreactivity in tumor samples positive (A1) and negative (B1) for nuclear survivin (400 X). Negative controls for each sample are adjacent to the corresponding image (A2 and B2)



- **Figure 5-2**. Ki-67 immunoreactivity in cystitis samples positive (A1) and negative (B1) for nuclear survivin (400 X). Negative controls for each sample are adjacent to corresponding image (A2 and B2)

DISCUSSION

The objective of this study was to evaluate the subcellular expression of survivin and its correlation with the cell proliferation marker Ki-67. With the results reported here, we accept our hypothesis – that nuclear survivin, but not cytoplasmic survivin, is correlated with Ki-67 immunoreactivity.

Nuclear survivin was significantly correlated with Ki-67 score in TCC and cystitis tissues (Table 5-2). Several studies in human cancer tissues have suggested a role for nuclear survivin in malignancy. Nuclear survivin has been found in human hepatocellular carcinomas and urinary bladder TCC, but their normal tissue counterparts did not have nuclear survivin immunoreactivity.[74,110] Nuclear survivin is associated with an unfavorable prognosis in ovarian carcinoma,[119] esophageal squamous cell carcinoma,[50] mantle cell lymphoma,[118] non-small cell lung cancer[120] and urinary bladder TCC.[57] In contrast, cytoplasmic survivin had no prognostic significance.[50,57,120]

Cytoplasmic survivin showed no significant correlation with cell proliferation in both TCC and normal bladder mucosa (Table 5-1). The lack of correlation between cytoplasmic survivin and cell proliferation is not surprising, given that survivin is thought to promote cell proliferation in the nucleus and control cell survival (but not cell proliferation) in the cytoplasm.[77] Studies in hepatic and urinary bladder tissue in humans also suggest that cytoplasmic survivin may have a role in normal tissue maintenance.[74,110]

It has been suggested that survivin, although classified as an inhibitor of apoptosis, has a more predominant role as a mitotic regulator.[14] This is supported by studies in human hepatocellular carcinoma, where cell proliferation index correlated with survivin expression and apoptotic index did not.[111,121,122] In addition, nuclear survivin is associated with increased cell proliferation in human non-small cell lung carcinoma,[69] hepatocellular carcinoma,[103] ovarian tumors,[123] esophageal squamous cell carcinoma,[50] and urinary bladder TCC.[57] Since survivin expression is greatest in the G_2/M phase of the cell cycle,[68] it has been proposed that nuclear survivin regulates the cell cycle, whereas cytoplasmic survivin regulates apoptosis.[103] The results of the current study support this

theory, given that nuclear survivin was correlated with Ki-67 expression, but cytoplasmic survivin was not. The results of this study and human tissue studies suggest that perhaps nuclear survivin may be a suitable target for therapy for TCC.

Splice variants of survivin with different apoptotic properties have been described in human tissues.[7,124] Survivin-Δex3 is found in the nucleus, has more anti-apoptotic properties than survivin-2B, which is found primarily in the cytoplasm.[7,10] In human hepatoma cell lines, nuclear accumulation of survivin-Δex3 was found in cells positive for Ki-67, but not in cells negative for Ki-67.[10] Although the splice variants in canine tissue have not been described, the polyclonal antibody used in this study should bind to all isoforms of the survivin protein. Therefore, while we can theorize that survivin- Δex3 may be the variant correlated with the higher Ki-67 index, further evaluation is necessary in canine tissues to determine the splice variant roles in the dog.

The presence of nuclear survivin and its correlation with cell proliferation index in cystitis tissues may demonstrate a link between hyperplasia and malignant transformation. Nuclear survivin is demonstrated in pre-neoplastic oral and cervical lesions in human tissue,[89,90] however, correlation with cell proliferation was not evaluated in those studies. The localization of survivin in hyperplastic human urinary bladder tissues is unknown. Given the link between cystitis and urinary bladder cancer in humans and similarities between human and canine TCC,[45] it is possible that cystitis may lead to TCC in dogs.

Interestingly, Ki-67 and nuclear survivin immunoreactivities were primarily in the basilar epithelium of cystitis tissues (Figure 5-2B), whereas with TCC tissue, the distribution was heterogenous (Figure 5-1). Given that basilar epithelium has a high

regenerative capacity, it is not surprising that Ki-67 expression was predominantly basilar. The nuclear survivin localization in the basilar epithelium is further evidence to support a role for nuclear survivin in cell proliferation.

The results of this study help define a role of nuclear survivin in canine urinary bladder tissues. We conclude that nuclear survivin, but not cytoplasmic survivin, is correlated with cell proliferation index. Because of the association with cell proliferation and prognosis in other tumors, it is probable that nuclear survivin may serve as an important prognostic tool in canine urinary bladder TCC. Prospective studies are warranted to determine the prognostic potential of nuclear survivin. In addition, because of nuclear survivin and cell proliferation, it is possible that nuclear survivin may serve as a therapeutic target for canine TCC. Lastly, the presence of nuclear survivin in cystitis may support a role of inflammatory hyperplasia in malignant transformation. Further investigation is necessary to define the role of nuclear survivin in a clinical setting.

CHAPTER 6

Conclusions and Future Directions

CONCLUSIONS

The objectives of this thesis were aimed to test the hypothesis that survivin is expressed in canine urinary bladder TCC, and therefore, survivin may participate in tumor development and growth. In the first part of this thesis, we showed that while survivin protein and mRNA is present in canine TCC and normal bladder tissue, the expression patterns were different; nuclear survivin was specific to TCC and not normal bladder. In the second chapter, we evaluated survivin in cystitis tissues and showed that nuclear survivin and survivin mRNA are present in inflammatory hyperplastic urinary bladder; therefore, survivin is not an optimal diagnostic or differentiating marker for TCC. However, this lead to investigation of survivin's role in cell proliferation in canine bladder tissue. We showed that survivin mRNA levels are not different between the small number of TCC, cystitis and normal bladder tissues evaluated in Chapter 4. Therefore, perhaps survivin mRNA expression does not correspond with survivin protein expression or function in canine tissues. The final part of the study addressed the role of survivin protein and showed that nuclear survivin, but not cytoplasmic survivin, correlates with cell proliferation index (assessed by Ki-67).

Based on the results of the body of work presented in this thesis, we cannot fully accept our hypothesis (that survivin is overexpressed in canine TCC). However, the work presented here helps us conclude that nuclear survivin is in canine TCC and cystitis tissues, but not normal tissue. In addition, the presence of survivin in canine TCC demonstrates further evidence that this tumor in the dog serves as a good model for the human disease. Because survivin is not specific to TCC compared to cystitis and normal tissues, it is unlikely to be a good diagnostic test for dogs in a clinical setting. Lastly, we

demonstrate that nuclear survivin is associated with higher Ki-67 expression; therefore, survivin may have a role in cell proliferation and perhaps bladder tumor growth and progression.

FUTURE DIRECTIONS

Because our results show that nuclear survivin is correlated with a higher cell proliferation index, future studies should evaluate the clinical significance of nuclear survivin. In human tumors, nuclear survivin is a negative prognostic indicator in a variety of tumors.[50,57,118-120] Prospective studies in urinary bladder cancer patients can help determine if nuclear survivin may be a useful prognostic tool, and if so, it may help clinicians alter their therapy for patients that have positivity for nuclear survivin.

That nuclear survivin is present in cystitis and TCC tissues may indicate that nuclear survivin can be a suitable target for therapy for both tumors and chronic cystitis. Because survivin-Δex3 localizes to the nucleus due to a nuclear localization signal in its carboxyl terminus,[125] a potential method to target nuclear survivin would involve RNA interference, which inhibits translation of mRNA into survivin protein. Such methods have been employed in targeting human bladder cancer cell lines *in vitro*.[42-44,58] Sequences of the interfering RNA can be constructed such that it can target the nuclear isoform. Although the splice variants have not been described in canine survivin, RNA interference may be a useful therapy in the future to complement standard therapy.

Other evidence has suggested that targeting survivin specifically in tumors or specific isoforms may not be necessary to prevent normal tissue toxicity.[126] General methods to utilize survivin as a therapeutic target include cytolytic T cells pulsed against

survivin peptides, ribozymes to degrade survivin mRNA, or inhibition of survivin phosphorylation with cyclin-dependent kinase inhibitors.[127] Given the high recurrence rate with canine TCC and an overall poor prognosis, survivin is a novel therapeutic target that shows potential to improve our treatment of this frustrating disease.

The subcellular localization of survivin in canine tissues, presence of splice variants in human tissues and the potential for targeting nuclear survivin demonstrates that further investigation of splice variants in canine tissues is essential to improve our understanding of this protein. Human survivin splice variants have been described in TCC[79] and different splice variants can correlate with prognosis in soft-tissue sarcomas and leukemia.[128,129] Should we find that splice variants exist in canine tissue, we may be able to use these for prognosis or therapy.

The work presented in this thesis contributes to our preliminary understanding of survivin's role in urinary bladder tissues. No other studies to date have evaluated survivin in canine urinary bladder tissues, therefore, this report opens a door for future research that may drastically improve our understanding of bladder carcinogenesis or progression. Most importantly, however, we hope that this research will contribute to our understanding of bladder cancer such that we can alter our standard therapy for patients with TCC to improve their overall survival.

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