SURVIVIN EXPRESSION IN CANINE AND FELINE CANCER

by

MELANIE E. JOHNSON

(Under the Direction of ELIZABETH W. HOWERTH)

ABSTRACT

Survivin is an inhibitor of apoptosis protein that is expressed during embryonic development and several human malignancies but is absent in most adult tissues. Its expression is often associated with a more malignant phenotype and decreased response to chemotherapeutics. Analysis of the expression profile of transcripts in various cancers identified survivin as one of the top four transcripts upregulated in malignancy. Four human survivin isoforms have been identified that differ in their ability to inhibit apoptosis. Thus, these isoforms may represent a regulatory balance between apoptosis and inhibition of apoptosis. Intense research is currently under way in human medicine to investigate the role of survivin in cancer. However, nothing is known in regards to survivin expression in veterinary species. In order to examine survivin expression in canine and feline tissues, immunohistochemistry (IHC) and RT-PCR were performed on dog and cat embryos, normal adult tissues, inflammatory and hyperplastic tissues, and a variety of neoplastic tissues. Survivin was expressed in fetal tissues, some hyperplastic, inflammatory, and neoplastic tissues, but was absent in most normal, adult tissues. To determine its significance as a prognostic parameter in canine cancer, survivin expression was determined in neoplastic lymph nodes from dogs undergoing chemotherapy for lymphosarcoma. Survivin expression was shown to be a negative predictor of survival in canine lymphosarcoma. Canine survivin isoforms have yet to be identified. To explore the possibility of differential expression of canine survivin isoforms, survivin subcellular localization, and proliferative and apoptotic markers were compared between canine mammary adenomas and carcinomas that were all positive for survivin by IHC. Differences in nuclear survivin expression, Ki-67, and caspase-3 between the tumor types were not observed, thus potential expression of different canine survivin isoforms was not apparent from our data. In conclusion, results of this work suggest that survivin expression in veterinary species needs to be further investigated, as this protein may prove to be useful in the diagnosis and prognosis of animal cancers. Furthermore, survivin could potentially become a therapeutic target in veterinary oncology in the future.

INDEX WORDS: apoptosis inhibitor, cancer, canine lymphosarcoma, canine mammary tumors, caspase-3, cell division, Ki-67, prognostic indicator, review, survivin

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MELANIE E. JOHNSON

BSA, 1989, The University of Georgia

DVM, 1994, The University of Georgia

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MELANIE E. JOHNSON

Major Professor: Elizabeth W. Howerth

Committee: Barry G. Harmon
Nicole Northrup
Elizabeth Uhl
Karen Cornell

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2005

DEDICATION

In memory of Jerry, a constant source of joy and inspiration.

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CHAPTER 1

INTRODUCTION

Survivin is a bifunctional protein, belonging to the inhibitor of apoptosis gene family, which is capable of suppressing apoptosis and is required for proper cell division.[11] In humans, it is widely distributed in early embryonal development, yet there is limited expression in terminally differentiated tissues.[1] It is overexpressed, however, in a wide variety of human cancers, suggesting reactivation of the survivin gene in neoplastic processes.[7] In addition, survivin expression in human cancers is often associated with decreased survival times and reduced response to chemotherapeutic drugs.[7] Its limited expression in normal tissues and its overexpression in a variety of neoplasms make survivin a potential therapeutic target in the treatment of cancer.

Survivin is unique among the inhibitor of apoptosis family of proteins in that it is cell cycle-regulated, with peak expression occurring during the G2/M phase of mitosis.[8] During cell division, it localizes both to various components of the mitotic apparatus and to the kinetochore, in association with Aurora B kinase and inner centromere protein (INCENP).[4] Survivin is essential for proper cell division, as revealed by survivin knockout mice that die within 5 days of embryonic development.[11] The observed disruption of the microtubule network, absence of mitotic spindles, and failure of cytokinesis with resultant multinucleation in these survivin -/- mice clearly establishes the essential role of survivin during cell division.

Although the exact mechanism behind the ability of survivin to inhibit apoptosis is still unclear, experimental evidence clearly supports the notion that survivin suppresses apoptosis in addition to being required for cell division.[2] The conflict surrounding its function in cell division, versus its role in inhibition of apoptosis, may lie in the fact that survivin exists in two distinct subcellular pools,[5] at least in humans, as the result of four alternatively spliced transcripts.[3,9] Several observations substantiate survivin's role as an inhibitor of apoptosis. Firstly, survivin overexpression has been linked with inhibition of cell death initiated via intrinsic and extrinsic apoptotic pathways.[7] In addition, transgenic expression of survivin results in inhibition of apoptosis *in vivo*, whereas heterozygous survivin +/- phenotypes exhibit increased susceptibility to apoptosis.[6] Finally, molecular antagonists of survivin result in caspase-dependent apoptosis.[10]

While intensive survivin research is currently ongoing in human medicine, its expression in companion animals has not been investigated. The first objective of this study was to determine survivin expression, by immunohistochemistry and RT-PCR, in canine and feline normal and abnormal tissues. The tissues examined included: embryos at varying stages of development, normal adult tissues, and a wide variety of inflammatory or hyperplastic tissues and their neoplastic counterparts. We hypothesized that survivin would be expressed during embryonal development and some neoplastic conditions, but would not be expressed in normal, terminally differentiated tissues, inflammatory tissues, or hyperplastic tissues. The second objective of this study was to determine survivin expression in neoplastic lymph nodes of dogs undergoing chemotherapy for lymphosarcoma. We hypothesized that some dog lymphosarcomas would be survivin-positive whereas others would be survivin-negative, and that survivin expression would be a negative predictor of survival. The final objective of this study was to establish whether there were differences in subcellular localization of survivin or proliferative and apoptotic indices between survivin-positive canine mammary adenomas versus carcinomas. We hypothesized that the carcinomas would have increased nuclear survivin expression, and increased proliferation/and or decreased apoptosis and that this could possibly be due to the differential expression of canine survivin isoforms.

Knowledge of the distribution of survivin in canine and feline tissues, and its role in neoplastic processes, is important. This information will help determine if survivin can be used as a prognostic parameter in veterinary medicine and whether developing therapies to target survivin would be of benefit in canine and feline cancer patients.

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CHAPTER 2

LITERATURE REVIEW

SURVIVIN: A BIFUNCTIONAL INHIBITOR OF APOPTOSIS PROTEIN[1]

[1] Johnson, M.E. and E.W. Howerth, *Vet Pathol* **41**:599-607, 2004.
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ABSTRACT: Survivin is a recently discovered protein belonging to the inhibitor of apoptosis (IAP) gene family. IAP molecules are characterized by both the presence of a zinc-binding fold termed the baculoviral IAP repeat (BIR) and the ability to suppress apoptosis. In addition to inhibiting apoptosis, survivin is essential for proper cell division. Survivin is expressed during embryonal development but is absent in most normal, terminally differentiated tissues. Survivin is also upregulated in a variety of human cancers, and its expression in tumors is associated with a more aggressive phenotype, shorter survival times, and a decreased response to chemotherapy. The exact mechanism behind the ability of survivin to inhibit apoptosis is still unclear. Furthermore, it is not known why this protein is upregulated in cancer. The purpose of this paper is to provide an overview of the current knowledge of survivin, including its role in cell division and its expression in normal and neoplastic tissues. Although much of the current research in this field has its focus in human medicine, this area also has potential significance for veterinary species.

Key words*:* survivin, inhibitor of apoptosis, cell division, cancer, review

INTRODUCTION

The inhibitor of apoptosis (IAP) proteins are a family of highly conserved cell death inhibitors that have been found in yeast, invertebrates, and vertebrates. IAPs were first discovered in baculoviruses, where they were shown to be involved in suppressing host cell death response to viral infection.[11,18] Survivin, a recently discovered IAP, is a bifunctional protein that regulates cell division and suppresses apoptosis.[5] Survivin is unique among the IAP proteins because it exhibits cell-cycle-regulated expression that peaks at mitosis.[41] Survivin is abundantly expressed in fetal tissues [1,5] yet undetectable in most normal, terminally differentiated adult tissues. [6] Survivin is also overexpressed in a variety of human neoplasms, suggesting that reactivation of the survivin gene frequently occurs in cancers. [6] Currently, survivin protein expression is being used as a prognostic factor in several human neoplasms.[6] High survivin expression by neoplasms correlates with more aggressive behavior, decreased response to chemotherapeutic agents, and shortened survival times, as compared to cancers that are survivin-negative. As its expression is among the most tumor-specific of all gene products, there is considerable biomedical interest in this protein. Manipulation of survivin regulation and expression may also lead to the development of new immunotherapy and gene therapy strategies for the treatment of cancer. The purpose of this review is to discuss the role of survivin in normal and neoplastic tissues, and in targeting survivin as a potential therapy in cancer.

Molecular Organization and Structure of Survivin

IAP molecules are characterized by the presence of one or more copies of an approximately 70 amino acid zinc-binding fold, termed the baculoviral IAP repeat (BIR), as well as the ability to inhibit apoptosis.[19] Survivin is the smallest member of the IAP family of proteins and has only a single N-terminal BIR domain, a long C-terminal alpha-helix coiled region, and a dimeric arrangement.[76] The BIR domain is thought to be critical for anti-apoptotic function while the coil-coiled domain probably interacts with tubulin structures. The survivin molecule contains three separate and chemically distinct surfaces, including acidic and basic regions on the BIR domain and a hydrophobic helical surface on alpha 6. This arrangement is consistent with functionally relevant protein-protein interaction surfaces.[76]

The murine survivin gene, located on chromosome 11E2, contains four exons which gives rise to three separate gene products through alternative splicing.[16] The longest open reading frame, containing all four exons, produces an 140 amino acid protein that is similar to human full-length survivin. A second isoform, lacking sequence derived from exon 2, gives rise to a 40 amino acid protein that lacks the BIR domain and the C-terminal coil-coiled domain. The third gene product results from the retention of intron 3, subsequently acquiring a new in-frame stop codon. The 121 amino acid protein produced contains the BIR domain but lacks the coil-coiled domain.

The human survivin gene, spanning 14.7 kb on telomeric position of chromosome 17, produces a 16.5 kDa protein.[7] Similar to murine survivin, the human survivin gene, as the result of three alternatively spliced transcripts, can give rise to three different isoforms of the protein[44] (Figure 2.1). These isoforms include full-length survivin, survivin-2B, and survivin-ΔEx-3. The full-length survivin gene consists of a three-intron, four-exon structure. The survivin-2B transcript results from the retention of a portion of intron 2, whereas the survivin-ΔEx-3 transcript results from the removal of exon 3. The sequence alterations produced from the splice variants results in marked changes in the corresponding protein structure and, subsequently, differences in their ability to inhibit apoptosis. Insertion of exon 2B in survivin-2B interrupts the essential BIR domain which leads to a marked decrease in the apoptotic activity of this isoform. The removal of exon 3 in survivin-ΔEx-3 likewise interrupts the BIR domain. However, this isoform of the protein retains its ability to suppress apoptosis. In addition, loss of this exon results in a frame shift in exon 4 generating a novel COOH-terminal. There is also different subcellular localization of survivin and its splice variants. Survivin and survivin-2B are predominantly cytoplasmic whereas survivin-ΔEx-3 is primarily nuclear.[43] These different isoforms of survivin and their varied locations in the cell may represent a regulatory balance between apoptosis and inhibition of apoptosis.[43]

The Role of Survivin in Cell Division

Survivin is essential for proper execution of mitosis and cell division.[59] Its specific expression in G2/M is transcriptionally controlled, [40] as is typical for mitotic genes.[81] During mitosis, survivin binds to the microtubules of the mitotic spindle through its carboxy-terminal alpha helices. Interference of survivin-microtubule interactions, by means of antisense-mediated reduction in the expression of survivin, results in failure of the anti-apoptotic function of survivin and an increase in caspase-3 activity with subsequent apoptosis.[6] Disruption of survivin function has likewise been linked with cell division defects exemplified by supernumerary centrosomes, formation of multipolar mitotic spindles, and failure of cytokinesis, with cells becoming polyploid and multinucleated.[14,39] Additional evidence for the crucial role of survivin in mitosis has been demonstrated in knock-out mice. Homozygous disruption of the survivin gene resulted in embryonic death at 4 to 5 days, with the null embryos revealing failed cytokinesis and disrupted microtubule formation.[75]

In addition to its role in maintaining a normal bipolar mitotic apparatus, survivin is also a kinetochore-associated chromosomal passenger protein[67] in concert with Aurora B kinase and inner centromere protein (INCENP).[12] Furthermore, Aurora B kinase activity is enhanced by survivin binding.[12] The mechanism behind survivin's ability to regulate Aurora B kinase activity and the exact interaction between these three chromosomal passenger proteins is speculative at this time. The discrepancy in regards to survivin's cytoplasmic versus nucleoplasmic location lies in the fact that survivin exists in two immunochemically distinct subcellular pools, possibly as a result of alternatively spliced transcripts.[21]

The Role of Survivin in Apoptosis

The IAP family of proteins is defined by the presence of at least one BIR domain and the ability to inhibit apoptosis. Using these criteria, humans have eight IAP family members: NAIP, cIAP1, cIAP2, XIAP, Ts-XIAP, ML-IAP, apollon, and survivin. Several IAPs in humans and *Drosophila* have been shown to directly bind and inhibit caspases via their BIR domains.[19,48] For example, XIAP binding and inhibition of caspase-9 is through its third BIR domain (BIR3), whereas caspase-3 and caspase-7 are suppressed via a region within the molecule between BIR1 and BIR2.[71] The precise mechanism by which survivin suppresses apoptosis, however, is still incompletely understood. Several mechanisms are theorized (Figure 2.2). Direct suppression of caspase-3 by survivin has been speculated by some investigators, yet survivin lacks structural components present in other IAPs that allow for their direct binding to caspase-3.[60] There is also speculation that survivin binds to caspase-9[52], but there are likewise problems with this theory. Phosphorylation of survivin on the threonine at position 34 (Thr^{34}) is critical for a functional survivin molecule. However, phosphorylation of survivin fails to explain why this would promote interaction with caspase-9.[66] Another possibility is that survivin requires the cofactor hepatitis B X-interacting protein (HBXIP) to bind pro-caspase -9, thus preventing apoptosis via the intrinsic pathway.[45] Finally, survivin may indirectly inhibit caspases via intermediate proteins. Survivin binds to Smac/DIABLO, which is a proapoptotic protein that binds IAPs and thus prevents them from inhibiting caspases.[20,77] During interphase, survivin co-localizes with Smac/DIABLO in the cytosol.[70] A point mutation at amino acid Asp-71 in survivin results in the failure of survivin to complex with Smac/DIABLO. Consequently, this was shown to abolish survivin's ability to protect against Taxol-induced apoptosis in HeLa cells.[70]

The role of survivin as an inhibitor of apotosis versus its function in cell division has been explored *in vivo* through the use of transgenic (K14-survivin) mice which constitutively express survivin in keratinocytes.[26] Overexpression of survivin in K14-survivin mice does not affect normal skin differentiation, nor is there an increase in basal keratinocyte proliferative rates between K14-survivin mice and controls upon induction of keratinocyte proliferation. However, K14-survivin mice had a 60% reduction in apoptosis compared to the control group following UVB exposure. These results demonstrate that constitutive expression of survivin inhibits keratinocyte apoptosis, yet cell proliferation remains unaffected. This implies that survivin expression in cancer does not simply reflect an increase in the number of mitotic tumor cells. In vitro, K14-survivin keratinocytes also have increased resistance to UVB-induced apoptosis. The transgenic cells, however, are more susceptible to Fas-mediated (extrinsic) apoptosis indicating that the cytoprotection offered by survivin is ineffective against the Fas-induced apoptotic pathway. K14-survivin mice crossed with p53-deficient mice, creating K14-survivin mice lacking one p53 allele (p53/-), are equally resistant to UVB-induced apoptosis as p53-/-

keratinocytes. Thus, it appears that survivin expression can substitute for the loss of one p53 allele in suppressing apoptosis.

Regulation of Survivin

Phosphorylation at Thr34 of survivin plays an essential role in its regulation. The cyclin-dependent kinase, p34cdc2-cyclinB1, is the only known kinase able to phosphorylate survivin and colocalizes with it on the mitotic apparatus.[51] Elevations in p34cdc2 kinase, induced by spindle checkpoint activation, result in increased survivin expression.[54] This increase in survivin does not result from a substantial change in RNA levels or promoter activity, suggesting post-transcriptional stability of survivin by p34cdc2 kinase. Indeed, nonphosphorylatable survivin, produced by a mutation of Thr34$\rightarrow$ Ala (T34A), is more rapidly cleared than wild-type survivin[54] by the ubiquitin-proteosome pathway.[79] The Thr34 phosphorylation site appears to be in a position to stabilize anti-apoptotic protein-protein interactions through the BIR domain of survivin.[76] Failure to phosphorylate Thr34, by the mutation T34A, leads to the dissociation of a survivin-caspase-9 complex and subsequent caspase-9 dependent apoptosis during cell division.[52] Apoptosis does not result from the inability to progress through the cell cycle, as cells expressing survivin T34A do not exhibit G_2/M arrest. Instead, apoptosis occurs upon entry and progression through mitosis. These findings indicate that phosphorylation of Thr34 is critical for survivin in its role as a suppressor of apoptosis.

The mechanisms behind the differential expression of survivin in neoplastic tissues versus normal, terminally differentiated adult tissues are largely unknown at this time. There is mounting evidence that a multitude of tumorigenic pathways are potentially involved in the upregulation of survivin in cancer (Figure 2.3). Wild-type p53 transcriptionally represses survivin,[29,46,79] and overexpression of survivin is able to counteract p53-dependent apoptosis induced by adriamycin[46] or ultraviolet light.[29] The means by which p53 inhibits survivin is still controversial. Whereas there is evidence which supports the direct binding of p53 to the survivin promoter[29], other data indicates that alteration of the chromatin configuration within the survivin promoter, with subsequent decreased access to the promoter, is responsible for the suppression of survivin by p53.[46] Inhibition of signal transducer and activator of transcription-3 (STAT3) signaling pathways has also been shown to result in the reduction of survivin expression in primary effusion lymphoma[10] (PEL) and astrocytomas[38], with subsequent induction of apoptosis. In addition, forced expression of survivin prevents apoptosis induced by STAT3 inhibition in PEL cells.[10] Thus constitutive STAT3 activation, which is frequently observed in cancer, may lead to survivin upregulation. Finally, survivin upregulation by c-H-Ras oncoprotein has been demonstrated, independent of cell cycle progression or cellular proliferation.[69] This supports the impression that elevated survivin levels is not simply due to an increased fraction of G2/M cells secondary to Ras-mediated cell cycle alteration.[69] Furthermore, inhibitors of phosphatidylinositol 3-kinase (P13-K) and MEK1 result in a reduction of survivin, suggesting that these intracellular Ras-pathways are involved in the regulation of survivin expression.[69]

The regulation of survivin is also being investigated in non-neoplastic cell lines (Figure 2.3). Survivin mRNA and protein expression increase in CD34+ bone marrow cells following incubation with a combination of the hematopoietic growth factors thrombopoietin, Flt3 ligand, and stem cell factor in a cell-cycle-independent manner.[23,24] Subsequent removal of these growth factors results in a reduction of survivin mRNA and protein levels, cell cycle arrest, and an increase in the number of apoptotic cells.[24] In addition, inhibition of PI3-K/AKT and mitogen-activated protein kinase ($MAPK^{p42/22}$) pathways result in reduced survivin expression in growth factor-stimulated CD34+ cells prior to arresting cell cycle progression.[23] Hence, it is likely that these pathways are involved in cytokine-regulated survivin expression of CD34+ cells and that the regulation by these growth factors is not simply a reflection of cell cycle progression.

Survivin is also induced in quiescent endothelial cells (EC) upon mitogenic stimulation with vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF).[53] Antisense targeting of survivin results in the loss of cytoprotection afforded by VEGF against ceramide or TNFα-induced apoptosis, suggesting that VEGF protects EC against apoptosis during angiogenesis by upregulating survivin.[46] VEGF-mediated cytoprotection of EC from the chemotherapeutic agents taxol and VBL, drugs which interfere with microtubule dynamics, result from the upregulation of survivin via activation of the PI3-K/PKB pathway.[74] Furthermore, induction of survivin by VEGF appears to protect cellular integrity against taxol and VBL by maintaining the microtubule integrity. Angiopoietin-1 has also been shown to upregulate survivin in EC, via a P13-K/AKT pathway, subsequently protecting EC from apoptosis-inducing stimuli.[56] Interference by a dominant negative survivin, however, results in the loss of angiopoitein-1 to protect EC from undergoing apoptosis. Thus targeting survivin, in addition to inhibiting tumor cell growth, may also prove to be beneficial in inducing apoptosis in proliferating EC of the tumor vasculature. Although upregulation of survivin in EC may be detrimental to the host by supporting tumor growth, survivin plays a crucial protective role in ischemic brain injury.[17] Following middle cerebral artery occlusion in mice, survivin can be detected in microvessels of the infarcted areas, apparently both by VEGF-dependent and independent mechanisms. In addition, heterozygous survivin deficient mice (survivin +/-) have significantly decreased vessel density in the infarcted areas as compared to the survivin +/+

mice. Thus upregulating survivin in EC may prove to be beneficial in promoting angiogenesis in ischemic conditions.

Expression of Survivin in Normal Tissues

Survivin is highly expressed during embryonic development and may be important in tissue homeostasis and differentiation. The gene is quiescent in most terminally differentiated tissues. Among the IAP family members, survivin exhibits the most restricted expression in adult tissues, but has been identified in several apoptosis-regulated fetal tissues.[1] In early murine embryogenesis, widespread distribution of survivin is observed, whereas, by late gestation, the protein is present in only limited locations. Normal adult human tissues that express survivin include thymus[6], CD34+ bone marrow stem cells[13], and basal colonic epithelium.[25] Survivin is also expressed in the gastric mucosa of adult humans and rats.[15]

Of human thymocyte subsets, double-positive (CD4+/CD8+) thymocytes express the highest levels of survivin, demonstrating upregulation in this subset of T lymphocytes.[37] There is downregulation of survivin, however, in mature single positive (CD4+ or CD8+) T cells, and peripheral T cells are negative for survivin. Thus, survivin may be important in T-cell development. Survivin is also normally expressed in CD34+ hematopoietic stem cells, which raises the concern that survivin-targeted therapy for cancer cells could disturb normal hematopoiesis.[24] CD34+ cells expressed survivin in all phases of the cell cycle, whereas cancer cells specifically expressed survivin in a G2/M-specific manner. Likewise, survivin upregulation in CD34+ cells was not as dramatic as in leukemic cells, implying dysregulation of survivin in leukemia. It is possible that CD34+ cells, under the regulation of hematopoietic growth factors, require only small amounts of survivin to undergo mitosis rather than apoptosis. Survivin expression can also be detected in normal colonic tissue, hyperplastic, premalignant, and malignant lesions of the colon.[25] This indicates that survivin cannot be used as a specific marker for colon cancer. Distribution of survivin within these tissues varies, however, with survivin expression restricted to the basal colonic crypts in normal colonic mucosa. Hyperplastic polyps have uniformly intense staining of all epithelial cells, whereas adenomas and adenocarcinomas are heterogeneous in their staining pattern. In humans and rats, there is strong nuclear expression of survivin in mucosal surface epithelial cells of the stomach. In addition, human chief and parietal cells show nuclear and cytoplasmic survivin expression. Thus, survivin may play a role in gastric mucosal integrity.

Expression of Survivin in Human Cancer

One of the clinically significant features of survivin is its differential distribution in many cancers compared to its limited expression in normal terminally differentiated tissues. In human medicine, detection of survivin expression by immunohistochemical staining is becoming an important prognostic parameter in a variety of cancers including carcinomas, sarcomas, and hematologic neoplasms. High survivin expression in tumors correlates with a more aggressive and invasive clinical phenotype. Subsequently, a poorer prognosis and a decreased responsiveness to chemotherapeutic agents can be expected.[2,3,4,31,35,36,50,63,72,73,78]

Several human carcinomas have been shown to express high levels of survivin: lung[49], breast[73], colon[36], stomach[42], esophagus[35], pancreas[64], bladder[72], uterus[62], ovary[78], liver[32], and nonmelanoma skin cancer.[29] The overexpression of survivin is consistently associated with more aggressive tumor types and a poorer prognosis than tumors that are negative for survivin. For example, normal oral mucosa and skin, including adnexal structures, are negative for survivin. In contrast, one study found that 56% and 64% of oral and cutaneous squamous cell carcinomas, respectively, were strongly positive for survivin.[51] Likewise, survivin expression correlated with larger, more poorly differentiated tumors, including those that had metastasized to lymph nodes. Detection of survivin in urine may also prove to be a diagnostic marker of bladder cancer.[68] Its presence in urine has been used to diagnose bladder cancer and to differentiate neoplastic lesions from inflammatory conditions with a sensitivity of 100% and specificity of 95%.

Overexpression of survivin has also been documented in leukemias[4,33], neuroblastoma[2,31], melanoma[28], soft tissue sarcoma[34], and high-grade-non-Hodgkin's lymphoma[6]. Low-grade-non-Hodgkin's lymphomas, however, do not express survivin.[6] Similar to the poor prognosis observed with its overexpression in carcinomas, high survivin expression in sarcomas is likewise a negative prognostic parameter. In a study involving patients with diffuse large B-cell lymphoma, survivin was shown to be an independent prognostic factor for poor prognosis in this cancer.[3]

Survivin expression associated with viral-induced neoplasms has also been observed and its expression may be an early indicator of malignancy.[22] Immunohistochemical evaluation of normal and abnormal human cervical squamous epithelium, including low and high-grade squamous intraepithelial lesions (LSILs, HSILs) and squamous cell carcinomas reveals nuclear staining in the normal epithelium, LSILs, and HSILs. Staining intensity is variable, however, with the greatest intensity of staining in the tissues containing human papillomavirus (HPV). In situ hybridization of these HPV positive tissues reveals colocalization of HPV DNA and survivin, suggesting that survivin plays a role in HPV-mediated cervical dysplasia. Survivin expression is also present in juvenile-onset recurrent respiratory papillomatosis.[57] In comparison to normal laryngeal tissue that is negative for survivin, viral-induced papillomas have increased mRNA and protein expression of survivin. Likewise, the strongest survivin expression is present in papillomas that have undergone malignant transformation.

Targeting Survivin in Cancer

The observation that survivin is expressed in neoplastic tissues, yet undetectable in most normal differentiated tissues, makes it a promising therapeutic target in cancer chemotherapy. Several experiments targeting survivin expression are currently under investigation. For example, a replication-deficient adenovirus encoding a survivin Thr34→ Ala (pAd-T34A) mutant to target tumor cells of breast, cervical, prostate, lung, and colorectal cancers causes the tumor cells to undergo apoptosis without affecting proliferation of normal fibroblasts, endothelium, or smooth muscle cells.[47] The combination of pAd-T34A with the chemotherapeutic agent, taxol, results in enhanced tumor cell death. Intratumor administration of pAd-T34A in mice tumors in vivo also inhibits tumor growth and induces apoptosis. Additional evidence that dysregulation of the survivin pathway leads to apoptosis in cancer is seen in melanoma cell lines transfected with T34A, which interferes with phosphorylation of endogenous survivin. This results in spontaneous apoptosis of the melanoma cell line and enhanced in vitro cell death by the chemotherapeutic agent cisplatin.[27] Finally, down-regulation of survivin expression using an antisense oligonucleotide within a lung carcinoma cell line induces apoptosis and inhibits growth of the neoplastic cells.[55] Furthermore, a synergistic effect was noted when this cell line was treated with antisense oligonucleotide and the chemotherapeutic agent, etopside. This finding demonstrates that targeting of the survivin pathway in cancer, alone or in conjunction with chemotherapeutic agents, has potential as a novel therapeutic regimen.

Immunotherapy also appears to be a plausible approach to treating survivin-positive tumors. Autoantibodies against survivin have been detected in humans with lung and colorectal cancer, making this a potential diagnostic tool in certain neoplasms.[61] In some of the lung cancer patients, anti-survivin reactivity was found prior to clinical disease. Thus, antibodies against survivin may prove to be an early predictive marker of cancer. Spontaneous cytotoxic T lymphocyte response to survivin, in a MHC class I-restricted manner, has been detected in patients with chronic lymphocytic leukemia, melanoma, and breast cancer.[8,9] In addition, in vitro cytolytic T cell induction against a survivin epitope results in cytolytic activity against a wide variety of human tumors, including renal cell carcinomas, breast cancer, colon cancer, multiple myeloma, and leukemias.[65] Hence, survivin appears to be a universal tumor antigen and immunotherapy a conceivable approach to treating survivin-positive tumors.

CONCLUSIONS

Survivin is a recently discovered inhibitor of apoptosis that is expressed in embryonal development and a variety of neoplastic conditions, with minimal expression observed in normal, terminally differentiated adult tissues. Survivin-positive neoplasms are often more aggressive and less responsive to chemotherapeutic agents, making survivin an independent negative prognostic parameter in a variety of human cancers. Survivin is also a potential therapeutic target for cancer chemotherapy, however, survivin is also necessary for proper cell division. Therefore, a thorough understanding of its role in normal tissues must be determined prior to targeting survivin pathways in cancer. Mechanisms by which survivin suppresses apoptosis are still under considerable debate and it is not known how survivin is upregulated in neoplastic cells.

While survivin research is under intense investigation in human medicine, little is known regarding its expression in domestic animals. Information on survivin expression in both normal and abnormal tissues in domestic animals is needed and will facilitate subsequent investigations into its potential utility in the diagnosis, prognosis, and therapy in animal neoplasms.

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FIGURE LEGENDS

Figure 2.1. Survivin Isoforms. Survivin exists in three isoform alternatively spliced transcripts. Survivin-ΔEx-3 is primarily nuclear in location, whereas full length survivin and survivin-2B are predominantly within the cytoplasm. These immunochemically distinct pools may explain the discrepancy in regards to survivin's ability to play the roles as both a mitotic spindle-associated protein and a kinetochore-associated chromosomal passenger protein.

Figure 2.2. Potential mechanisms by which survivin inhibit apoptosis. Survivin may inhibit apoptosis by binding caspase 9 or it may block SMAC, thus preventing this proapoptotic protein from blocking inhibitor of apoptosis proteins.

Figure 2.3. Regulation of survivin. Multiple tumorigenic pathways are potentially involved in the overexpression of survivin in cancer. Wild-type p53 transcriptionally represses survivin, whereas mutant p53 may contribute to upregulation of survivin. Constitutive STAT3 activation and upregulation by c-H-Ras oncoprotein may also result in survivin overexpression. In nontransformed cell lines, upregulation of survivin in endothelial cells is observed following stimulation with VEGF, bFGF, and angiopoietin-1. In CD34+ hematopoietic stem cells, there is upregulation of survivin following incubation with a combination of thrombopoietin, Flt3 ligand, and stem cell factor.

FIGURE 2.1

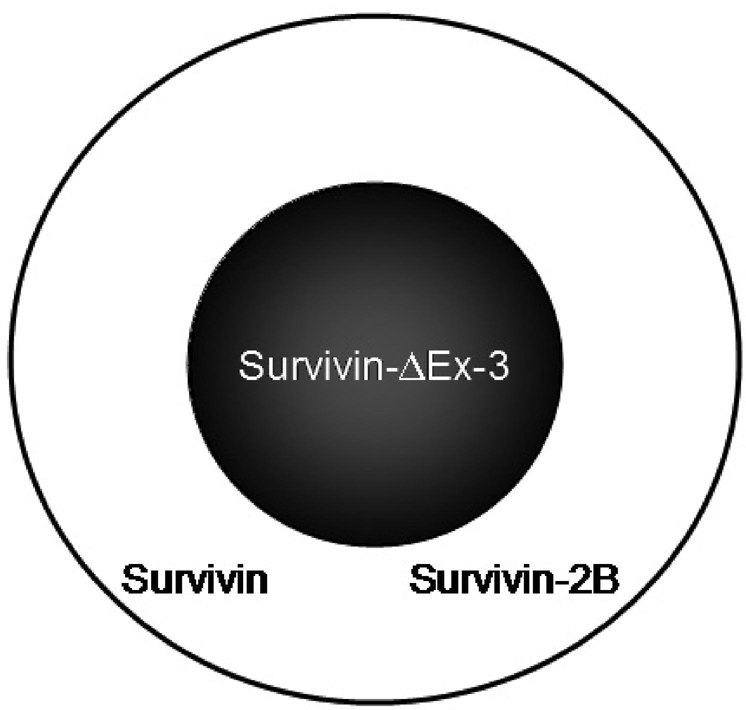
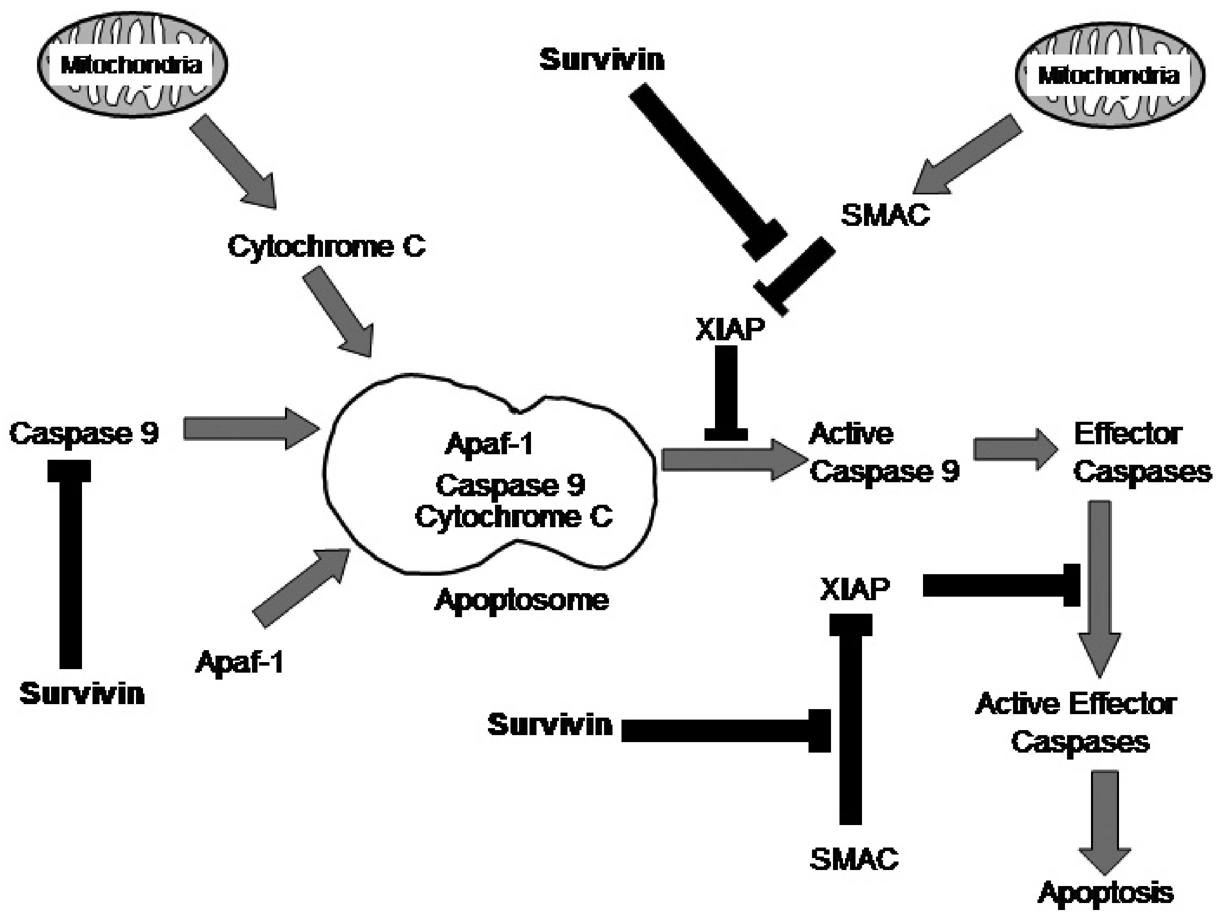


FIGURE 2.2


Mitochondria
Survivin
Mitochondria
SMAC
Cytochrome C
XIAP
Caspase 9
Apaf-1
Caspase 9
Cytochrome C
Active
Caspase 9
Effector
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XIAP
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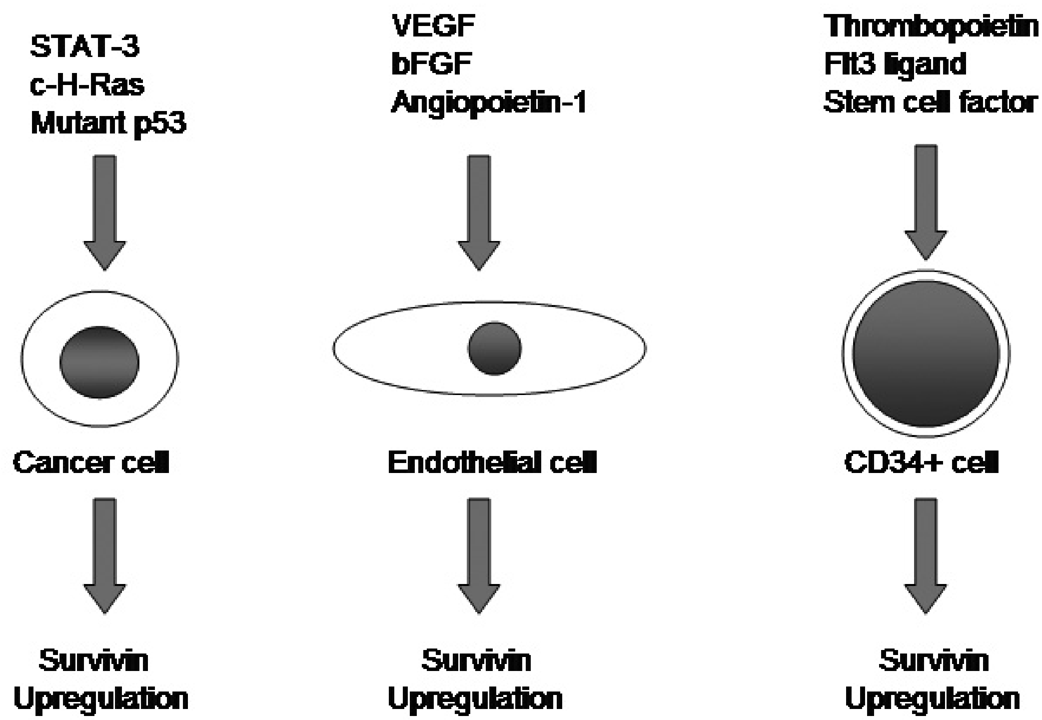
Survivin
Active Effector
Caspases
Survivin
SMAC
Apoptosis

FIGURE 2.3


STAT-3
c-H-Ras
Mutant p53
VEGF
bFGF
Angiopoietin-1
Thrombopoietin
Flt3 ligand
Stem cell factor
Cancer cell
Endothelial cell
CD34+ cell
Survivin
Upregulation
Survivin
Upregulation
Survivin
Upregulation

CHAPTER 3

A SURVEY OF SURVIVIN EXPRESSION IN CANINE AND FELINE NORMAL, HYPERPLASTIC, AND NEOPLASTIC TISSUES[1]

[1] Johnson, M.E.and E.W. Howerth. To be submitted to *Veterinary Pathology*.

ABSTRACT: Survivin is a bifunctional protein required for cell division and capable of suppressing apoptosis. It is expressed in embryonal development and in a variety of human cancer cells, but is absent in most human adult tissues, suggesting reactivation of the survivin gene in neoplastic processes. There is no information describing survivin expression in canine or feline normal, hyperplastic, or neoplastic tissues. In this study, immunohistochemical detection of survivin was performed in the following canine and feline tissues: embryonal tissues at different stages of development, normal adult tissues, and a variety of hyperplastic tissues and their neoplastic counterparts. Several embryonal tissues expressed survivin in early gestation, with survivin virtually absent by late gestation. Normal adult tissues minimally expressed survivin. Many hyperplastic and neoplastic tissues expressed survivin, but within a particular tumor type, some tumors were survivin-positive whereas others were survivin-negative. Survivin is also present in reactive fibroblasts, endothelial cells, and lymphocytes, which suggests that survivin is upregulated during inflammation and wound healing.

RT-PCR was used to detect mRNA in canine and feline fetal tissues, normal tissues, and neoplastic tissues. Protein expression, detected by immunohistochemistry, usually positively correlated with mRNA expression in the samples examined. Feline fetal survivin mRNA was partially sequenced and had 99% homology to a published feline survivin sequence and 94% homology to the published canine sequences. Canine survivin mRNA from DH82 cells had 100% homology to published dog sequences.

INTRODUCTION

Survivin is a recently discovered protein belonging to the inhibitor of apoptosis (IAP) gene family. It is the smallest member of the IAP family of proteins and is unique among these proteins in that it exhibits cell-cycle-regulated expression that peaks during mitosis.[24] Survivin is required for embryonal development and is expressed in a variety of human cancers, but is absent in most normal, differentiated tissues.[1,2,4] In several human cancers, high survivin expression correlates with more aggressive behavior and decreased response to chemotherapy, as compared to the more benign survivin-negative counterparts. This makes survivin a potential prognostic indicator and a possible target for cancer chemotherapy. However, survivin also plays a critical role in cell division, necessitating a thorough understanding of its function in normal tissues prior to developing novel chemotherapeutic agents for cancer through survivin targeting. Information on its expression in veterinary species is lacking and is vital in determining if survivin, similar to what is observed in humans, is a protein that is expressed in canine and feline cancers. If so, survivin expression in canine and feline cancers may prove to be a prognostic parameter and a novel target for treatment. The purpose of this study was to provide a survey of survivin distribution in a wide variety of canine and feline normal and abnormal tissues.

MATERIALS AND METHODS

Tissue Samples

All animal use in this study was approved by The University of Georgia Institutional Animal Care and Use Committee (IACUC # A2003-10163-c1). Fetal tissues, ranging from approximately 3 to 8 weeks of gestation, were obtained from pregnant dogs and cats undergoing elective ovariohysterectomies. Tissues were formalin-fixed and embedded in paraffin within forty-eight hours with the exception of some fetal tissues, which were subjected to formalin fixation one to five days prior to being embedded in paraffin. The increased formalin fixation time was performed to determine if the additional time in formalin would have any adverse affects on epitope availability.

Immnohistochemical detection of survivin was performed in the following canine and feline tissues: embryonal tissues at varying stages of development, normal adult tissues, and a wide variety of hyperplastic tissues and their neoplastic counterparts. Normal adult tissues examined included: brain (cerebrum and cerebellum), eye, nasal epithelium (dog only), thyroid (cat only), heart, lung, stomach, small and large intestine, pancreas, liver, spleen, bladder, adrenal gland (cat only), ovary, uterus, testicle, skeletal muscle, and skin. The hyperplastic and neoplastic canine conditions included: hyperplastic nasal epithelium and nasal carcinoma, mammary adenoma and mammary adenocarcinoma, rectal polyp and rectal carcinoma, transitional cell epithelial hyperplasia and transitional cell carcinoma (TCC), reactive bone and osteosarcoma (OSA), mesothelial cell hyperplasia and mesothelioma, hemangioma and hemangiosarcoma (HSA), lymphoid hyperplasia and lymphoma (LSA), fibroplasia and fibrosarcoma (FSA), squamous hyperplasia and squamous cell carcinoma (SCC), and hemangiopericytoma. The hyperplastic and neoplastic feline conditions included: lymphoplasmacytic enteritis and gastrointestinal lymphoma, fibroplasia and fibrosarcoma, squamous hyperplasia and squamous cell carcinoma, and inflammatory ear polyp and ceruminous gland carcinoma.

RT-PCR for survivin was performed on a variety of normal canine and feline tissues, including: entire canine and feline embryos at approximately 4 weeks gestation; individual feline fetal tissues at approximately mid gestation including liver, thymus, heart, brain, intestine, and kidney; adult canine bone marrow and corpus luteum; and adult feline liver and testicle. RT-PCR for survivin was also performed on several neoplastic tissues including: canine nasal carcinoma, canine mammary carcinoma, canine synovial cell carcinoma, canine chondrosarcoma, feline fibrosarcoma, and DH82 cells[34], a canine macrophage-monocyte cell line. Fresh tissues were immediately snap frozen in liquid nitrogen and then stored at -80 degrees F until RNA extraction was performed.

Immunohistochemical Staining for Survivin

Affinity purified rabbit anti-human survivin antibody (R&D Systems, AF886, Minneapolis, MN) was used for immunohistochemical detection of survivin protein. This antibody is made against the full-length human survivin peptide and recognizes all three alternatively spliced variants of the human survivin protein. Formalin-fixed, paraffin-embedded tissues were sectioned at 3-5 µm, deparaffinized in xylene, and rehydrated through a graded alcohol series. Antigen retrieval was done by heating the slides to boiling in a microwave in 0.01M citrate pH 6.0 for ten minutes and then allowing the slides to cool for 15 minutes. The slides were then washed in phosphate-buffered saline (PBS) and endogenous peroxidase was blocked with a 3% hydrogen peroxide/methanol solution for 20 minutes. Protein Block Serum-Free (Dako) was used to block non-specific antibody binding and slides were incubated with rabbit anti-human survivin antibody, diluted 1:150 with PBS with 3% bovine serum albumin, for one hour at room temperature. Slides were then incubated with polymer-linked horseradish peroxidase (Dako) for 40 minutes at room temperature, washed with PBS, and then 3,3'-diaminobenzidine (DAB) was used as the chromagen (Vector Laboratories). Slides were counterstained with hematoxylin and coverslipped. The positive control consisted of either a canine nasal adenocarcinoma or canine transitional cell carcinoma that consistently produced intense staining. The negative control consisted of the canine tumors mentioned above with substitution of the primary antibody with Rabbit Super Sensitive Negative Control (Biogenex HK408-7R).

Assessment of Survivin Expression

Immunohistochemical detection of survivin in embryonic and adult normal tissues was recorded as either positive or negative for each tissue examined. In the hyperplastic and neoplastic tissues, the percentage and intensity of staining (1-4) for survivin was recorded. Distribution of survivin within the cytoplasm and/or nucleus was also noted.

RT-PCR for Survivin

Tissues were lysed with RNA-Bee (TEL-TEST, INC.) and total RNA was extracted with phenol and chloroform. A one step-RT-PCR kit (Roche) was used for all reactions. The RT-PCR mixture contained 0.4 μM of the upstream primer, 0.4 μM of the downstream primer, 0.2mM each of the 4 deoxynucleotides, 1 μg of RNA, 2.5 units of *Taq* polymerase, 25 units of Mulv reverse transcriptase, 40 U of RNAse inhibitor, 0.05mM of $MgCl_2$, 12.5mM of DTT, 10 μl of 5x PCR buffer, and 25.5 μl of RNAse-free water for a total volume of 50 μl. The survivin PCR product was obtained by RT-PCR using forward primer 5'-AAGGACCACCGCATCTCTACATTC-3' and reverse primer 5'-CGCACTTTCTTCGCAGTTTCCTCA-3'. This primer pair was designed based on a published sequence of dog survivin (GenBank Accession Number ABO95108) and was predicted to detect only full length survivin. The expected size of the survivin PCR product was 352 bp. As an internal control, GAPDH was amplified by using forward primer 5'-AGTCAACGGATTTGGCCGTAT-3' and reverse primer 5'-

AGCCTTCTCGGTGGTGAAGAC-3’, using the same RT-PCR protocol. The GAPDH primer pair was designed based on a published article[17] that was subsequently modified for canine and feline sequences (GenBank Accession Numbers AF097177 and AB038240). The expected size of the GAPDH product was 303 bp. RT-PCR was performed at 50° C for one hour, followed by denaturation at 94° C for one minute. Forty cycles of 94° C for 30 seconds, 55° C for 30 seconds, and 68° C for 100 seconds were followed by a final extension at 68° C for 7 minutes. All reactions were performed in a thermal cycler. The PCR products were separated on a 1.5% agarose gel containing 7 μl of ethidium bromide. The positive control was from a survivin-positive human T-cell leukemia. The negative controls consisted of a water control and a canine synovial cell carcinoma that was negative for survivin by immunohistochemistry.

After separation on agarose gel, the survivin band PCR products from the fetal feline tissue and the DH82 cells were extracted using the QIAquick gel extraction kit (Qiagen Q28704) and submitted to the University of Georgia Molecular Genetics Instrumentation Facility for sequencing. Sequences were compared with known sequences of this region.

RESULTS

Canine and Feline Embryonal and Normal Adult Tissues

Survivin expression in canine and feline embryonic tissues was similar in distribution and stage of gestation at which the protein was expressed. In the first trimester, survivin was detected by immunohistochemistry (IHC) in multiple organs. Cytoplasmic staining of survivin was observed in hematopoietic cells in the liver, chondrocytes, mucosal epithelial cells of the gastrointestinal tract, renal tubular epithelial cells, epidermis, cells of neuroectoderm origin and mesenchymal cells (Figure. 3.1). Survivin expression was detected, by IHC, in mid-gestation in all organs examined, including: heart, thymus, liver, kidney, intestine, and brain. By late gestation, survivin expression, by IHC, was virtually absent in all organs examined except for cytoplasmic staining in renal tubular epithelium, epidermis, and hematopoietic cells in the bone marrow. Immunohistochemical detection of survivin was consistently reproduced in tissues fixed for as long as five days in formalin prior to placing in paraffin.

Adult canine tissues were negative for survivin with the exception of individual hematopoietic cells in the bone marrow and the corpus luteum of the ovary. Adult feline tissues were negative with the exception of individual hematopoietic cells of the bone marrow, corpus luteum of the ovary, neuroendocrine cells of the gastrointestinal tract, and endometrial glands within the uterus.

Canine Hyperplastic and Neoplastic Tissues

The findings in canine neoplastic and hyperplastic tissues, including the number of samples stained, and the percent and intensity of staining within the survivin-positive tissues are summarized in Table 3.1. Survivin was present in several of the canine hyperplastic tissues and their neoplastic counterparts, and within areas of inflammation in the hyperplastic tissues. Staining was predominately cytoplasmic, however, nuclear staining was also observed in some of the tissues.

Although all canine nasal carcinomas were positive, intensity and percentage of staining varied. One of the tumors had less than 10% positive cells and the weakest staining (2) of all nasal carcinomas, while the other nasal carcinomas that had at least 80% staining and a staining intensity of ≥ 2. Survivin was observed exclusively in the cytoplasm of all tumors, with the exception of the least positive staining tumor, that also had 10% nuclear staining in addition to cytoplasmic staining. Hyperplastic nasal epithelium also expressed survivin, with 100% positivity in 3 of 5 of the tissues and ≤ 50% positivity in the remaining two tissues. Staining intensity overall was less than the neoplastic tissues, with the greatest intensity of staining, 2.5, in one sample with 100% staining. Survivin was localized exclusively in the cytoplasm in all hyperplastic nasal tissues.

All canine mammary carcinomas expressed survivin, however, the percent, intensity, and location of staining was variable (Figures 3.2, 3.3). The two mammary carcinomas with the greatest percent of staining (90-100%) also had the most intense staining (3.5-4.0) and this was predominantly cytoplasmic. Nuclear staining predominated in the mammary carcinoma with the least percent of staining (50%) and lowest intensity score (2). All mammary adenomas were positive; however, one adenoma had less than 10% positive staining and low (1) staining intensity that was exclusively nuclear in location. The remaining adenomas ranged from 75-100% staining, but staining intensity was less than that of the carcinomas with the exception of one adenoma that scored 3.5. In these adenomas, staining was predominantly cytoplasmic, but some nuclear staining was also present (Figure 3.4).

Only 2 of 5 canine rectal carcinomas were positive, but both had a high percentage and intensity of staining. The majority of this staining was cytoplasmic, with ≤ 20% nuclear staining. Survivin was detected in 3 of 5 of the rectal polyps; however, percent staining was no greater than 50%, with one polyp having less than 10% staining. Intensity of staining was consistently less than the rectal carcinomas, with the highest score of 1.5. Similar to the rectal carcinomas, staining was predominantly cytoplasmic.

Although most canine TCC were positive, percent and intensity of staining varied widely (Figure 3.5). Two of the TCC had ≤ 30% staining and intensity of staining ≤ 1; staining was predominantly cytoplasmic but nuclear staining was the greatest (25%) in these two TCC. One TCC had 60% staining with an intensity score of 3.5, whereas the TCC with 90% staining had an intensity score of 4. Hyperplastic transitional epithelium was invariably positive but the intensity of staining varied widely. All of the hyperplastic epithelium had cytoplasmic staining ranging from 75-100%, however, two of the tissues also contained large numbers of cells with positive (75-95%) nuclear staining (Figure 3.6).

Two of 3 positive canine SCC had a low percentage of staining (<20%) and a low intensity score. One of these had only nuclear staining (100%), with rare cytoplasmic staining while the other had a large amount of both cytoplasmic staining (75%) and nuclear staining (50%). The SCC with the most diffuse and intense staining had 100% cytoplasmic staining with only 30% nuclear staining. The two samples of hyperplastic squamous epithelium with the least intense staining had predominantly nuclear staining, with rare cytoplasmic positivity. On the other hand, the most intense staining sample had an equal percent (75%) of nuclear and cytoplasmic staining.

All 4 positive canine OSA had at least 75% staining, but the intensity of staining varied greatly. The two OSA with the least intense staining had no nuclear staining, whereas the second most intensely staining tumor (2.5) had 20% nuclear staining and 100% cytoplasmic staining. This was in contrast to the most intense staining OSA that had 100% nuclear staining and only 50% cytoplasmic staining. All 3 of the reactive bone samples were positive; however, one of these had minimal (<10%) staining, whereas, the other two samples had at least 75% staining. The proliferative bone with the least percent of staining had predominantly cytoplasmic staining, whereas the other two specimens had 100% percent nuclear staining and no greater than 20% cytoplasmic staining.

In both of the positive canine LSA, cytoplasmic staining predominated, but nuclear staining was detected in both tissues (Figures 3.7, 3.8). All hyperplastic lymph nodes were positive, but the percentage of staining ranged widely. The intensity of staining was also variable, and did not correlate with the percent of staining. In contrast to the LSA, nuclear staining was often as prominent as the cytoplasmic staining. The distribution of staining in the hyperplastic lymph nodes was also variable, with survivin-positive lymphocytes primarily in the germinal centers in some samples, whereas the paracortex was primarily positive in other samples.

Only 3 of the 5 canine fibrosarcomas were positive and the percent staining was variable and $\leq$ 50%. The intensity of staining was very low and was exclusively cytoplasmic in distribution. On the other hand, all of the fibroblastic tissues were positive, with $\geq$ 50% of the fibroblasts usually staining with intensity scores greater than the neoplastic tissues. Similar to the neoplastic tissues, only cytoplasmic staining was observed in fibroblasts. Some of the fibroblastic tissues also contained lymphocytes and reactive endothelial cells that were survivin-positive.

Of 4 positive hemangiopericytomas, one tumor had less than 10% staining that was exclusively nuclear in distribution. The other 3 had staining percentages ranging from 30-50% and intensity scores of 1-2.5; staining was cytoplasmic with one also having significant nuclear staining.

Three positive canine hemangiosarcomas had a similar percent and intensity of staining. Equal distribution of cytoplasmic and nuclear staining was observed in two of the tumors, whereas, one hemangiosarcoma had primarily cytoplasmic staining. Most of the hemangiomas were positive; however, they had lower percent staining and intensity of staining than the hemangiosarcomas. All of the hemangiomas had exclusively cytoplasmic staining.

Only two mesotheliomas were available for this study, and both were similar in percent and intensity of staining and had relatively equal distribution of cytoplasmic versus nuclear staining. Only one of the hyperplastic mesothelial tissues was positive, and had more diffuse staining than its neoplastic counterparts, but of equal staining intensity.

Feline Hyperplastic and Neoplastic Tissues

The findings in feline neoplastic and hyperplastic tissues, including the numbers of samples stained, and the percent and intensity of staining within the survivin-positive tissues are summarized in Table 3.2. Survivin was present in several of the feline hyperplastic tissues and their neoplastic counterparts, and within areas of inflammation in the hyperplastic tissues. Staining was predominately cytoplasmic, however, nuclear staining was also observed in some of the tissues.

The10 feline SCC were from three anatomical locations: skin (7), oral cavity (2), and nasal cavity (1). Of SCC from the skin, 5 of 7 were positive. The majority of the skin SCC had at least 75% staining, with only one tumor having 25% staining, and an intensity range of 2-4. Cytoplasmic staining was 100% in all of the skin tumors and 3 also had at least 50% nuclear staining. Both oral SCC (one gingival and one from hard palate) were positive, with 80-90% staining. In the SCC from the hard palate, intensity of staining was 2.5, with 100% cytoplasmic positivity and only rare nuclear staining. Staining in the gingival SCC, however, was predominantly nuclear in location (75%), with only 25% cytoplasmic staining. The nuclear staining was very intense (3.5), whereas the cytoplasmic staining was very weak (1). The nasal

SCC had the least percent staining of all feline SCC, with only 20% positive-staining cells. Although there was 100% cytoplasmic and nuclear staining, the nuclear staining was intense, whereas the cytoplasmic staining was very weak.

One feline ceruminous gland carcinoma out of three was positive, with a large percentage of staining that was very intense (Figure 3.9). The distribution was predominantly cytoplasmic, with less than 10% nuclear staining. None of the positive inflammatory polyps had staining greater than 20% or an intensity score greater than 1.5 in the epithelium. In two of the positive samples, nuclear staining predominated, whereas, one of the samples had predominantly cytoplasmic staining. Positive staining fibroblasts and lymphocytes also were noted within the polyps.

One feline gastrointestinal LSA out of five was positive. It was a gastric LSA that stained diffusely and intensely for survivin. There was 100% cytoplasmic staining and 80% nuclear staining. In addition to the neoplastic lymphocytes in the mucosa, a hemangiosarcoma was present in the submucosa and the neoplastic endothelial cells also had strong cytoplasmic survivin staining. None of the lymphocytic enteritis samples stained positive.

The two positive vaccine-induced feline FSA had only 10% staining and an intensity score of ≤ 1, whereas the oral FSA had 75% staining and an intensity score of 2. Nuclear staining predominated in the vaccine-induced tumors, with one having less than 10% cytoplasmic staining. The oral FSA had an equal distribution of nuclear and cytoplasmic staining. The 2 positive fibroblastic tissues varied greatly, with one having 100% staining whereas the other had less than 10% staining; however, the staining intensities of the two samples were similar. The distribution of staining varied between the two, with nuclear staining predominating in the diffuse staining tissue versus cytoplasmic staining predominating in the other.

RT-PCR of Canine and Feline Survivin

First trimester canine and feline fetal tissues were positive for survivin by RT-PCR. Survivin expression was detected in all mid-gestation feline fetal organs examined, including: heart, thymus, liver, kidney, intestine, and brain (Figure 3.11). Although the normal adult canine bone marrow and corpus luteum were positive by IHC, they were negative by RT-PCR. As there were only a small number of hematopoietic cells in the bone marrow that were positive for survivin by IHC, RT-PCR may not have been sensitive enough to detect this. It is unknown why the corpus luteum was not positive by RT-PCR, as the positive reaction observed by IHC did not appear to be due to non-specific binding. The normal adult feline liver and testicle were negative for survivin both by IHC and RT-PCR. The canine mammary tumor and canine chondrosarcoma were positive for survivin IHC and RT-PCR. The canine nasal carcinoma, canine synovial cell carcinoma, and the feline fibrosarcoma were negative for survivin by IHC and RT-PCR. GAPDH was positive for all of the samples amplified by RT-PCR. The size of the normal canine and feline bands were similar to those of the tumor bands.

Survivin mRNA from feline fetal tissue had 99% homology to published cat sequence (GenBank AB182320) and 93-94% homology to published dog sequences (GenBank NM00100348, AB180206, AY741504, NM 001003019, AB095108). The sequence obtained from feline fetal survivin mRNA is as follows:

5’-CCTCAAATTCTTTCTGTTTATGGTTGGTTTCCTTTGCAATTTTGTTCTTG
GCTCTCTCTTTGTCCAGTTTCAAAAATTCACTGAGGGTTAATTCTTCAAA
CTGCTTCTTGACAGAAAGAAAGGCACAACCAGATGAATGCTTCTTATGTT
CCTCTATGGGGTCATCGTCTGGCTCCCAGCCTTCCAGCTCCTTGAAGCAG
AAGAAACACTGAGCCAAATCGGGCTCGTTCTCAGTGGGACAGTGGATGAA

GCCGGCCTCGGCCATCCGCTCCGGGGTGCAGGCGCAGCCCTCCAAGAACG

GCCAGTTCTTGAATGT-3’

Survivin mRNA from DH82 cells had 100% homology to dog sequences (GenBank NM001003348, AB180206, AY741504), 97% homology to dog sequence (GenBank AB095108), and 94 % homology to cat sequence AB182320. The sequence obtained from the DH82 survivin mRNA is as follows:

5’-ACATTCAAGAACTGGCCGTTCCTGGAGGGCTGCGCCTGCACCCCGGACCG

GATGGCAGAGGCCGGCTTCATCCACTGTCCCACTGAGAACGAGCCAGACT

TGGCCCAGTGTTTCTTCTGCTTCAAGGAGCTGGAAGGCTGGGAGCCAGAT

GATGACCCTATAGAGGAGCATAAAAAACATTCATCTGGTTGTGCTTTCCT

TTCTGTCAAGAAGCAGTTTGAAGAATTAACCCTCAGTGAATTTTTGAAAC

TGGACAAAGAAAGAGCCAAGAACAAAATTGCAAAGGAAACCAACAACAAG

CAGAAAGAATTTGAGGAA-3’

DISCUSSION

Our findings in canine and feline embryonal tissues are consistent with the human literature, in that survivin is widely distributed in early embryonal development, decreasing to discrete locations in mid-gestation, but virtually absent by late gestation.[1]

Recently it was shown that survivin is expressed in mouse oocytes and throughout all stages of preimplantation embryos.[18] Antisense targeting of survivin results in arrested development by the early blastocyst stage, nuclear defects, loss of the mitotic spindle and tubulin network, with the embryos subsequently undergoing apoptosis via a pathway involving caspase-3 and –9.[18] These findings are in agreement with a previous report, in which mice embryos with homozygous disruption of the survivin gene die within 4 to 5 days of embryonic development, exhibiting failed cytokinesis and disruption of the microtubule network.[33] Thus, survivin plays a critical role in cell division in early embryonal development, being essential for chromosome alignment, sister chromatid segregation, and cytokinesis. Recently, survivin was shown to be necessary for sustained checkpoint arrest in response to lack of tension at the kinetochore, ensuring symmetrical transmission of chromosomes.[22] It is speculated that survivin, in association with the chromosomal passengers Aurora B kinase (AIM-1) and inner centromere protein (INCENP), are key regulators of microtubule attachment to kinetochores during mitosis.[6] Furthermore, the binding of survivin to Aurora B kinase has been shown to increase the activity of this enzyme.[6] Overexpression of Aurora-B kinase, like survivin, is observed in several human cancers.[16,32] Increased mitotic phosphorylation of histone H3, secondary to Aurora-B kinase overexpression, results in chromosomal instability which may contribute to carcinogenesis.[30] The overexpression of survivin, in relationship to Aurora-B kinase, in tumors has not been investigated.

Survivin expression in adult canine tissues was limited to individual hematopoietic cells in the bone marrow and the corpus luteum of the ovary. The protein was likewise expressed in individual hematopoietic cells of the feline bone marrow and the corpus luteum. Survivin expression can also be detected in neuroendocrine cells throughout the gastrointestinal tract and within the endometrial glands in cats. In humans, adult tissues that express survivin include: CD34+ hematopoietic progenitor cells,[12] lymphocytes in the thymus,[20] basal colonic epithelial cells,[13] and gastric mucosal cells.[7] In CD34+ bone marrow cells, survivin expression can be induced by a combination of the hematopoietic growth factors, thrombopoietin, Flt3 ligand, and stem cell factor.[11,12] Upregulation of survivin in CD34+ cells occurs during all phases of the cell cycle, unlike cancer cells where survivin expression specifically occurs during the G2/M phase of the cell cycle. In addition, survivin upregulation in CD34+ cells is mild in comparison to leukemic cells, implying loss of survivin regulation in this cancer.[12] The presence of survivin in normal hematopoietic cells raises the concern that targeting survivin in cancer therapy may possibly interfere with normal hematopoiesis.

The majority of canine carcinomas in each category, with the exception of the rectal carcinomas, were survivin-positive. However, of the positive tumors within each group, the percent and intensity of staining was variable. There were fewer positive canine sarcomas, with approximately 60% of all sarcomas staining positive for survivin. Similar to the carcinomas, there was both a wide range of percent and intensity of staining. Feline squamous cell carcinomas expressed survivin most frequently, whereas ceruminous carcinomas and gastrointestinal lymphomas infrequently expressed survivin. Feline fibrosarcomas often expressed survivin, however, the vaccine-induced fibrosarcomas had a low percentage of staining as compared to the oral fibrosarcoma. Cytoplasmic staining predominated in the vast majority of canine and feline tumors, with predominant nuclear staining being uncommon. Further studies are necessary to determine if cytoplasmic versus nuclear survivin staining is associated with tumor behavior in dog and cat cancers.

Survivin is expressed by virtually all types of human malignancies.[23] The overexpression of survivin in human tumors, including carcinomas and sarcomas, is almost always associated with a poorer prognosis than tumors of the same type that are survivin-negative.[23] The observation that both survivin-positive and survivin-negative tumors exist within most canine and feline tumors types raises the question of whether or not these survivin-positive tumors represent a more aggressive phenotype, as is documented in human medicine. Additional studies are needed to determine if survivin expression in canine and feline cancers is a negative predictor of survival.

Although the vast majority of evidence suggests that survivin expression is a negative prognostic parameter in a wide variety of human tumors, exceptions to this general observation do exist.[3,19,31] Nuclear expression of survivin in gastric carcinomas, mammary carcinomas, and pediatric ependymomas and choroid plexus tumors has been associated with a favorable prognosis. In one study of gastric carcinomas,[31] exclusive nuclear staining for survivin had a good prognosis, and correlated with younger patients and lower occurrence of vessel cancer invasion. In contrast, cytoplasmic staining correlated only with older patients, but no association with progression of the tumor or prognosis. Another report, in a series of mammary carcinomas, showed nuclear expression of survivin was most commonly observed and proved to be an independent prognostic parameter of good prognosis. [19] Finally, a recent report has demonstrated that normal ependyma and choroid plexus of the perinatal and pediatric brain tissue express high levels of both nuclear and cytoplasmic survivin.[3] Pediatric ependymomas and choroid plexus tumors that lack nuclear survivin expression are associated with higher grade, less differentiated tumors. These observations suggest that nuclear survivin may prevent tumor progression.

Survivin-positive hyperplastic epithelial and mesenchymal cells were detected in many canine and feline tissues, often expressing survivin as diffusely and/or intensely as the neoplastic counterparts. The majority of the canine and feline hyperplastic and neoplastic tissues that expressed survivin had primarily cytoplasmic staining, with lesser amounts of nuclear staining. Rarely, nuclear staining predominated. This variation in subcellular location of survivin could be related to the expression of different isoforms.

Two distinct subcellular pools of survivin observed in humans are the result of three alternatively spliced transcripts that can give rise to three different isoforms: full-length survivin, survivin-2B, and survivin-ΔEx-3.[8] The full-length survivin transcript consists of three-introns and four-exons which produces a 16.5 kDa protein. The survivin-2B transcript retains a portion of intron 2, which consequently interrupts the BIR domain, resulting in a reduction of the apoptotic activity of this isoform. The survivin-ΔEx-3 transcript is produced by the removal of exon three, resulting in a frame shift that generates a novel COOH-terminal.[26,27] While survivin and survivin-2B are primarily present in the cytoplasm, survivin-ΔEx-3 is nuclear in location.[10] Recently, it was shown that survivin-2B is positively regulated by p53, with its overexpression playing a role in sensitizing leukemia cells to doxorubricin-induced apoptosis. In contrast, full-length survivin and survivin-ΔEx-3 were decreased by p53.[35] Thus, these different isoforms may provide a regulatory balance between apoptosis and inhibition of apoptosis. Additional studies are needed to determine if survivin isoforms exist in the dog and cat, and if so, whether there is differential expression of the isoforms in canine and feline normal and hyperplastic tissues versus neoplastic tissues.

There is a paucity of information regarding survivin expression in human hyperplastic tissues. Survivin has been detected in normal, hyperplastic, and neoplastic colonic mucosa.[13] However, the staining pattern of survivin is different among these various colonic tissues. Multiple pathways are potentially involved in the overexpression of survivin in cancer, including loss of p53,[14,15,25,29] constitutive signal transducer and activator of transcription-3 (STAT3) activation,[5,21] and upregulation by c-H-Ras oncoprotein.[31] It remains to be determined whether the mechanisms behind the upregulation of survivin in cancer differs from pathways involved in its upregulation in hyperplastic tissues.

Lymphocytes, fibroblasts, and reactive endothelial cells within inflammatory tissues were often strongly positive for survivin, with the exception of feline lymphoplasmacytic enteritis. Endothelial cells express survivin when stimulated with vascular endothelial growth factor.[28] Antisense targeting of survivin in reactive endothelial cells results in cell death upon apoptotic stimuli, suggesting survivin protects endothelial cells against apoptosis during angiogenesis.[30] In addition, upregulation of survivin in endothelial cells following ischemic conditions is thought to promote angiogenesis.[9] Thus, survivin upregulation is not unique to cancer cells and may be an important protein in hyperplastic tissues, inflammatory conditions and wound healing.

In conclusion, survivin is expressed in a wide variety of canine and feline tissues, including embryonal, hyperplastic, inflammatory, and neoplastic tissues. There is only minimal expression of the protein, however, in normal adult canine and feline tissues. Formalin fixation of tissues for as long as five days prior to immunohistochemical detection of survivin has no adverse effect on staining, thus epitope stability of survivin makes immunohistochemistry a reliable detection method. The close homology between canine and feline mRNA sequences suggests that this protein is highly conserved and will function similarly in these two species. Further studies are necessary to determine if survivin expression in canine and feline cancers will be of prognostic value, and if so, targeting survivin pathways in these species may be a novel therapeutic regimen in veterinary oncology. We have shown that survivin is expressed in a wide variety of canine and feline hyperplastic and neoplastic tissues. Therefore, survivin may be a good candidate protein to evaluate as a prognostic indicator.

Table 3.1. Survivin expression in canine neoplastic and hyperplastic tissues as determined by immunohistochemistry.

Lesion	Number of positive staining tissues/ number examined (%)	Percent staining of positive staining tissues	Intensity (1-4) of positive staining tissues
Nasal carcinoma	5/5	10-100	2-4
Hyperplastic nasal epithelium	5/5	10-100	0.5-2.5
Mammary carcinoma	5/5	50-100	2-4
Mammary adenoma	5/5	10-100	1-3.5
Hemangiopericytoma	4/7	10-50	1-2.5
Rectal carcinoma	2/5	75-90	2.5-3
Rectal polyp	3/5	10-50	1-1.5
Transitional cell carcinoma	5/6	10-90	0.5-4
Hyperplastic transitional epithelium	6/6	85-100	1-4
Osteosarcoma	4/5	75	1-3.5
Reactive bone	3/3	10-100	1-3.5
Mesothelioma	2/2	30-50	1-2
Mesothelial hyperplasia	1/5	100	2
Lymphosarcoma	2/5	50-75	2-3
Lymphoid hyperplasia	5/5	10-75	1-3.5
Fibrosarcoma	3/5	10-50	2.5
Fibroplasia	5/5	10-100	1-3.5
Hemangiosarcoma	3/5	75	2.5
Hemangioma	4/5	10	1-2
Squamous cell carcinoma	3/4	10-90	1-4
Squamous hyperplasia	3/5	10-30	1-2.5

Table 3.2. Survivin expression in feline neoplastic and hyperplastic tissues as determined by immunohistochemistry.

Lesion	Number of positive staining tissues/ number examined (%)	Percent staining of positive staining tissues	Intensity (1-4) of positive staining tissues
Squamous cell carcinoma	8/10	20-100	1-4
Squamous hyperplasia	2/6	75-100	2-4
Fibrosarcoma	3/5	10-75	0.5-2
Fibroplasia	2/5	10-100	1-2
Ceruminous carcinoma	1/3	75	3.5
Inflammatory ear polyp	3/5	10-20	1-1.5
Gastrointestinal lymphoma	1/5	100	4
Lymphocytic enteritis	0/4	-	-

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FIGURE LEGENDS

Figure 3.1. Feline fetus, first trimester: Survivin staining in mesenchymal and epithelial embryonal tissue.

Figure 3.2. Canine mammary adenocarcinoma: Diffuse cytoplasmic survivin staining of the neoplastic epithelial cells and rare nuclear staining.

Figure 3.3. Canine mammary adenocarcinoma: Weak cytoplasmic survivin staining and prominent nuclear staining.

Figure 3.4. Canine mammary adenoma: Diffuse cytoplasmic survivin staining of the neoplastic epithelial cells and occasional nuclear staining.

Figure 3.5. Canine transitional cell carcinoma: Diffuse cytoplasmic survivin staining of the neoplastic epithelial cells and infrequent nuclear staining.

Figure 3.6. Canine hyperplastic transitional epithelium: Diffuse cytoplasmic survivin staining of hyperplastic epithelial cells and frequent nuclear staining.

Figure 3.7. Canine lymphosarcoma: Diffuse cytoplasmic survivin staining of the neoplastic lymphocytes and occasional nuclear staining.

Figure 3.8. Canine lymphosarcoma: Rare survivin-positive lymphocytes.

Figure 3.9. Feline ceruminous gland carcinoma: Diffuse cytoplasmic survivin staining of the neoplastic epithelial cells and no nuclear staining.

Figure 3.10. Feline fibrosarcoma: Negative staining for survivin with the exception of one mitotic figure that is survivin-positive.

Figure 3.11. Agarose gel electrophoresis of feline fetal tissues, examined for survivin by RT-PCR: molecular weight marker (lane 1), positive control (lane 2), positive feline fetal kidney, heart, intestines, brain, thymus, liver (lanes 3,5,7,9,11,12), GAPDH of kidney, heart, intestines, and brain (lanes 4,6,8,10). These fetal tissues were also positive for survivin by IHC.

FIGURES 3.1 – 3.10

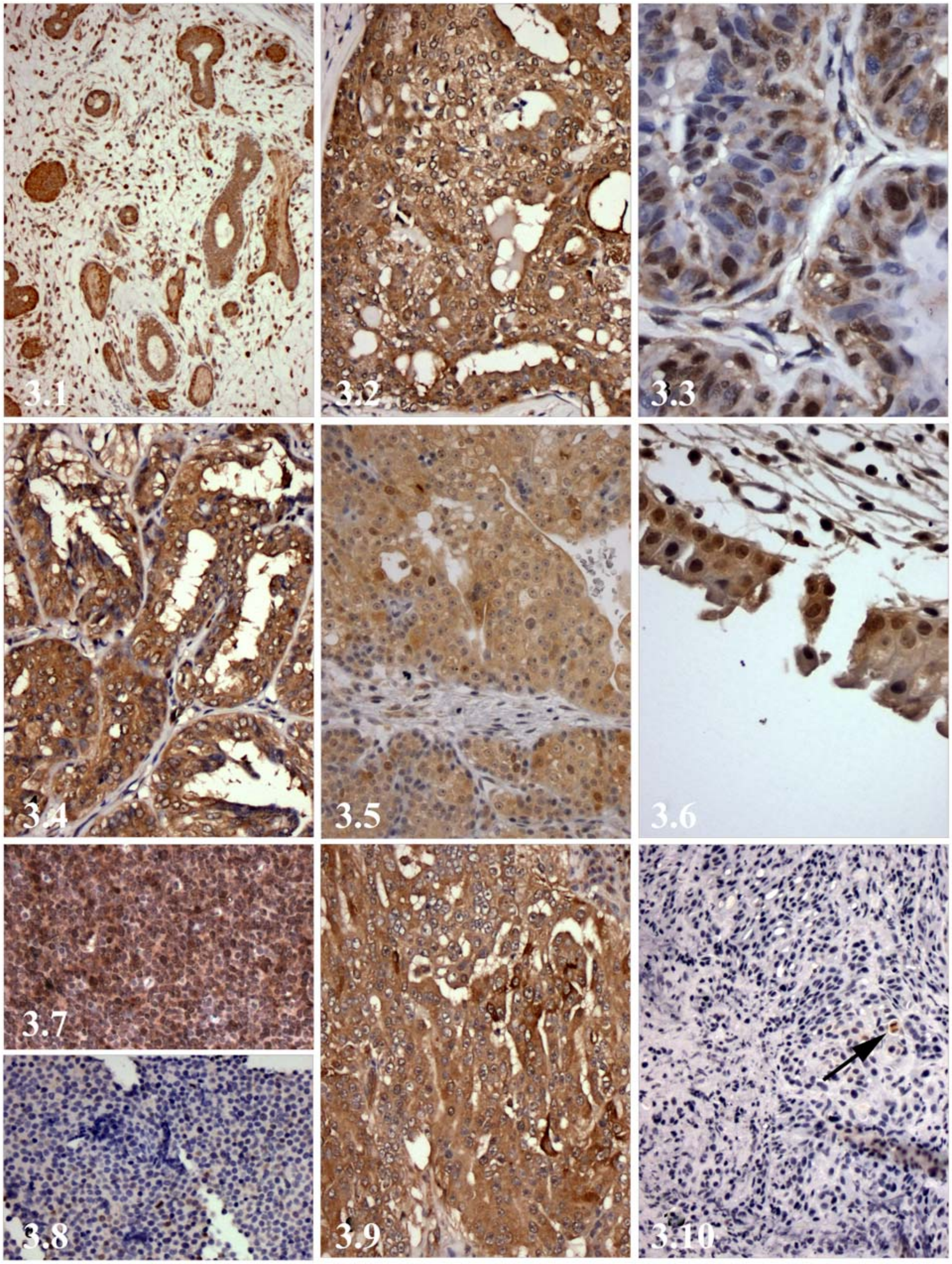
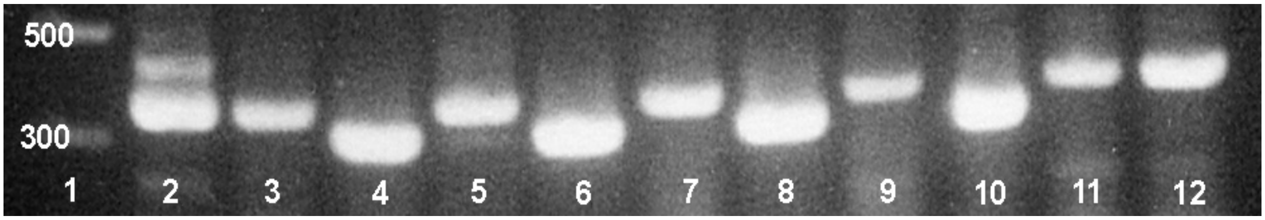


FIGURE 3.11



CHAPTER 4

SURVIVIN EXPRESSION IN CANINE LYMPHOSARCOMA[1]

[1] Johnson, M.E, E.W. Howerth and N. Northrup. To be submitted to *Veterinary Pathology*

ABSTRACT

Survivin is a protein belonging to the inhibitor of apoptosis (IAP) gene family that is absent in most normal, terminally differentiated tissues but is overexpressed in a variety of human cancers. Its expression in tumors often correlates with a more aggressive phenotype and decreased response to chemotherapeutics. Although understanding the role of survivin in human cancer is currently under intense investigation, the significance of survivin expression in domestic animal species remains unknown. The purpose of this study was to determine if survivin expression is a prognostic parameter in canine lymphosarcoma (LSA). Survivin expression was evaluated by immunohistochemistry (IHC) in neoplastic lymph nodes from 14 dogs with LSA undergoing treatment with the same chemotherapeutic protocol. Survivin was expressed in the tumor cells of eight dogs, with the percentage of survivin-positive tumor cells ranging from 25-100%. The survivin-positive LSA included 6 B-cell and 2 T-cell immunophenotypes and were from five dogs in WHO substage A and 3 in substage B. Of the six dogs whose LSA was survivin-negative, 4 had B-cell and 2 had T-cell LSA and 4 were in WHO substage A and 2 in substage B. An unpaired Student's T-test comparing survivin expression to overall survival time identified survivin as a negative prognostic factor in canine LSA (P = 0.002). Larger numbers of dogs with LSA of the T-cell immunophenotype and in WHO substage B need to be examined before it can be determined whether or not the influence of survivin expression on overall survival time is independent of these other prognostic parameters.

Key words: Canine, inhibitor of apoptosis, lymphosarcoma, prognostic indicator, survivin

INTRODUCTION

Survivin is an inhibitor of apoptosis protein that has a dual role, including regulation of the cell cycle and suppression of apoptosis.[7] It is expressed in embryonal development and virtually all human malignancies, but is undetectable in most adult tissues.[1,6] This suggests that reactivation of the survivin gene frequently occurs in cancers. In addition, survivin expression in tumors often correlates with more aggressive behavior, decreased response to chemotherapeutic agents, and shortened survival times, as compared to similar tumor types that are negative for survivin.[6] Thus, its expression in cancer may prove to be an important negative prognostic parameter. The fact that survivin is upregulated in several cancers, yet absent in most normal adult tissues, also makes this protein an attractive target in cancer treatment. Experiments targeting survivin in neoplastic cells, including a replication-deficient adenovirus encoding a mutated survivin protein[9] and downregulation of survivin expression using an antisense oligonucleotide,[11] are currently under investigation. Results of these experiments suggest that targeting of the survivin pathway in cancer has potential as a novel therapeutic regimen.

Information describing survivin expression in normal and neoplastic tissues of domestic animal species, however, is lacking. Canine lymphosarcoma (LSA) is a common neoplasia in dogs, accounting for approximately 20% of all canine neoplasms.[5,10] Untreated, the majority of dogs will die of this disease within six weeks.[8] Chemotherapy, however, induces complete remission in greater than 60% of dogs, with median survival times of 6 to 12 months.[16] Although treatment is rarely curable, complete response and a good quality of life until relapse occurs is common. Thus, owners are willing to pursue treatment in order to gain additional time with their pet. Chemotherapy, however, is a costly procedure and the success of treatment is variable. Thus, prognostic parameters are important in determining which dogs are good candidates for treatment and in the future, treatment may be tailored to the individual dog based on prognostic factors. Currently, the two most consistent factors described as having prognostic importance are immunophenotype and WHO substage.[4,12,14]

The goal of this study was to determine whether survivin expression in canine LSA is a negative prognostic parameter. We hypothesized that some LSA will express survivin, whereas others will be survivin-negative. In addition, the survivin-positive dogs will have shorter survival times when compared to those that are survivin negative. Several of the currently available chemotherapeutic drugs induce apoptosis in cancer cells. If survivin expression is associated with shorter survival times and decreased response to chemotherapeutics, then potentially dogs with LSA that express survivin could be treated with drugs that are not apoptotic-dependent for their effect on cancer cells.

MATERIALS AND METHODS

Tissue Samples

All animal use in this study was approved by the University of Georgia Institutional Animal Care and Use Committee (IACUC # A2003-10163-cl). Lymph node biopsies were from 14 dogs undergoing chemotherapy for LSA at the University of Georgia (UGA) Small Animal Teaching Hospital. Immunohistochemistry was performed for the B and T cell markers (CD79 and CD3, respectively), and survivin and was performed prior to the administration of chemotherapeutic drugs. All dogs were treated with the Modified Madison-Wisconsin protocol.[3]

Immunohistochemistry

Immunohistochemistry for survivin, CD3, and CD79 was done on a DAKO Autostainer using a similar protocol. Formalin-fixed, paraffin-embedded tissues were dewaxed, dehydrated, and heated to boiling for ten minutes in 0.01M citrate (pH 6.0) for antigen retrieval. Endogenous peroxidase was blocked with a 3% hydrogen peroxide/methanol for 20 minutes. Protein Block Serum-Free (Dako) was used to block non-specific antibody binding. Affinity-purified rabbit anti-survivin polyclonal antibody (R&D Systems, Minneapolis, MN), at a dilution of 1:150, was used for immunohistochemical detection of survivin protein. Rabbit anti-CD3 (Dako), at a dilution of 1:400, and mouse anti-CD79 (Dako), at a dilution of 1:200, were used for immunohistochemical detection of CD3 and CD79, respectively. Primary antibodies were allowed to incubate for one hour. After incubation, the slides were incubated with polymer-linked horseradish peroxidase (Dako) for either 10 minutes (CD3, CD79) or for 40 minutes (survivin). 3,3'-diaminobenzidine (DAB) was used as the chromagen (Vector Laboratories). Slides were counterstained with hematoxylin and coverslipped. The positive control for survivin was a canine transitional cell carcinoma that consistently produced intense staining. The positive control for CD3 and CD79 was normal canine lymph node. The negative control was the positive control slide with substitution of the primary antibody with Rabbit or Mouse Super Sensitive Negative Control (Biogenex). The percentage of survivin staining was recorded for each lymph node.

Statistical Analysis

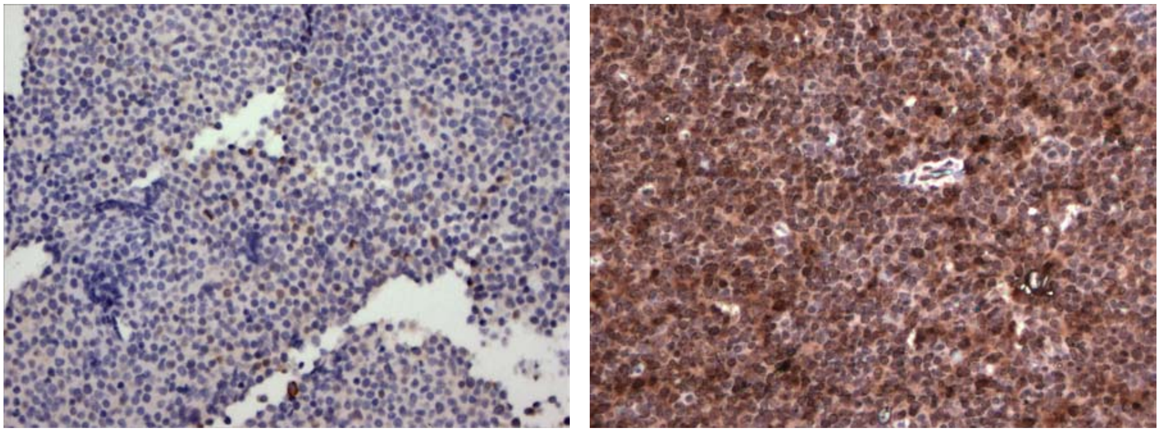
Overall survival time was measured from the onset of treatment to the time of euthanasia due to progressive disease. An unpaired Student's t-test was used for statistical analysis, comparing survivin-positive versus survivin-negative lymph nodes to the months of overall survival time. The statistical calculations were performed using Microsoft Excel. $P<0.05$ was considered significant.

RESULTS

Table 1 shows the percent of survivin expression in the neoplastic lymph nodes, the immunophenotype of each tumor, and the survival time and WHO substage of each dog. Neoplastic lymph nodes of eight of the 14 dogs (57%) expressed survivin (Figure 4.1). The survivin-positive lymph nodes varied in their percent of survivin expression, ranging from 25-100% staining of positive tumor cells. The survivin-positive LSA included 6 B-cell and 2 T-cell immunophenotyes and were from five dogs in WHO substage A and 3 in substage B. Neoplastic lymph nodes of six of the 14 dogs (43%) were negative for survivin expression. The survivin-negative lymph nodes included 4 B-cell and 2 T-cell immunophenotypes and 4 were in WHO substage A and 2 in substage B. The mean survival time (± SD) for the survivin-positive lymph nodes was 3.25 (±2.51). The mean survival time (± SD) for the survivin-negative lymph nodes was 11.42 (± 5.28). Using an unpaired Student's t-Test comparing survivin expression to the months of overall survival time, survivin was identified as a negative prognostic factor in canine LSA ($P=0.002$), (Figure 4.2)

Table 4.1. Survivin Expression in Canine Lymphosarcoma as determined by immunohistochemistry

% Survivin Expression	Survival Time	Immunophenotype	WHO Substage
100	7	B	A
100	1	B	A
90	6	B	A
80	5	B	A
75	0.5	T	B
50	1	T	B
50	2	B	B
25	3.5	B	A
0	5.5	B	B
0	18	B	A
0	11	B	A
0	11	B	A
0	17	T	A
0	6	T	B



A B

Figure 4.1: Examples of differential expression of survivin in lymph nodes from dogs with lymphosarcoma. (A) B-cell LSA negative for survivin expression. (B) B-cell LSA positive for survivin expression. IHC was performed using an immunoperoxidase method with DAB. Original magnification, x200

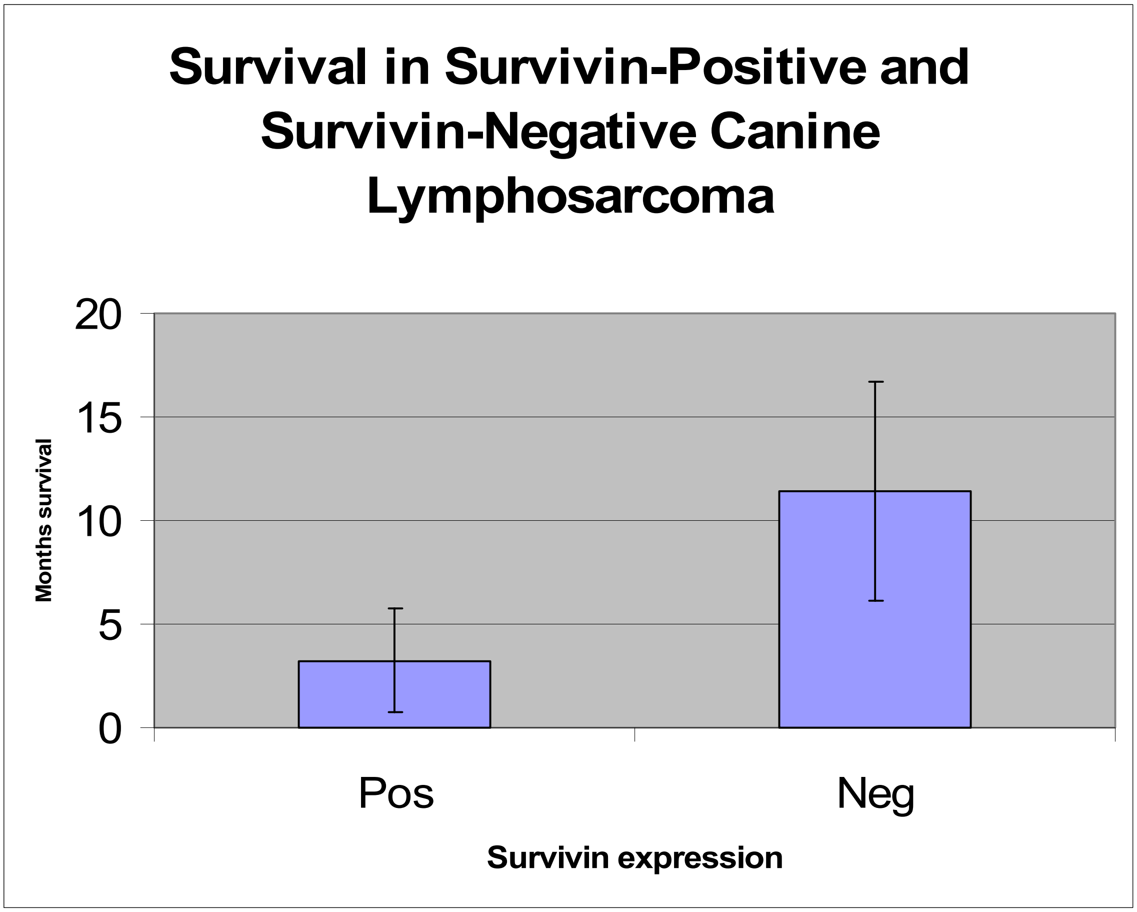


Figure 4.2: Mean survival time (± SD), in months, of dogs with survivin-positive (n=8) and survivin-negative (n=6) LSA.

DISCUSSION

We found that survivin expression predicted a shorter overall survival time in dogs with LSA receiving the same standard chemotherapeutic treatment. A number of prognostic factors are currently in use to predict survival times in canine LSA. Of these, the two most consistent factors described as having significant prognostic importance are immunophenotype and WHO substage.[4,12,14] Dogs with T-cell LSA and those presenting with substage B disease (with systemic signs) normally experience shorter remissions and decreased survival times.[4,14,15] There were too few T cell LSA and WHO substage B patients in this study to perform multivariate analyses to determine if the influence of survivin expression on overall survival time was independent of these other prognostic factors. However, from the limited numbers examined, there were survivin-positive dogs that should have had a more favorable prognosis because of either expressing the B-cell immunophenotype or being classified as WHO substage A. Likewise, there were survivin-negative dogs that should have had a poorer response because of either expressing the T-cell immunophenotype or being classified as WHO substage B. Larger numbers of cases need to be evaluated to determine if survivin expression is an independent negative prognostic factor in canine LSA.

Survivin is expressed in virtually all human malignancies and is often associated with more aggressive behavior and decreased response to chemotherapeutics.[6] In humans with diffuse large B-cell lymphoma[2] and anaplastic large-cell lymphoma,[13] survivin expression was shown to be an independent negative prognostic parameter; our findings in dogs with LSA were similar. It is possible that survivin expression could replace some of the more classic prognostic factors that are currently used for canine LSA. In particular, survivin could be a substitute for the more invasive prognostic factors, such as bone marrow evaluation for WHO staging.

In conclusion, we have shown survivin expression to be a prognostic factor for poor survival rates in canine LSA. Multivariate analyses, to determine if other classical prognostic parameters are associated with the expression of survivin, still need to be performed. Although the number of patients included in this study was small, our findings are consistent with the human literature in that survivin is often a negative predictor of survival in human cancers. To our knowledge, this is the first report that has correlated survival times with survivin expression in a veterinary species. Further studies evaluating survivin expression in veterinary species are warranted.

ACKNOWLEDGEMENTS

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CHAPTER 5

SURVIVIN EXPRESSION IN CANINE MAMMARY ADENOMAS AND CARCINOMAS AND ITS CORRELATION WITH APOPTOTIC AND PROLIFERATIVE INDICES[1]

[1] Johnson, M.E.and E.W. Howerth. To be submitted to *Oncogene.*

Survivin is an inhibitor of apoptosis (IAP) protein that is essential for normal cell division and has the ability to inhibit apoptosis. Survivin is expressed in a variety of human tumors and its expression is often associated with a more aggressive phenotype. Studies evaluating survivin expression in canine neoplasms have not been conducted. The initial goal of this study was to evaluate survivin expression in canine mammary tumors to determine if survivin expression correlated with a more malignant phenotype and could potentially be used as a prognostic parameter. A total of 15 canine mammary tumors, including 7 adenomas and 8 carcinomas, were evaluated for survivin expression by immunohistochemistry (IHC). Survivin expression was positive in 15/15 (100%) of the tumors, with the percent of survivin staining ranging from 50-100%. Cytoplasmic survivin staining predominated in all tumors except one mammary carcinoma. In humans, survivin isoforms exist which differ in their ability to inhibit apoptosis. These isoforms may represent a regulatory balance between apoptosis and inhibition of apoptosis. Although it is unknown whether canine survivin isoforms exist, we hypothesized that survivin expression in both benign and malignant mammary tumors was due to differential expression of canine survivin isoforms. To indirectly explore this possibility, nuclear survivin staining was compared between the adenomas and carcinomas. In addition, immunohistochemical detection of Ki-67 and cleaved caspase-3, for determination of proliferative rate and the apoptotic index, respectively, were compared between the adenomas and carcinomas. Differences in nuclear staining and Ki-67 and cleaved caspase-3 activity between the tumor types were not observed. In conclusion, we found no differences in the percent expression of survivin, nuclear survivin expression, Ki-67 or cleaved caspase-3 activity between the

adenomas and carcinomas. Our results do not provide strong evidence to support the existence of canine isoforms.

Keywords; canine mammary tumors; caspase-3; Ki-67; nuclear; survivin

Introduction

Survivin is a bifunctional molecule, belonging to the inhibitor of apoptosis (IAP) family of proteins, which is capable of counteracting apoptotic cell death in addition to regulating the cell cycle in the G2/M phase.[11] Survivin is expressed during fetal development but is almost completely down-regulated in most normal, terminally differentiated tissues.[1] It is overexpressed, however, in a variety of human cancers and has been shown to be a negative prognostic parameter in several human tumors.[9] Upregulation of survivin in cancer, compared to its limited expression in normal tissues, suggests that this protein plays an important role in the development of cancer. Thus, targeting survivin could potentially prove to be beneficial in cancer chemotherapy.[9] However, its requirement in normal cell division necessitates an understanding of its regulatory role in the cell cycle before survivin pathways can be manipulated for the treatment of cancer.

Uncontrolled cell proliferation and/or inhibition of apoptosis can contribute to the development of cancer. Because survivin functions both as a regulator of the cell cycle and as an inhibitor of apoptosis, it could potentially contribute to tumorigenesis by promoting cell proliferation or by reducing apoptosis. The human survivin gene, as a result of alternatively spliced transcripts, can give rise to four different isoforms.[2,13] The resultant survivin isoforms vary in their ability to inhibit apoptosis and also show different subcellular localization.[12,13] It is feasible that the survivin isoforms represent a regulatory balance between apoptosis and inhibition of apoptosis. Although differential expression of survivin isoforms have been associated with differences in apoptotic indices,[23] no reports in the literature have found a direct association of survivin isotypes with proliferative indices.

The purpose of this study was two-fold. The first objective was to examine survivin expression, in canine mammary tumors, to determine if survivin expression correlated with malignancy. As we found survivin to be equally expressed in the benign mammary tumors and their malignant counterparts, the second objective of this study was to determine if there were differences between nuclear survivin expression and proliferation and apoptotic indices between the benign and malignant tumor types. Differences in these indices could potentially be due to the expression of functionally different canine survivin isoforms.

Results

Tables 5.1 and 5.2 summarize the findings in the canine mammary adenomas and carcinomas, respectively, including the percent of survivin staining, the percent of cytoplasmic and nuclear survivin staining, the proliferative index as measured by the percentage of Ki-67-positive cells, and the apoptotic index as measured by the percentage of caspase-3-positive cells. Diffuse cytoplasmic staining predominated in all the tumors except one mammary carcinoma, which showed diffuse nuclear staining and infrequent cytoplasmic staining. Nuclear staining of the tumors was variable, ranging from 0-90%.

Table 5.1. Survivin, Ki-67, and cleaved caspase-3 expression in canine mammary adenomas by immunohistochemistry

Percent survivin staining	Percent cytoplasmic survivin staining	Percent nuclear survivin staining	Percent positive Ki-67 cells	Percent positive caspase-3 cells
100	100	0	27	0
100	100	30	10	0
100	100	10	05	0
100	100	0	27	2
100	100	0	05	4
90	100	0	27	2
75	100	25	20	1

Table 5.2. Survivin, Ki-67, and cleaved caspase-3 expression in canine mammary carcinomas by immunohistochemistry

Percent survivin staining	Percent cytoplasmic survivin staining	Percent nuclear survivin staining	Percent positive Ki-67 cells	Percent positive caspase-3 cells
100	100	0	17	0
100	100	10	47	1
90	100	25	01	1
90	100	10	08	0
90	100	0	84	4
75	100	50	10	2
50	10	90	05	1
50	100	10	07	3

Survivin is equally expressed in canine mammary adenomas and carcinomas

All of the mammary adenomas (7/7) and carcinomas (8/8) expressed survivin. There was no difference in percent staining between the two as evident by an unpaired t-test (P=0.113). This suggests that survivin expression can not be used to differentiate benign from malignant canine mammary tumors.

Differences in Ki-67 and caspase-3 between the adenomas and carcinomas were not observed (Figure 5.1).

The percent positive Ki-67 cells ranged from 5-27% in the adenomas and 1-84% in the carcinomas. The percent positive caspase-3 cells ranged from 0-4% in the adenomas and 0-4% in the carcinomas. The results of an unpaired t-test show no difference in Ki-67 staining (P=0.666) or caspase staining (P=0.780) between the adenomas and the carcinomas. The existence of different canine isoforms in the adenomas versus the carcinomas, differing in their affect on cell proliferation and apoptosis, can not be demonstrated from these findings.

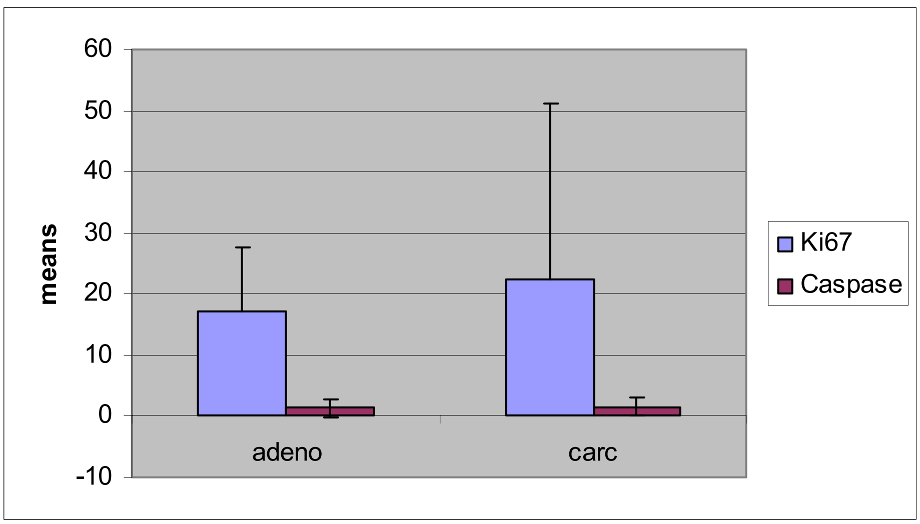

Figure 5.1: Mean (± SD) for Ki-67 and cleaved caspase-3. The mean (± SD) for Ki-67 in the adenomas was 17.3 (± 10.4).. The mean (± SD) for Ki-67 in the carcinomas was 22.4 (± 28.8). The mean (± SD) for cleaved caspase-3 in the adenomas was 1.29 (± 1.50). The mean (± SD) for cleaved caspase-3 in the carcinomas was 1.50 (± 1.41).

Differences in nuclear survivin expression between the adenomas and carcinomas were not observed.

Three of the 7 adenomas had nuclear staining, ranging from 10-30%. Six of the eight carcinomas had nuclear staining, ranging from 10-90%. There was no difference in nuclear staining between the two as evident by an unpaired t-test (P=0.255). This suggests that nuclear expression of survivin can not be used to differentiate benign from malignant canine mammary tumors.

Discussion

In the present study, all benign and malignant canine mammary tumors were positive for survivin, by IHC, using a polyclonal anti-survivin antibody for use in humans. Histologic grade has been shown to be a prognostic factor in canine mammary tumors, with simple carcinomas carrying a poorer prognosis than complex or tubular/papillary carcinomas.[8,22] Therefore, although the clinical outcome of the dogs in this study was not determined following mastectomy, it is probable that the carcinomas represented a more aggressive phenotype than the adenomas. The observation that the adenomas and carcinomas were equally positive for survivin suggests that survivin expression in canine mammary tumors, at least with the polyclonal antibody used in this study, will not be useful in identifying mammary tumors with a greater malignant potential.

Although survivin expression has been shown to be a negative predictor of survival in several human malignancies, some studies have found no correlation between survivin expression and prognosis.[3,15] Contradictory results have been reported in regards to survivin expression as a marker of malignancy in human mammary tumors. Similar to our results, one study of human mammary tumors found no correlation between survivin expression and tumor grade.[18] However, another group demonstrated survivin to be an independent prognostic factor of poor prognosis.[19] The reason for the contradictions in the human literature is unknown.

One study of human mammary tumors actually found nuclear expression of survivin to be an independent prognostic parameter of good prognosis in mammary carcinomas, but was not associated with histologic grade.[6] In our study, there was no difference in nuclear staining between the adenomas and carcinomas, suggesting nuclear staining can not be used to differentiate benign versus malignant tumors. Perhaps if this study had a larger data set and prognostic data, nuclear staining might have been significant. It would be interesting to examine larger numbers of carcinomas, with varying degrees of nuclear staining, to determine if nuclear staining is associated with survival times in canine malignant mammary tumors. However, the determination of nuclear versus cytoplasmic expression of survivin by IHC should be interpreted with caution, as IHC results may lead to misinterpretation of the expression pattern due to differences in tissue processing.[10] This could result in either false positive or false negative staining. To avoid this potential problem, it is recommended that an alternative approach such as Western blots, using cell lysates from the fractional cytoplasm and nuclei, are performed to confirm survivin localization.[10]

The human survivin gene, as the result of alternatively spliced transcripts, can give rise to four isoforms of the protein: full-length survivin, survivin-2B, survivin-ΔEx-3, and the most recently identified splice variant, survivin-3B. [2,13] Full-length survivin and survivin-2B are primarily found in the cytoplasm, whereas survivin-ΔEx-3 is predominantly present in the nucleus.[12] Full length survivin and survivin-ΔEx-3 can inhibit apoptosis, whereas survivin-2B shows marked reduction in apoptotic activity. Recently it was shown that p53 downregulates survivin and survivin-ΔEx-3 while upregulating survivin-2B in leukemia cells.[23] In addition, survivin-2B inhibited cell growth and made the leukemia cells more sensitive to doxorubicin-induced apoptosis.[23] This suggests that survivin-2B is proapoptotic, acting as an antagonist to full length survivin and survivin-ΔEx-3. A fourth isoform, survivin-3B, has recently been discovered.[2] This isoform most likely has the capability to inhibit apoptosis, however, its involvement in cell division is questionable. Currently it is unknown whether canine survivin isoforms exist. If the canine isoforms are similar to the human isoforms in their distribution pattern, specific isoforms cannot be differentiated solely by their nuclear or cytoplasmic distribution. When this study was designed, identification of specific isoforms by their presence in the nucleus versus the cytoplasm, seemed plausible. Only recently has it been demonstrated that the different isoforms are not necessarily restricted to one subcellular location.[10] Thus, it is unknown in this study if the survivin expressed in the nucleus represented a different canine isoform than the survivin observed in the cytoplasm.

In the canine tumors examined, Ki-67 expression was not higher in the carcinomas. Thus, differential expression of a survivin isoform responsible for increased cell proliferation in the carcinomas over the adenomas, cannot be explained from our data. Although several studies have found a positive correlation between survivin expression and proliferative activity in human malignancies,[5,7,14,20,21] no studies have been performed on dogs. In this study, survivin expression was compared between two groups of canine mammary tumors based on their histologic appearance, rather than clinical outcome, of the dogs following mastectomy. It is possible that some of the malignant tumors were not as aggressive as they appeared histologically.

Ki-67 labeling index has been shown to be of prognostic value in benign and malignant canine mammary tumors.[16] We, however, did not find an association between proliferative index, based on Ki-67, and benign versus malignant tumors in this study. Constitutive expression of survivin in transgenic mice resulted in reduced apoptosis when compared to control mice, whereas there was no difference in cell proliferation between the groups.[4] Thus, survivin expression in neoplastic tissues is not simply a reflection of increased mitotic rate of tumor cells. Additional studies, examining the correlation between proliferative activity and the individual survivin isoforms, may provide further insight into survivin's role in cell proliferation.

In the canine tumors examined, caspase-3 expression was not lower in the carcinomas. Thus, differential expression of a survivin isoform responsible for decreased apoptosis in the carcinomas over the adenomas, can not be explained from our data. In the human literature, several reports have found a positive correlation between survivin expression and reduced apoptotic activity.[5,14,17] Survivin-positive tumors showed decreased apoptosis when compared to survivin-negative tumors of the same type. These observations seem plausible, as survivin has definitely been shown to play a role in the inhibition of apoptosis. However, in a study of human mammary tumors examining the differential expression of survivin variants, two of the survivin splice variants, survivin and survivin-ΔEx-3, positively correlated with apoptosis whereas no correlation was found between survivin-2B and apoptosis.[18] An inverse relationship was noted, however, between survivin-ΔEx-3, tumor size, and number of nodal metastases. Thus, the survivin isoforms may be influenced by other factors, implying that reduction of the apoptotic index cannot simply be explained by the differential expression of the anti-apoptotic survivin variants.

In conclusion, we found no differences in overall survivin expression, nuclear survivin expression, Ki-67 or cleaved-caspase-3 activity, between the adenomas and carcinomas, suggesting that these markers can not be used to differentiate benign from malignant canine mammary tumors. The significance of survivin expression in these tumors remains unknown.

The number of tumors evaluated in this study, however, was small and a larger study is needed before conclusions can be drawn. In our study, we were evaluating adenomas and carcinomas that equally expressed survivin, and attempted to demonstrate the existence of canine survivin isoforms via differences in proliferative and apoptotic indices and subcellular localization. Preferably, it should be determined whether or not canine survivin isoforms exist. If so, antibodies can then be developed against the specific splice variants to directly determine if differences in the isoforms exist. The differential expression of these survivin isoforms in normal and neoplastic canine tissues could then be explored directly.

Materials and methods

Tissue Samples

All animal use in this study was approved by The University of Georgia Institutional Animal Care and Use Committee (IACUC # A2003-10163-c1). The canine mammary tumors were either obtained from dogs undergoing mastectomy at the University of Georgia Teaching Hospital, College of Veterinary Medicine, University of Georgia, or from mammary tumors submitted to the Athens Diagnostic Laboratory, College of Veterinary Medicine, University of Georgia. The 7 benign tumors included simple mammary adenomas and cystadenomas. The 8 malignant tumors were all simple mammary carcinomas. All slides were formalin-fixed, paraffin-embedded, dewaxed, and dehydrated prior to IHC.

Immunohistochemistry

Immunohistochemistry was performed on replicate sections for survivin, Ki-67, and cleaved caspase-3. The DAKO Autostainer was used for the detection of survivin and Ki-67. Slides were hand-stained for caspase-3. All negative controls consisted of substitution of the primary antibody with Rabbit or Mouse Super Sensitive Negative Control (Biogenex). Slides were counterstained with hematoxylin and coverslipped.

For survivin, slides were heated to boiling, for 10 minutes, in 0.01M citrate pH 6.0. Endogenous peroxidase was blocked with a 3% hydrogen peroxide/methanol solution for 20 minutes. Protein Block Serum-Free (DAKO) was applied for 7 minutes, followed by rabbit anti-survivin antibody, diluted 1:150, for one hour. Polymer-linked horseradish peroxidase (DAKO) was then applied for 40 minutes. 3,3'-diaminobenzidine (DAB) was used as the chromagen (Vector Laboratories). The positive control consisted of a canine nasal adenocarcinoma that consistently produced intense staining.

For Ki-67, slides were heated to boiling, for 15 minutes, in 0.01M citrate pH 6.0. Endogenous peroxidase was blocked with a 3% hydrogen peroxide/methanol solution for 5 minutes. Biotin Blocking System (DAKO) was applied for 20 minutes, followed by Protein Block Serum-Free for 5 minutes. Prediluted mouse anti-Ki-67 antibody (DAKO) was placed on the slides for 45 minutes. LSAB2 biotinylated link HRP (DAKO) was applied for 25 minutes, followed by LSAB2 StreptAvidin HRP (DAKO) for 25 minutes. DAB was used as the chromagen. The positive control consisted of a canine hepatocellular carcinoma that had a high percentage of Ki-67- positive cells.

For cleaved caspase-3, slides were placed in BORG Decloaker (Biocare Medical) and steamed for 60 minutes. Protein Block Serum-Free was applied for 7 minutes, followed by rabbit anti-caspase-3 cleaved antibody (Biocare Medical), diluted 1:100, for one hour. MACH 3 Rabbit Probe (Biocare Medical) was applied for 20 minutes, followed by incubation for 20 minutes with MACH 3 R-Polymer ALP (Biocare Medical). Vulcan Fast Red (Biocare Medical) was used as the chromagen. The positive control was canine colon that had approximately 5% caspase-3 -positive epithelial cells.

Statistical Analysis

An unpaired t-test was used to compare nuclear survivin staining, Ki-67, and cleaved caspase-3 staining between the adenomas and carcinomas. The statistical calculations were performed using Microsoft Excel. $P<0.05$ was considered significant.

Antibodies

Antibody against survivin was obtained from R&D Systems (AF886). Antibody against Ki-67 was obtained from DAKO (M7240). Antibody against cleaved caspase-3 was obtained from Biocare Medical (CP229A).

Scoring Criteria

Survivin expression was recorded as the percentage of positive staining cells within the entire tumor and the percentage of positive cytoplasmic and nuclear staining. For Ki-67 and caspase-3 expression, 100 cells were counted in five areas, chosen from areas with the highest percentage of positive Ki-67 and caspase-3 cells. The proliferative and apoptotic fraction of epithelial cells were expressed as the ratio of cells positively staining for Ki-67 and caspase-3 to the 500 cells counted, given as a percentage for each case.

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CHAPTER 6

DISCUSSION

Survivin Expression in Normal and Abnormal Canine and Feline Tissues

Survivin is an inhibitor of apoptosis protein that is necessary for proper cell division and has the ability to inhibit apoptosis. Its expression in embryonal development and virtually all human cancers, but its limited expression in most human terminally differentiated tissues, suggests that reactivation of the survivin gene frequently occurs in neoplastic processes. In addition, human tumors that are survivin positive are often more aggressive and show decreased response to chemotherapy when compared to similar tumor types that are survivin negative. Thus, intense investigations are currently under way in human medicine to determine the role of survivin in human neoplasms, as this protein could potentially be a therapeutic target in cancer.

Nothing is known, however, regarding survivin expression in canine and feline normal or abnormal tissues. Survivin expression was examined in canine and feline embryos, normal adult tissues, inflammatory lesions, a variety of hyperplastic tissues and their neoplastic counterparts, and DH82 cells, a canine macrophage-monocyte cell line. Survivin protein was detected, by immunohistochemistry (IHC), using a polyclonal human anti-survivin antibody. Survivin mRNA was detected by RT-PCR. There was usually a positive correlation between survivin protein expression and survivin mRNA expression. The RT-PCR product for feline fetal survivin had 99% homology to a published cat sequence (GenBank AB182320) and 93-94% homology to several published dog sequences. The RT-PCR product for DH82 cells had 100% homology to published dog sequences.

Consistent with the human literature, survivin was widely distributed in early embryonal development, but was virtually absent by late gestation. Survivin has been shown to be essential for embryonal development. Homozygous disruption of the survivin gene results in the death of mice embryos within 5 days of embryonic development. The cells exhibit failed cytokinesis and disruption of the microtubule network. Survivin expression in adult canine tissues was limited to individual hematopoietic cells in the bone marrow and the corpus luteum of the ovary. Adult feline tissues were negative for survivin with the exception of individual hematopoietic cells of the bone marrow, corpus luteum of the ovary, neuroendocrine cells of the gastrointestinal tract, and endometrial glands within the uterus. This limited distribution of survivin in normal, terminally differentiated tissues is similar to observations in the human literature. In humans, survivin is normally expressed in CD34+ hematopoietic stem cells. Although the individual hematopoietic cells that expressed survivin in adult canine and feline fetal tissues were not immunophenotyped, the limited numbers of bone marrow cells positive for survivin is suggestive of a stem cell population. The fact that survivin is normally expressed in human and animal hematopoietic cells raises the concern, both in human and veterinary medicine, that survivin-targeted therapy for cancer cells could potentially disturb normal hematopoiesis.

The majority of canine and feline carcinomas in each type examined were positive for survivin, with the exception of canine rectal carcinomas and feline ceruminous carcinomas, which were usually negative for survivin. There were fewer positive canine and feline sarcomas than carcinomas. Survivin is expressed in virtually all human malignancies, including carcinomas and sarcomas. In human cancers, survivin expression is almost always associated with a poorer prognosis than tumors of the same type that are survivin-negative. The observation that both survivin-positive and survivin-negative tumors exist within most canine and feline tumor types raises the question of whether or not these survivin-positive tumors represent a more aggressive phenotype. Additional studies are needed to determine if survivin expression in canine and feline cancers is a negative predictor of survival, as is documented in human medicine.

Survivin-positive hyperplastic epithelial and mesenchymal cells were detected in many canine and feline tissues, often expressing the protein as diffusely and/or intensely as their neoplastic counterparts. Lymphocytes, fibroblasts, and reactive endothelial cells within inflammatory tissues were also often strongly positive for survivin. Thus, survivin upregulation is not unique to cancer cells, and may be an important protein in hyperplastic tissues, inflammatory conditions, and wound healing. Multiple pathways are potentially involved in the overexpression of survivin in cancer. It remains to be determined whether the mechanism behind the upregulation of survivin in cancer differs from pathways involved in its upregulation in hyperplastic and inflammatory conditions.

The majority of canine and feline hyperplastic and neoplastic tissues had primarily cytoplasmic staining, with nuclear staining less commonly observed. This variation in subcellular location of survivin could possibly be related to the expression of different isoforms. In humans, two distinct subcellular pools of survivin exist as the result of alternatively spliced variants. In addition, these isoforms vary in there ability to inhibit apoptosis, thus they may provide a regulatory balance between apoptosis and inhibition of apoptosis. Additional studies are needed to determine if survivin isoforms exist in the dog and cat, and if so, whether there is differential expression of these isoforms in canine and feline normal and hyperplastic tissues versus neoplastic tissues.

Survivin Expression in Canine Lymphosarcoma

To find out if survivin is a negative prognostic parameter in canine cancer, a retrospective study was conducted to determine survivin expression in neoplastic lymph nodes of dogs with lymphosarcoma (LSA). All the dogs had undergone chemotherapy at the University of Georgia Small Animal Teaching Hospital, receiving the same chemotherapeutic protocol. There were approximately equal numbers of dogs with survivin-positive versus survivin-negative neoplastic lymph nodes. Survivin expression was shown to be a negative predictor of survival in canine lymphosarcoma, using a student T-test (P=0.002). Survivin expression was not always associated with a particular immunophenotype or WHO substage. These are currently the prognostic factors that are considered the most significant in canine LSA. Multivariate analysis still needs to be performed to determine whether these other prognostic factors influence survivin expression, or if survivin is an independent prognostic factor.

Correlation of Survivin with Subcellular Localization and Apoptotic and Proliferative Indices

The human survivin gene, as the result of four alternatively spliced transcripts, can give rise to four isoforms of the protein. These isoforms vary in their ability to inhibit apoptosis and in their subcellular localization. These isoforms may represent a regulatory balance between apoptosis and inhibition of apoptosis. Presently, little is known in regards to the roles played by these splice variants. A better understanding of the interaction of these isoforms could potentially allow for selective targeting of specific survivin isoforms, resulting in the apoptotic death of cancer cells while not affecting normal cell division.

Currently it is unknown whether canine survivin isoforms exist. To indirectly explore the possibility of the differential expression of canine survivin isoforms, differences between survivin subcellular localization, and proliferative and apoptotic indices were compared between canine mammary adenomas and carcinomas that were all positive for survivin by immunohistochemistry. Differences in nuclear survivin expression or the proliferative rate and/or the apoptotic index between the mammary carcinomas and adenomas could suggest the existence of differentially expressed canine isoforms that vary in their subcellular localization and their ability to affect cell proliferation and/or inhibit apoptosis. The proliferative and apoptotic indices were measured by immunohistochemical detection of Ki-67 and caspase-3, respectively. Differences in nuclear survivin expression, Ki-67, and caspase-3 expression between the benign and malignant tumors were not observed. Thus, from this experiment, strong evidence was not present to support the existence of canine isoforms. If the canine isoforms are similar to the human isoforms in their distribution pattern, specific isoforms cannot be differentiated solely by their nuclear or cytoplasmic distribution. When this study was designed, identification of specific isoforms by their presence in the nucleus versus the cytoplasm, seemed plausible. Only recently has it been demonstrated that the different isoforms are not necessarily restricted to one subcellular location. Thus, it is unknown in this study if the survivin expressed in the nucleus represented a different canine isoform than the survivin observed in the cytoplasm. Recently, it was shown that the survivin isoforms can form dimers. It is possible that the interaction of these isoforms, not just the concentration of the isoforms, determines the role survivin plays in apoptosis. Thus, it would be beneficial to determine if canine survivin isoforms exist prior to determining the roles they may play in cell proliferation and apoptosis.

In conclusion, the information obtained from this research project is similar to findings in the human literature. Continued investigations into survivin expression in veterinary species are needed. This protein may prove to be a useful prognostic parameter in canine and feline cancer, and in the future may be a potential target in the treatment of cancer in these species.

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