Expression and Function of Survivin in Canine Osteosarcoma

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Abstract

Osteosarcoma has a high mortality rate and remains in need of more effective therapeutic approaches. Survivin is an inhibitor of apoptosis family member protein that blocks apoptosis and drives proliferation in human cancer cells where it is commonly elevated. In this study, we illustrate the superiority of a canine osteosarcoma model as a translational tool for evaluating survivin-directed therapies, owing to the striking similarities in gross and microscopic appearance, biologic behavior, gene expression, and signaling pathway alterations. Elevated survivin expression in primary canine osteosarcoma tissue correlated with increased histologic grade and mitotic index and a decreased disease-free interval (DFI). Survivin attenuation in canine osteosarcoma cells inhibited cell-cycle progression, increased apoptosis, mitotic arrest, and chemosensitivity, and cooperated with chemotherapy to significantly improve in vivo tumor control. Our findings illustrate the utility of a canine system to more accurately model human osteosarcoma and strongly suggest that survivin-directed therapies might be highly effective in its treatment. Cancer Res; 72(1); 249–59. ©2011 AACR.

Introduction

Osteosarcoma is the most common primary bone tumor in dogs and humans (1–3) and is characterized by both aggressive local tissue infiltration and a very high metastatic rate. Despite the use of neoadjuvant and adjuvant chemotherapy, the 5-year survival rate is only 60% in humans and the 2-year survival rate is only 20% in dogs, illustrating that new therapeutics are needed (1).

The dog is a well-established model for spontaneous osteosarcoma in humans, owing to striking similarity in biology and gene expression (3, 4). The large size of dogs, relative outbreeding, and immunocompetence increase their model potential. Furthermore, dogs with spontaneous tumors naturally develop therapy resistance and metastasis. In addition, tumor burdens in spontaneously arising cancers of dogs are more similar to humans than the experimentally induced tumors found in murine models, which may be important with regard to biologic factors such as hypoxia and clonal variation. The size of canine tumors also allows for serial imaging and tissue collection over time (3, 4).

Survivin is a 16.5-kD protein belonging to the inhibitor of apoptosis (IAP) family (5). The survivin gene has 5 known mRNA splice variants: survivin, survivin-2B, survivin-ΔEx3, survivin-3B, and survivin-26 (6). Unlike other IAP family members, survivin has 2 known functions in cells: regulation of cell division and inhibition of apoptosis (7). Survivin is found as a monomer in the chromosomal passenger complex (CPC) and binds via its C terminal to the microtubules of the mitotic spindle during mitosis (8). Survivin is dimeric in its role as an IAP molecule (9) and is thought to have targets upstream of effector caspases or target effector caspases themselves (10, 11). Evidence suggests that survivin plays a role in inhibiting both caspase-dependent and caspase-independent apoptosis (12, 13). Survivin may also indirectly inhibit apoptosis, via binding to the proapoptotic protein Smac/DIABLO, preventing it from binding to and inhibiting other IAP molecules (14). Some survivin is localized to the mitochondria and is thought to prevent apoptosis upon release as well as block the release of apoptosis-inducing factor (AIF; ref. 13). Both cytoplasmic and mitochondrial survivin are thought to inhibit apoptosis via binding to other proteins, possibly the effector caspases or other associated proteins (10, 15). More recent evidence suggests that survivin also enhances telomerase activity and may play a role in chemotherapy resistance and metastasis (16–19).

Normal cells do not require survivin for survival (20). In cancer cells, however, survivin is critical for its roles in cell division and inhibition of apoptosis (5). It also seems to have roles in tumorigenesis (15, 21) and drug resistance (17, 18). Hematopoietic progenitor cells, thymocytes, and T cells express survivin at low levels for cell proliferation (22–24); however, differentiated cells do not express survivin. More specific to the purpose of our study, survivin is only expressed at very low levels in normal osteoblasts (25). Most types of cancer express survivin at very high levels and depend on it for continued proliferation (5, 20).

Survivin expression has prognostic significance in many types of human cancer (26). Small studies in human
osteosarcoma have suggested that survivin may be useful in determining prognosis and degree of malignancy (27–29); however, definitive studies about the role of survivin in human osteosarcoma are lacking. Survivin expression is a negative prognostic factor in dogs with B-cell lymphoma (30), as has been shown in human B-cell lymphoma (31), and survivin expression has been identified in other canine neoplasms (30).

In this study, we sought to determine the effects of survivin inhibition in canine osteosarcoma cell lines and to evaluate a correlation between survivin expression and outcome in canine patients with osteosarcoma. We hypothesized, as observed in human osteosarcoma, that survivin inhibition would decrease cell proliferation and increase apoptosis and chemo sensitivity in canine osteosarcoma cells and that increased survivin expression would correlate with a poor prognosis in canine patients with osteosarcoma.

Materials and Methods

Cell lines and conditions

The Abrams canine osteosarcoma cell line was provided by Dr. William Dernell, and the D17 canine osteosarcoma cell line was purchased from American Type Culture Collection. Both cell lines were serially passaged by trypsinization and maintained in C/10 media [Minimum Essential Medium (MEM); Lonza, supplemented with 1 \times \text{MEM vitamin solution (Cellgro), 2 mmol/L L-glutamine (Cellgro), 1 mmol/L sodium pyruvate (Cellgro), 1 \times \text{nontessential amino acid solution (Cellgro), 1 \times \text{antibiotic/antimycotic (Cellgro), and 16\% heat-inactivated FBS (Atlas)}]. Cells were grown in a humidified incubator with 5% CO2 at 37°C. Both cell lines were confirmed to be of canine origin by multispecies multiplex PCR and identified by short tandem repeat analysis as described (32).

siRNA transfection

A custom siRNA against canine survivin (430) and a scrambled siRNA control were designed with an online resource (BLOCK-IT RNA Designer; Invitrogen) and purchased from Invitrogen. Transfection was accomplished with HiPerFect transfection reagent (Qiagen) in 100 \muL Opti-mem media (Invitrogen). Transfected cells were harvested for analysis 24 to 72 hours following transfection. Detailed transfection methods are included in the Supplementary Data.

Survivin expression

Detailed methods for the techniques described later are included in the Supplementary Data.

Quantitative reverse transcriptase PCR. We evaluated survivin mRNA in the survivin siRNA–transfected, sham-transfected, and control cells at 48 hours posttransfection by real-time reverse transcriptase (RT)-PCR. Primers for canine survivin and the housekeeping gene, hypoxanthine phosphoribosyltransferase, were designed with Integrated DNA Technologies’ (IDT) website and purchased from IDT (Supplementary Table S1).

Immunofluorescence. Transfected cells were washed, fixed with methanol, and air-dried, followed by incubation in rabbit polyclonal anti-survivin antibody (Novus Biologicals) followed by Alexa Fluor 594 goat anti-rabbit IgG (H + L; Invitrogen). The slides were washed again, then mounted with VectaShield plus 4,6-diamidino-2-phenylindole (DAPI) mounting medium (Vector Laboratories). Images were acquired with a Zeiss Axioplan 2 imaging microscope and Axio Vision Release 4.6 software (Carl Zeiss Micro-Imaging Inc).

Western blotting

At 48 hours posttransfection, the cells were harvested, protein quantified, and equal amounts loaded onto a 4% to 12% Bis-Tris gel (Invitrogen), followed by electrophoresis and transfer to a polyvinylidene difluoride (PVDF) membrane. The membrane was then blocked in 5% nonfat dry milk in TBS with Tween-20 (TBST), followed by incubation with rabbit polyclonal anti-survivin (Novus) followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Pierce). Protein bands were detected with SuperSignal West Femto Maximum Sensitivity ECL Substrate (Thermo Scientific) using radiographic film (Kodak). The images were scanned and pixel density assessed with Image J (NIH, Bethesda, MD).

Cell number and viability

To determine cell numbers, total and live/dead cell numbers were counted in triplicate at 24, 48, 72 hours posttransfection with trypan blue.

Apoptosis

Caspase-3/7 assay. To determine levels of apoptosis, we used a Sensolyte Homogenous AMC Caspase-3/7 Assay Kit (AnaSpec). At 48 hours posttransfection, the cells and their supernatants from each individual well were harvested from 6-well plates and centrifuged at 1,500 rpm for 7 minutes. The media was aspirated off, and the cells were lysed with 1 \times lysis buffer (AnaSpec). Next, the lysates were transferred to eppendorf tubes and inverted for 30 minutes at 4°C. Then, the lysates were centrifuged at 2,500 \times g for 10 minutes at 4°C. The supernatant was collected from each sample and transferred at 60 \muL per well to a 384-well black-walled plate in duplicate. Twenty microliters of caspase-3/7 assay reagent mix was added to each well, and the plate was put on a plate shaker for approximately 60 minutes at 100 to 200 rpm in the dark at room temperature. The plate was then read on a microplate reader (Synergy HT, Bio-Tek) to measure fluorescence intensity at 360/460 nm. Fluorescence intensity measurements were termed "Relative Fluorescent Units" or RFUs in graphical presentations.

TUNEL assay. For further analysis of apoptosis, we used a commercial terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) kit (In Situ Cell Death Detection Kit; Roche Diagnostics). At 48 hours posttransfection, 2-well chamber slides were rinsed in PBS and air dried overnight followed by fixation (4% paraformaldehyde in 1 \times PBS) for 1 hour at room temperature. The slides were rinsed in 1 \times PBS and incubated in permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate in 1 \times PBS) for 2 minutes at 4°C. The slides were rinsed 2 more times in 1 \times PBS, then 200...
μL of TUNEL reaction mixture was added. Slides were incubated for 60 minutes in the dark at 37°C, rinsed 3 times in 1× PBS, and mounted with VectaShield plus DAPI mounting medium. Microscopic images of random 20× fields were obtained.

**Cell-cycle analysis**

Propidium iodide (PI) staining and flow cytometry was used to evaluate changes in cell-cycle distribution following siRNA transfection. The cells were trypsinized, resuspended in 1.5 mL 1× PBS, and mounted with VectaShield plus DAPI mounting medium. The cells were then placed on ice for 30 minutes or overnight at −20°C. After this incubation, the cells were centrifuged at 1,500 rpm for 7 minutes, washed once in PBS, then resuspended in 250 μL 1× PBS. Two hundred μL of extraction buffer (192 μL of 0.2 mol/L Na2HPO4, 8 μL of 0.1 mol/L citric acid), followed by 500 μL PI-RNase reagent (50 μg/mL PI, 125 Worthington U/mL RNase) was added. Cells were filtered through a 40-μm nylon cell strainer and incubated at 37°C for 30 minutes. Samples were then run on a FACScan flow cytometer (BD Biosciences). Cell-cycle analysis on samples was conducted with FlowJo Software (Tree Star).

**Chemosensitivity**

To determine sensitivity to carboplatin (Amatheon) or doxorubicin (Bedford Laboratories), cells were incubated with no drug, 21.55 μmol/L carboplatin, or 125 nmol/L doxorubicin. Drug was added for 36 to 48 hours directly after a 24-hour transfection. Cells were then harvested from their individual wells with their supernatants, and their individual wells were counted. Four RFUs for each sample were normalized to cell count (RFU/cell) and results expressed as fold change versus control (untreated cells). Degree of synergy between survivin knockdown and chemotherapy was evaluated using the Bliss independence model (see Supplementary Data).

**Murine xenograft experiment**

Female 6- to 8-week-old nu/nu mice were purchased from the NIH. EZN-3042, a survivin inhibiting locked nucleic acid antisense oligonucleotide (33, 34), and EZN-3046 (scrambled control oligonucleotide) were supplied by Enzon Pharmaceuticals. After demonstration of gene knockdown efficacy in vitro (not shown), mice were injected subcutaneously with 2 × 10⁶ Abrams osteosarcoma cells. Tumors were grown to 7-mm diameter, size matched, and then allocated into 4 groups (n = 8 per group). Group 1 received saline and EZN-3046, group 2 received doxorubicin (Bedford) and EZN-3046, group 3 received saline and EZN-3042, and group 4 received doxorubicin and EZN-3042. EZN-3042 and EZN-3046 were administered intraperitoneally at 100 mg/kg every 3 days starting day 0 until the end of the study (day 55). Doxorubicin or an equivalent volume of saline was administered intravenously at 3 mg/kg on day 4 and repeated every 2 weeks until the end of the study. Mice were weighed and tumors measured every 3 days. Mice were sacrificed at 15-mm tumor diameter or at the end of the study if they were still alive. A separate group of tumor-bearing mice were randomized similarly at 10-mm tumor diameter. They received EZN-3042 or EZN-3046 on days 0 and 3 (a.m.) and doxorubicin or saline on day 3 (p.m.) and sacrificed on day 4. Tumors were harvested and snap-frozen or paraffin embedded for analysis of survivin expression by qRT-PCR as described earlier and immunohistochemistry as described later.

**Canine osteosarcoma patient population**

The population of canine patients with appendicular osteosarcoma that was studied was a subset of patients from a previously reported randomized, prospective clinical trial (35). The study was approved by the Institutional Animal Care and Use Committees of the participating institutions. All dogs underwent amputation followed by 5 cycles of adjuvant doxorubicin, with or without an investigational matrix metalloprotease inhibitor and had decalcified primary tumor tissue blocks available for analysis. Inclusion/exclusion criteria, staging, and follow-up procedures were standardized as previously reported (35). Histologic grading (from 1–3) was carried out in a subset of cases by one author (B.E. Powers) using a published schema incorporating amount of matrix, percentage of necrosis, nuclear pleomorphism, nuclear size/number, and mitosis score (35). Mitotic index was also calculated by counting the number of mitotic figures per 10 random 400× fields.

**Survivin immunohistochemistry**

Slides of canine osteosarcoma tissues were prepared from paraffin blocks. Slides were put through a hydration process of xylene baths to graded alcohol, then immersed in Target Retrieval Solution (DakoCytomation) and put through a pressure cooker cycle and cooled to room temperature. The slides were then washed in TBST, blocked with Biocare Sniper (Biocare Medical) for 10 minutes, then washed again. Incubation in primary rabbit polyclonal anti-survivin antibody, at 1:600 dilution occurred overnight at 4°C. The slides were washed 3 times before a 15-minute incubation in 3% hydrogen peroxide at room temperature and washed 3 additional times. Incubation in secondary antibody, Envision + Dual Link System Peroxidase (Dako) for 30 minutes occurred at 4°C. The slides were washed 3 more times, chromogen stained for 10 minutes by DAB Peroxidase Substrate Kit (Vector), washed once more, and lightly counterstained with hematoxylin. The slides were graded on the basis of survivin stain intensity (0 = negative, 1 = weak, 2 = moderate, 3 = strong, and 4 = intense) and proportion of cells with positive survivin staining (0.0%; 1, 1%–10%; 2, 10%–25%; 3, 25%–50%; 4, >50%). A final immunoreactivity score for each tissue sample was calculated by multiplying the percentage score by the intensity grade (possible score of 0–16). Scoring was carried out by 2 individuals blinded as to patient outcome and the final survivin score averaged across the 2 raters. This scoring system has previously been used in immunohistochemical scoring of canine osteosarcoma samples (36) and for survivin scoring in canine lymphoma (30).
**Statistical analysis**

Statistical analysis of *in vitro* data were conducted with GraphPad Prism for Macintosh Version 5.0b (GraphPad Software). Survivin expression levels were summarized by standard descriptive statistics in terms of means and SDs. The comparisons of survivin expression, cell numbers, caspase activity, and apoptosis levels between experimental conditions was carried out using a 2-sample *t* test with a 2-sided significance level. Tumor growth between groups was evaluated using a one-way ANOVA. The Shapiro–Wilk test was used to verify the normality assumption. *P* values of less than 0.05 were considered statistically significant.

The Intraclass correlation coefficient (ICC) was used to evaluate the reproducibility of the survivin immunoreactivity scores between the 2 independent raters. The ICC can be interpreted as follows: 0–0.2 (slight), 0.2–0.4 (fair), 0.4–0.6 (moderate), 0.6–0.8 (substantial), and 0.8–1.0 (almost perfect; ref. 37).

Statistical analysis of survival data were conducted with a combination of Prism and SAS software version 9.2 (SAS Institute). Correlations between survivin expression levels and other markers on a continuous scale were evaluated using linear regression analysis. ANOVA was used to evaluate the association between survivin expression levels and categorical markers. The median disease-free interval (DFI) was estimated using the Kaplan–Meier method. Markers were categorized into a low risk and high risk group (with respect to predicting DFI) using the recursive portioning method (38). This method selects the best predictor variables using recursive splitting. It starts with the best possible predictor from the data set and successively splits the data into categories predicted to observe the event or not. As a splitting method, the exponential scaling method was used. The splitting process stopped when a minimum of 5 patients per group was reached or when there was no further decrease in prediction error. The associations between the categorized markers and DFI were evaluated using the log-rank test. Multivariate Cox proportional hazard regression analysis was used to determine the prognostic significance of the markers for predictive value of DFI. Predictive markers were selected by backward selection procedure with a *P* value cut off of less than 0.10. A previously deleted variable was allowed to reenter the final model if its *P* value was less than 0.05. The likelihood ratio test was used to compare various models. The proportional hazard assumption was verified using plots of the log(-log) survival curves and Schoenfeld residuals.

**Results**

**siRNA-mediated knockdown decreases survivin expression**

After preliminary validation of survivin as a viable target by confirming elevated survivin expression in 4 of 4 canine osteosarcoma cell lines by Western blot analysis (data not shown), we proceeded with survivin knockdown experiments. To verify the efficiency of the survivin knockdown in our survivin siRNA transfection, we harvested mRNA from the survivin knockdown, sham knockdown, and control cells at 48 hours post siRNA transfection. Analysis of the qRT-PCR data revealed approximately 20-fold and approximately 9-fold decreases in survivin mRNA expression in Abrams and D17, respectively, in the survivin knockdown cells when compared with control and sham knockdown cells (Fig. 1A).

Because inhibition of survivin mRNA expression does not confirm decreased survivin protein, we carried out further experiments to verify decreased protein expression. Western blot analysis of Abrams and D17 cells confirmed approximately 85% reduction in protein expression in survivin siRNA–transfected groups compared with the sham-transfected and control groups (Fig. 1B and C). Immunofluorescence cytochemistry further confirmed reduced survivin protein expression. On the basis of immunofluorescence assessment, knockdown efficacy was 80.2% ± 7.1% for Abrams and 76.2% ± 9.4% for D17 (Supplementary Fig. S1).

**Survivin inhibition decreases total cell number and cell viability**

Total and live/dead cell counts were conducted at 24, 48, and 72 hours post survivin knockdown in both cell lines. At all 3 time points, there were significantly decreased total cell numbers and significantly higher percentage of dead cells in the survivin siRNA–transfected compared with both the sham-transfected and control groups (Supplementary Fig. S2).

**Survivin inhibition increases apoptosis**

To determine whether the cell death observed was via apoptosis, we evaluated caspase-3/7 activity in the survivin siRNA–transfected, sham-transfected, and control cells 48 hours posttransfection. There was a significant increase in caspase activity in survivin siRNA–transfected cells compared with their respective sham-transfected and control cells (Fig. 2A). This was confirmed by observation of increased DNA fragmentation 48 hours post siRNA transfection, as assessed via TUNEL (Fig. 2B).

**Survivin inhibition results in failure of normal mitosis**

We used flow cytometry to analyze cell-cycle distribution 48 hours post siRNA transfection. In addition to an increase in the sub-G1 population consistent with previous observations about apoptosis, we observed an increase in a 4N population of cells, consistent with failure of mitosis, in survivin siRNA–transfected cells. Compared with control-transfected cells, the sub-G1 population increased an average of 3.0- and 1.9-fold in Abrams and D17, respectively, and the super-G2 (4N) population increased an average of 3.5- and 2.5-fold in Abrams (Fig. 3A and B) and D17 (Fig. 3C), respectively.

**Survivin inhibition increases chemosensitivity**

Doxorubicin and platinum drugs form the mainstays of medical therapy for the treatment of both canine and human osteosarcoma. To determine whether survivin was important in mediating resistance to chemotherapy in canine osteosarcoma, we incubated canine osteosarcoma cells with carboplatin or doxorubicin, with or without concurrent survivin or sham siRNA transfection. Survivin knockdown increased chemotherapy resistance in canine osteosarcoma in vitro.
caspase activity in both cell lines in the presence of doxorubicin and carboplatin (Fig. 4).

Survivin inhibition in canine osteosarcoma xenografts results in increased doxorubicin sensitivity
Survivin knockdown in vivo was accomplished using the locked nucleic acid antisense inhibitor EZN-3042. Mice bearing established xenografts of Abrams canine osteosarcoma cells were randomized to receive doxorubicin (DOX) or saline, EZN-3042 or the scrambled control oligo EZN-3046. Knockdown was confirmed in vivo by both qRT-PCR (not shown) and immunohistochemistry following 2 intravenous oligo treatments (Fig. 5A). Notably, there was a significant increase in survivin protein expression in both cell lines, 430, survivin siRNA; scrm, sham knockdown (scrambled) siRNA. Error bars in (C) represent SD. 430, survivin siRNA; scrm, sham knockdown (scrambled) siRNA. Error bars in (C) represent SD. *P < 0.05 versus control and sham transfection.

Figure 1. Efficiency of survivin gene knockdown in canine osteosarcoma cells. A, qRT-PCR for survivin mRNA in Abrams (Ab) and D17 cells showed an approximately 20-fold and approximately 9-fold decrease, respectively, in survivin siRNA when compared with the control (cntrl) and sham knockdown groups. B, Western blot analysis confirms survivin knockdown in both cell lines. C, using ImageJ analysis, we measured the integrated density of the survivin protein bands following 3 separate survivin siRNA transfections. There was approximately 85% reduction in survivin protein expression in both cell lines, 430, survivin siRNA; scrm, sham knockdown (scrambled) siRNA. Error bars in (C) represent SD. *P < 0.05 versus control and sham transfection.

Survivin in Canine Osteosarcoma
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Survivin protein expression in canine osteosarcoma tissues correlates with histologic features and clinical outcome
Survivin expression was studied via immunohistochemistry in 67 primary canine osteosarcoma tissues from dogs that underwent standardized staging, treatment, and follow-up as part of a previously reported prospective clinical trial (35). The ICC of the final survivin immunoreactivity score between the 2 independent raters was 0.90 [95% confidence interval (CI); 0.84–0.93], indicating a very high level of reproducibility. Demographic information about the patient population is reported in Supplementary Table S2. Survivin was expressed in 65 of 67 cases evaluated, with expression intensity ranging from modest to heavy (Supplementary Fig. S3). Median survivin immunoreactivity score was 5 (range, 0–12). Staining was predominantly nuclear, although a combination of nuclear and cytoplasmic staining was observed in most samples. Survivin immunoreactivity score correlated positively with both histologic grade and mitotic index (Figs. 6A and B).

The overall median DFI in the studied patient population was 211 days (range, 43–1,393 ± d). Upon univariate analysis, histologic grade (1/2 vs. 3), bone-specific alkaline phosphatase (BALP) activity (≤48 vs. >48), and survivin immunoreactivity score (≤2.75 vs. >2.75) were identified as significant predictors of DFI (Table 1, Fig. 6C). The median DFI in patients with a lower survivin immunoreactivity score (≤2.75) was 331 days versus 173 days in patients with a higher survivin immunoreactivity score (>2.75; P = 0.046).

As observed in the original clinical trial (35), drug assignment did not impact DFI. Upon multivariate analysis, BALP and histologic grade retained prognostic significance (Table 1). Survivin immunoreactivity was not identified as an independent significant predictor of DFI in the multivariate analysis, owing in large part to the strong positive correlation between survivin immunoreactivity and histologic grade.

Discussion
To assess the utility of canine osteosarcoma as a potential model for survivin-directed therapeutics, we sought to determine the impact of survivin inhibition on canine osteosarcoma cell lines in vitro, as well as the prognostic significance of
Survivin expression in primary canine osteosarcoma treated with surgery and chemotherapy. Survivin inhibition in Abrams and D17 canine osteosarcoma cell lines induced apoptosis, mitotic arrest, and increased caspase activity in the presence of carboplatin and doxorubicin. Furthermore, inhibition of survivin was associated with an increase in the efficacy of doxorubicin in a murine osteosarcoma xenograft. Other research groups have reported similar results when indirectly and directly inhibiting survivin in osteosarcoma. In one recent article, inhibition of STAT3 activity (which caused downregulation of survivin expression) in canine and human osteosarcoma decreased cell proliferation and viability and induced caspase-3/7-mediated apoptosis in treated cells (39). Another group inhibited survivin in HeLa cells and observed caspase-dependent cell death as well as mitotic failure, resulting in multinucleated cells, up to 8 and 16N (40). This observed increase in 4N(+) population could be attributed to the importance of survivin in the chromosomal passenger complex (8) and its association to the mitotic spindle during mitosis (7, 20). In addition, siRNA-mediated survivin inhibition in human MG-63 osteosarcoma cells and short hairpin RNA (shRNA)-mediated survivin inhibition of human SAOS2 osteosarcoma cells enhanced sensitivity to cisplatin and doxorubicin (41, 42).

We observed significantly increased apoptosis in survivin knockdown compared with the sham knockdown and control cells in both canine osteosarcoma cell lines in the absence of any proapoptotic stimulus (e.g., serum withdrawal or chemotherapy). There was also modestly increased apoptosis in the sham knockdown compared with the control for both cell lines. We speculate that the enhanced basal apoptosis observed in the survivin knockdown was possibly due to the cellular stress imparted by the siRNA transfection process combined with the survivin inhibition. The modest increase in apoptosis observed in the sham knockdown cells supports this observation.

Figure 2. Survivin knockdown induces apoptosis in canine osteosarcoma cells. A, forty-eight hours following survivin siRNA knockdown, activated caspase-3/7 activity (A) and TUNEL staining (B) were evaluated using ELISA and immunofluorescence, respectively. A significant increase in caspase activity and TUNEL reactivity was observed following survivin knockdown. Error bars indicate SD. *, P < 0.0001 versus control and sham transfection. scrm, sham knockdown (scrambled) siRNA.
It is interesting to note that the growth curves in the murine xenografts did not begin to diverge until after the second doxorubicin dose. It is possible that multiple EZN-3042 treatments enhanced gene knockdown beyond what was observed at the time we evaluated expression (following 2 injections), leading to a more pronounced effect on survivin expression. It is also worth noting that cotreatment with EZN-3042 and doxorubicin resulted in tumor stabilization rather than regression in the Abrams xenograft model. This could be a function of the doxorubicin dose intensity (supported by the lack of single-agent effect of doxorubicin in this experiment) or dosage and scheduling of EZN-3042 treatments relative to doxorubicin.

Elevated survivin protein immunoreactivity in canine osteosarcoma tissue samples correlated with increased histologic grade and mitotic index as well as decreased DFI upon univariate analysis. Survivin immunoreactivity lost prognostic significance upon multivariate analysis owing to a strong correlation between survivin score and histologic grade. The correlation of increased survivin protein immunoreactivity to increased mitotic index is not surprising considering our in

Figure 3. Cell-cycle changes following survivin knockdown in canine osteosarcoma cells. Cell-cycle analysis was conducted 48 hours following survivin knockdown. A representative histogram for the Abrams cell line is shown in (A) showing increases in both the sub-G₁ (apoptotic) and super-G₂ (4N) populations, as indicated by arrows. B, Means (± SD) of 4 independent transfections showing significantly increased sub-G₁ and super-G₂ populations following survivin knockdown in the Abrams cell line. Error bars indicate SD. * P < 0.05 compared with control and sham-transfected cells. C, Means (± SD) of 4 independent transfections showing significantly increased super-G₂ population following survivin knockdown in D17 cell line. There is also a trend toward increased sub-G₁ populations following survivin knockdown. Error bars indicate SD. * P < 0.05 compared with control and sham-transfected cells. scrm, sham knockdown (scrambled) siRNA.
in vitro findings that survivin inhibition caused mitotic failure and survivin’s known roles in mitosis and the cell cycle (6).

Our results in canine osteosarcoma are comparable with the limited information about survivin expression and outcome in human osteosarcoma. One group has reported that nuclear localization of survivin correlated with a positive outcome but did not report whether overall survivin expression had an impact on DFI or survival (27). Another group associated survivin expression with histologic grade, differentiation, and proliferation index (28). Most recently, high survivin mRNA expression has been correlated with both presence of metastasis and overall survival (29).

Survivin is a viable target for therapy. YM155, a small-molecule suppressor of survivin, is currently in phase II clinical trials in human cancer. Single-agent objective responses have been observed in patients with melanoma and non–small cell lung cancer (43, 44) as well as regression of established human hormone refractory prostate cancer in xenograft models (45). Studies in combination with chemotherapy are ongoing. EZN-3042, the antisense oligonucleotide used in these in vivo experiments, is capable of inhibiting survivin expression and tumor growth in vivo (33) and improves chemotherapeutic response in vitro (46). EZN-3042 is currently in phase I clinical trials in human cancer. Survivin is also being considered as an

Figure 4. Survivin knockdown increases canine osteosarcoma chemosensitivity. Abrams and D17 cells were treated for 48 hours with 21.55 μmol/L carboplatin (Carbo) or 125 nmol/L doxorubicin, and survivin knockdown. Caspase-3/7 activity was then determined by ELISA. Survivin knockdown significantly enhanced caspase activity in cells exposed to carboplatin and doxorubicin.

Degree of synergy was assessed with Bliss analysis. *, additive enhancement of caspase activity; **, synergistic enhancement of caspase activity. scrm, sham knockdown (scrambled) siRNA.

Figure 5. Survivin inhibition in vivo results in decreased tumor growth when combined with doxorubicin. A, EZN-3042, a survivin inhibiting locked nucleic acid antisense oligonucleotide, caused a significant decrease in survivin protein expression in Abrams canine osteosarcoma xenografts compared with tumors treated with a scrambled control oligonucleotide (EZN-3046). *, P < 0.01 compared with saline/EZN-3046. **, P < 0.05 compared with saline/EZN-3046 and DOX/EZN-3046. B, mice treated with DOX/EZN-3042 had significantly decreased tumor growth when compared with other treatment groups. Arrows represent doxorubicin treatments.

Survivin immunoreactivity correlates with grade, mitotic index, and outcome in canine osteosarcoma. A, primary canine appendicular osteosarcomas of histologic grade 2 or 3 had higher survivin immunoreactivity scores than did grade 1 tumors. B, there was a significant correlation between survivin immunoreactivity and mitotic index in primary canine osteosarcomas. C, canine patients with osteosarcoma treated with amputation and doxorubicin with high survivin immunoreactivity scores had a significantly inferior DFI on univariate analysis. hpf, high-power field.

Table 1. Univariate/multivariate analysis of factors associated with outcome in dogs with appendicular osteosarcoma treated with amputation and doxorubicin

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<td>0.487</td>
<td>0.0316</td>
<td>0.25–0.96</td>
</tr>
<tr>
<td>3</td>
<td>153 (70–486)</td>
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<table>
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<tr>
<th>Multivariate analysis</th>
<th>HR</th>
<th>P</th>
<th>95% CI</th>
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<tr>
<td>BALP</td>
<td></td>
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<tr>
<td>≤48</td>
<td>0.272</td>
<td>0.0052</td>
<td>0.11–0.69</td>
</tr>
<tr>
<td>&gt;48</td>
<td></td>
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<tr>
<td>Histologic grade</td>
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<tr>
<td>1/2</td>
<td>0.275</td>
<td>0.0088</td>
<td>0.11–0.72</td>
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</table>
immunotherapy target (47, 48). Phase I and II clinical trials of survivin-targeted vaccines are currently under way.

In conclusion, we have shown that transient survivin knockdown in canine osteosarcoma cells results in decreased total and viable cell numbers, increased apoptosis and mitotic arrest, and enhanced sensitivity to carboplatin and doxorubicin. Furthermore, elevated survivin expression in canine osteosarcoma tissue correlates with increased histologic grade, increased mitotic index, and decreased DFI. These findings are consistent with those in human osteosarcoma and indicate that survivin may be a viable therapeutic target for evaluation in canine osteosarcoma as a preclinical model for human osteosarcoma. There remains substantial room for improvement in the medical therapy for osteosarcoma, and canine osteosarcoma may provide a novel translational model for the investigation of survivin-directed therapeutics.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

6. Li F, Ling X. Survivin study; and update of “what is the next wave?” J Cell Physiol 2006;208:476–86.


