Prediction of chemosensitivity of feline injection site sarcomas based on expression of DNA damage checkpoint and repair proteins in tumor tissue: a pilot study

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Abstract

Fibrosarcomas arise at sites commonly used for injection in cats, such as subcutaneously or intramuscular in the interscapular region and thigh. Various studies demonstrated a relation between the tumors at sites of injections and vaccinations, and it is suggested a species specific local immune response, (inflammatory reaction to) adjuvants and host factors contribute to the pathogenesis of tumor development. Prevalence of injection site sarcoma (ISS) in the US and the Netherlands is relatively low. However, this type of cancer affects significantly younger cats and their tumors are significantly larger than with other types of tumors. Injection induced sarcomas grow invasively and recur more rapidly and frequently than sarcomas at other locations. Metastatic potential varies from 0 to 28% in most cases. Unfortunately, most current available treatment options for feline ISS, including surgery and adjuvant radiation therapy or adjuvant chemotherapy, show high recurrence rates ranging from 41% to 45%. Therefore, additional therapies are needed. Doxorubicin and Carboplatin are commonly used chemotherapeutic agents that have shown the most promise in feline ISS.

The goal of this pilot study was to determine if expression of DNA damage checkpoint and repair proteins correlates with chemosensitivity of feline injection site sarcomas. It was hypothesized that alterations in DNA damage response mechanisms modulate chemosensitivity of feline injection site sarcomas, explaining the inconsistent benefit of adjuvant chemotherapy and the low response rates noted for macroscopic disease.

Biopsies from sarcomas on three different cats were used in this project, one of which was not an injection site sarcoma but a fast growing recurrent sarcoma on the head with similar characteristics. This cat was included for purposes of comparison. Cells were grown in primary cell culture, showing variable in vitro growth patterns. Colony forming assays using several cell densities were done to determine optimum cell density for further colony forming assays (CFAs). We determined the optimal density for CFAs to be 1,000 cells/ml. This density was used in pilot CFA's treated with Carboplatin at two different dosages. Dosages of 20 µg/ml Carboplatin and 40µg/l Carboplatin were tested. Cells from all three cat sarcomas failed to grow colonies at 20µg/ml and 40µg/ml, however positive control did show colony formation. We determined the dosage of Carboplatin would need to be less than 20µg/ml in CFAs in order to detect a difference in chemosensitivity between sarcoma cells derived from different feline patients. HE staining of both true ISSs was done to study tumor morphology. The tumor cells showed similar morphology with intertwining spindle shaped cells, most of these had one ovoid basophilic nucleus and pale eosinophilic cytoplasm. This is conform what has been described on tumor morphology of feline ISS. Immunohistochemistry was performed with antibodies against γH2AX , checkpoint proteins Atm an p53, and DNA damage proteins ERCC1 and Rad51. IHC for γH2AX stained positive, indicating presence of DNA damage in the sarcoma cells of the feline patients in this study. IHC for Atm showed pervasive staining. IHC for p53, ERCC1 and Rad51 did not stain positive in any slide, but no positive control was used.

From the results of this research project, it could be concluded that sarcoma cells derived from different feline patients show variable in vitro growth patterns. DNA damage was detected in feline sarcomas, based on positive IHC staining of γH2AX in these cells. It is unknown whether there is upregulation of Atm or if optimalization of the IHC protocol is needed. IHC for p53, ERCC1 and Rad51 did not stain positive in any slide. It is unknown if antibodies used could not detect feline protein, or

if optimalization of the IHC protocol is needed. Many methods of the study need to be optimalized in order to obtain useful results, and evaluation of additional samples is needed and is going. Ultimately we hope expression of these proteins can be used to target patients to specific chemotherapy regimens.

Introduction

Feline injection site sarcomas (ISS)

Epidemiology

Two decades ago veterinarians got alerted to the fact that (fibro)sarcomas could arise at sites commonly used for injection in cats, such as subcutaneously or intramuscular in the interscapular region and thigh.[1-3,7-10] Various studies demonstrated a relation between the tumors at sites of injections and vaccinations.[1,3,7,8] Prevalence of ISS in the US and Canada is determined in numerous studies within different vaccinated groups and varies from 0.63 per 10,000 cats to 13 per 10,000 cats.[1,8] In contrast, the incidence of feline ISS in The Netherlands is extremely low.[4] It is difficult to determine the exact incidence of ISS in cats since the latency period after injection is extremely variable.[1,3,8]

It seems that significantly younger cats suffer from ISS than seen with sarcomas at other sites, and their tumors are significantly larger. The latter could be due to rapid growth of the tumor.[1-4,11] The average age at which cats develop an ISS has a bimodal distribution with a peak at 6-7 years as well as 10-11 years.[3] On palpation, strands of tumor tissues could be felt extending to the underlying musculature and/or dorsal spinal processes.[1] These injection induced sarcomas grow invasively and recur more rapidly and frequently than sarcomas at other locations. Sarcoma development after vaccination occurs with an interval that varies from 1 month to 10 years. Metastases develop in 0 to 28% of the cases (including treated cats) and could be seen most often in lungs, but also lymph nodes, other internal tissues such as mediastinum, pericardium and liver but also pelvis. Metastatic rate is low in most cases.[1-4,7-9,11]

Etiopathogenesis

It is suggested a species specific local immune response, (inflammatory reaction to) adjuvants and host factors (e.g. mutations in tumor suppressor genes) contribute to the pathogenesis of tumor development.[1-4,7-9] Cats tend to react with a more vigorous (chronic) inflammatory response to injection than other domestic species. The inflammatory reaction has been described as granulomatous. Inflammatory reactions such as inflammatory capsule or inflammatory infiltrate are much more common in ISS than in other sarcoma types and growth factors elucidated at this site are suspected of interacting with surrounding cells to promote oncogenesis. Growth factors demonstrated to be present in injection site sarcomas in cats are epidermal growth factor (EGF), platelet-derived growth factor (PDGF), tumor growth factor (TGF) and fibroblast growth factors (FGF-a and FGF-b).[1,3,7,8] In addition to these growth factors, cytokines and mutations or over-expression of oncogenes and tumor suppressor genes are mentioned to play a role in the promotion of malignant transformation of cells in this type of tumor.[3,7,8,9]

FeLV (killed), rabies (killed), panleukopenia (killed) and respiratory virus (killed) vaccines have been suggested to increase the risk of sarcoma development in cats. Rabies and FeLV vaccines have been strongly associated with development of ISS in cats in several studies. Reactions are seen with vaccines without and with adjuvant, but reactions at the vaccine site are more often seen in adjuvant

vaccines than non-adjuvant vaccines. Vaccines containing multiple antigens are more likely to cause a vaccine site reaction.[1,3,7,8] Multiple injections administered at the same site increases the risk as well.[3,7,8] In addition, it is important to realize that these sarcomas could also develop after other types of injections than vaccines.[1,7-9] Even other foreign material in the subcutis or muscle such as microchips and non-absorbable suture material have been associated with these tumors.[3,7,9]

Diagnosis

Feline ISS can be diagnosed based on signalment, location (common injection site) and growth pattern (rapid growth) of the mass and injection history. Within a few weeks, the tumor diameter could grow several centimeters.[3] Diagnosis should be confirmed by histological (incisional) biopsy because the inflammation hampers interpretation of tumor cytology. Pathological reports describe these tumors as anaplastic sarcomas, mesenchymal types of tumors. The cell origin of these injection associated sarcomas is usually fibroblast or myofibroblast. Different histotypes can be observed, such as fibrosarcoma (most frequently seen), malignant fibrous histiocytoma, osteosarcoma, chondrosarcoma, rhabdomyosarcoma, liposarcoma and undifferentiated sarcoma. On histopathology, vaccine site sarcomas differ from other types of sarcomas in various aspects. They are mostly located subcutaneously, necrosis (in the center of the tumor, due to the rapid growth) is more often seen, and other malignant features such as cellular pleomorphism and high mitotic index are more often present. An inflammatory infiltrate is common predominantly consisting of lymphocytes but also macrophages. In several reports a gray-brown material was sometimes seen inside macrophages, determined to be aluminium from the adjuvans of a vaccine.[1,3,7-9]

Treatment

The use of contrast CT images is important to determine the tumor volume and invasion into surrounding tissue, as palpation is found to be inadequate. Additional diagnostic imaging (radiographs, ultrasound) could be used to perform a metastasis check. Treatment plans could be formulated based on the CT and additional diagnostic images.[1,3,7,8] A multimodal approach of feline ISS is widely recognized to lead to better results.[3] Excessive surgical excision has been the preferred treatment method in feline ISS immediately after diagnosis when there is no evidence for metastases. This consists of tumor excision with 5 cm margins and a minimum of one fascial plane deep to the sarcoma. Resection of skeletal structures should be performed if the tumor is involved or close to these structures.[1-3,7-9] Surgery alone to treat these tumors has been proven inadequate. Recurrence rates of 30-70% have been noted even if surgery has been more radical, resulting from incomplete excised tumors with dirty margins. Histopathologically clean margins after extensive surgery do not prevent recurrence of the tumor either; in 19% to 50% of these type of cases recurrence was seen. It did take longer for the tumor to recur in these cases.[3,7,8] Radiation therapy added to surgical excision (preoperative as well as postoperative) has lead to more promising outcomes in median survival compared to other treatment protocols, but recurrence of the tumor is still seen. Median survival time is longer in cases of complete excision with adjuvant radiation therapy. Complete surgical excision of the tumor mostly leads to better results.[1,3,7,8,12] Radiation therapy as treatment on its own is considered not curative, only palliative. The same is true for chemotherapy, which is also not curative when applied solely.[7,8] Chemotherapy with chemotherapeutic agents Doxorubicin, Carboplatin, Vincristine, Mitoxantrone and/or

Cyclophosphamide might be beneficial as adjuvant therapy, but studies show varied results.[1-4,7-9] Partial responses (i.e. reduction in size) and delay of recurrence have been mentioned, but also complete remission was achieved with a chemotherapy protocol. Some ISS in cats do respond to chemotherapeutic agents but some do not, which makes prediction of outcome and prognosis difficult.[1,3,7-9,13] Doxorubicin and Carboplatin are commonly used chemotherapeutic agents that have shown the most promise in feline ISS. Unfortunately all current available treatment options for feline ISS, including surgery and adjuvant radiation therapy or adjuvant chemotherapy, show high recurrence rates ranging from 41% to 45%.[1-4,7-9,13] Therefore, additional therapy protocols are needed.

DNA damage response mechanisms

DNA damage

DNA in each cell is damaged every day, resulting in lesions. The types of lesions seen are those that impair base pairing, block DNA transcription and replication, base loss, single-strand breaks (SSBs) or double-strand breaks (DSBs). DSBs are formed when two SSBs are close to each other or a SSB or certain other lesion is encountered during DNA replication. The double strand breaks are very toxic for the cell and difficult to repair, but are not formed frequently. The above mentioned lesions could lead to blocking of genome transcription and replication but could also lead to mutations or more cell or organism viability threatening aberrations in the genome if not repaired or not correctly repaired. DNA aberrations could arise via physiological processes but also caused by reactive oxygen or nitrogen compounds. The physiological processes include DNA mismatches that sometimes occur during DNA replication, topoisomerase I and II activity induced DNA strands breaks, and DNA base-lesions caused by non-enzymatic methylations and hydrolytic reactions. Reactive oxygen arises as by product of oxidative respiration or redox-cycling (with environmental toxins) and Fenton reactions (with heavy metals), but is also produced during inflammation or infection by macrophages and neutrophils. Macrophages and neutrophils also produce reactive nitrogen compounds at these sites.[5]

Response mechanisms

DNA damage response (DDR) mechanisms function to detect lesions in DNA, signal their presence and stimulate their repair. A diversity of DDR mechanisms exist to react upon or support the repair of a wide variety in types of DNA lesions (see figure 1). Some lesions will undergo direct protein mediated reversal, whereas most others undergo a series of catalytic events mediated by several proteins to be repaired. Multiple repair systems exist, each acting upon different types of lesions by means of different key proteins (see table 1).[5] Several of the many mechanisms and key protein components in DDR will be addressed in this research. Focus is on components in DDR that are likely to modulate sensitivity or resistance to chemotherapeutics Doxorubicin and Carboplatin in feline ISS. Checkpoint proteins Atm and p53 will be addressed, as well as repair proteins ERCC1 and Rad51.

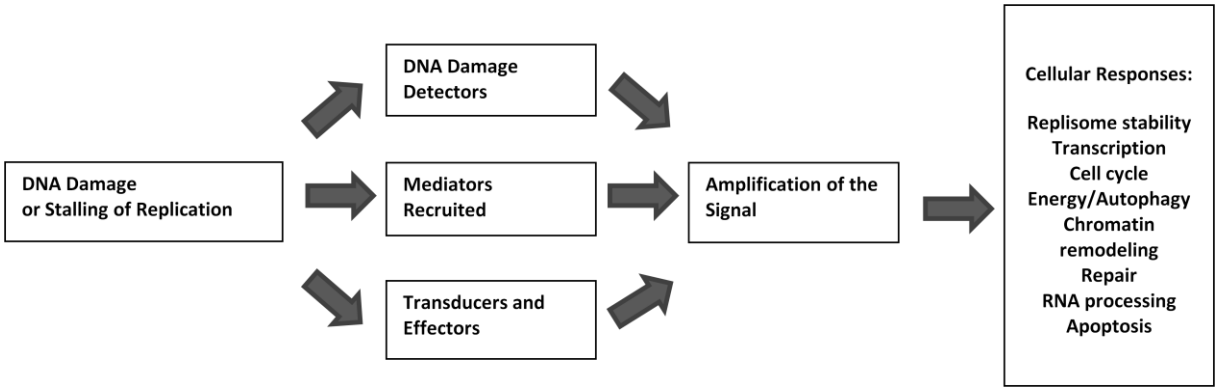


Figure 1. Model for the DDR[5]
The presence of a DNA lesion could lead to replication stalling. Various sensor proteins recognize these processes and initiate signaling pathways that lead to a variety of cellular responses.

Table 1. Several of many DDR mechanisms and components[5] Mechanisms and proteins addressed in this study are highlighted.

Mechanisms	Lesions	Key Proteins
Base Excision Repair (BER) and Single-Strand Break Repair (SSBR)	Abnormal DNA bases Simple base adducts, SSBs generated as BER intermediates	DNA glycosylases (sensors), APE1 endonuclease, DNA polymerases (β, δ, ε) and associated factors, flap endonuclease FEN1, ligase I or ligase III. Polymerase β lyase activity, XRCC1, PARP-1, PARP-2, polynucleotide kinase (PNK) and aprataxin (APTX)
Nucleotide Excision Repair (NER)	Lesions that disrupt the DNA double-helix	Sensors elongating RNA polymerase, XPC-HR23B and DDB1/2, plus XPA, XPE, XPF/**ERCC1**, XPG, CSA, CSB, TFIIH, DNA polymerases and associated factors, RPA, ligase I
Non-Homologous Endjoining (NHEJ)	Radiation- or chemically-induced DSBs V(D)J and CSR Intermediates	Sensors Ku and DNA-PKcs plus XRCC4, XLF/Cernunnos and ligase IV. Can also employ the MRE11-RAD50-NBS1 complex, Artemis nuclease, PNK, Aprataxin and polymerases μ and λ
Homologous Recombination (HR)	DSBs Stalled replication forks Inter-strand DNA cross-links Sites of meiotic recombination and abortive Topoisomerase II action	**RAD51**, RAD51-related proteins (XRCC2, XRCC3, RAD51B, RAD51C, RAD51D, DMC1), RAD52, RAD54, BRCA2, RPA, FEN1, DNA polymerase and associated factors. Promoted by MRN, CtIP, BRCA1, and the ATM signalling pathway
ATM-Mediated DDR Signaling	DSBs	**ATM**, MRN and CHK2. Promoted by mediator proteins such as MDC1, 53BP1 MCPH1/BRIT1, and by ubiquitin ligases RNF8, RNF168/RIDDLIN and BRCA1
ATR-Mediated DDR Signaling	ssDNA, resected DSBs	Sensors ATR ATRIP and RPA plus the RAD9-RAD1-HUS1 (911) complex, RAD17 (RFC1-like) and CHK1. Promoted by MRN, CtIP and mediator proteins such as TOPBP1, Claspin, MCPH1/BRIT1 and BRCA1

Checkpoint signaling proteins – Atm and p53

Atm and Atr are key checkpoint protein kinases signaling in presence of double-strand breaks (DSBs) and replication protein A-coated ssDNA respectively. They are recruited to and activated by these type of lesions. Target proteins CHK1 and CHK2 act together with Atm and Atr via multiple mechanisms on reduction of cyclin-dependent kinase (CDK) activity. Activation of the p53 transcription factor mediates some of these mechanisms. When CDKs are inhibited cell-cycle progression is slowed down or arrests at certain cell cycle checkpoints, i.e. at the G1-S, intra-S and G2-M points of the cell cycle. This is probably to ensure more time is available for DNA repair before the next phase in replication or mitosis is ensued. In addition to this CDK inhibition, DNA repair is enhanced by induction of DNA-repair proteins (both on transcriptional and post-transcriptional level), recruiting of repair factors, and/or activation of DNA-repair proteins. Atm and Atm are thought to modulate even more cellular processes via Atm/Atr-mediated phosporylation. After activation of the DDR system the following outcomes are possible: DNA is effectively repaired after which DDR is inactivated and the cell will function normally again, or if DNA is not effectively repaired chronic signaling by the DDR system will lead to cell death via apoptosis or senescence.[5,6] The p53 pathway makes a contribution in cell cycle arrest, senescence and apoptosis. Through these mechanisms it is thought p53 could prevent potentially malignant cells to expand and form tumors. Other undetermined factors are thought to additionally play a role in tumor suppression by p53 as well.[6,14] Also, one study discovered cell cycle arrest, senescence and apoptosis mediated by p53 are not essential in the suppression of early spontaneous tumor formation.[14]

Atm and p53 both are well known tumor suppressor genes, which have been implicated as possible mediators of chemosensitivity and chemoresistance in other tumor types. It has been shown in human studies that Atm deficient tumors are sensitive to chemotherapeutics or radiotherapy that induce DSBs.[6] A large number of other studies however show that tumors which lost Atm are more resistant to chemotherapy. For p53, the same positive and negative outcomes are seen. A study investigating mice and human tumor cells shows that inhibition of Atm in cancer cells that have normal p53 function results in chemoresistance because p53 dependent apoptosis is not executed. In the same study Atm suppressed tumorcells with inhibited p53 function are sensitized for chemotherapy induced cell death. So these results implicate that combined Atm/p53 status could predict the results of treatment with chemotherapeutics.[6] Therefore it is hypothesized they may also mediate chemosensitivity of FISS. p53 has been noted to be abnormally expressed in FISS cells commonly.[15,16] Expression of p53 in cytoplasm instead of nucleus in these cells may characterize functional inactivation. This abnormal cytoplasmic expression is associated with earlier tumor recurrence in FISS as well as several human types of cancer, and this mutated p53 is frequently associated with chemoresistence in human tumor cells.[15,17]

H2AX

Atm, Atr and DNA-PK mediate phosphorylation of serine 139 of H2AX (which is a histone H2A variant), this occurs at chromatins around sites where DSBs are located. As a consequence the chromatin structure plays a role in DDR because it is modulated when DNA damage is apparent. DSB signaling and repair is thought to be promoted by ubiquitin-adduct formation, recruiting of DDR factors and other chromatin-modifying components. In addition, the activation of Atm induces

chromatin relaxation at DSB sites and phosphorylation of H2AX tyrosine 142 has a function in the DDR. Thus phosphorylated(γ) H2AX plays an early signaling role in the DDR for DSBs induced by DNA damage.[5,6,18]

DNA damage repair proteins - Rad51 and ERCC1

ERCC1 is one of the key repair proteins together with XPF in nucleotide excision repair (NER) which recognizes and acts upon helix-distorting base lesions.[5] Assembled at the site of DNA damage in a complex with XPF, it makes the 5' incision to excise the damage out of a DNA strand that has the configuration of a loop or bubble structure.[19,20] The expression of ERCC1 has been demonstrated to be associated with chemoresistance the platinum drug Cisplatin in human studies, since the NER system plays a role in repairing by platinum damaged DNA.[17]

Rad51 is a key protein in the DDR mechanism of homologous recombination (HR) that acts upon DSBs.[5,18] The protein binds single strand DNA at the 3'overhang and here catalyses searching for target sequences that are homologous, and also starts DNA synthesis invading the sister chromatid at the site of homology to use as template.[18] Rad51 is shown to be over expressed in human soft tissue sarcoma (STS) cells, and is possibly linked to chemoresistance to Doxorubicin in these cells.[21] These STS that are also of mesenchymal origin have similar characteristics for recurrence, survival and chemoresistance as feline ISS.[22]

Chemotherapeutics and chemosensitivitiy of cancer cells

The mechanism of action for chemotherapy is that it induces DNA damage and bulky adduct lesions in DNA of cells. This could lead to either DNA repair or cytotoxicity via apoptosis (see figure 2). Because of the damage occurring in all tissue, toxicity in normal tissue is dose-limiting. But as a result of often impaired DDR mechanisms in cancer cells and more rapid proliferation than normal cells it can still be effective. On the other hand, DDR also often acts as a mechanism for chemotherapy-resistance. [5] Insight into the mechanisms that modulate chemoresistance or chemosensitivity in FISS cells is needed to be able to predict if and what type of chemotherapy would be effective in a patient.

Two mechanisms of resistance for chemotherapy in cancer cells are could broadly be defined. One is characterized by drugs not reaching their main intracellular target by a variety of mechanisms due to characteristics of the tumor. The other is tumor cells surviving after damage from chemotherapy through enhanced DNA damage repair or inactivation of pathways that would lead to cell death.[17] The latter mechanism is focus of this research, to determine how chemosensitivity or chemoresistance are influenced by components of the DDR pathways.

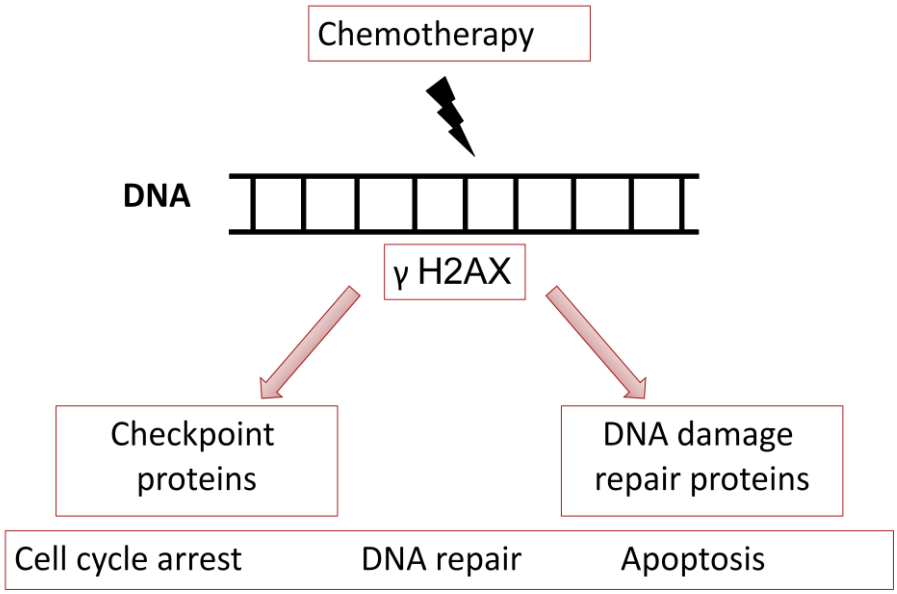


Figure 2. Chemotherapy induces DNA damage and bulky adduct lesions, which could lead to either DNA repair or cytotoxicity via apoptosis.

Mechanism of action - Doxorubicin

Doxorubicin is an anthracycline antibiotic chemotherapeutic isolated from cultures of *Streptomyces peucetius* var. *caesius.* [13,23] It is an effective chemotherapeutic agent in humans, dogs and cats, but more toxicity is reported in dogs and humans than in cats.[23] Its primary antitumor working mechanism is based on nucleotide base intercalation, interaction with topoisomerase II and also free radical production. These mechanisms result in inhibition of nucleotide replication and formation of DNA-cleavable complexes. A chronic adverse effect of doxorubicin treatment reported in cats is nefrotoxicity, but also anorexia, gastrointestinal toxicity, myelosuppression and cardiotoxicity.[13,23]

Mechanism of action – Carboplatin

Carboplatin is an alkylating (second generation) platinum containing compound within the group of metal chemotherapeutics.[17,24,25] It has less nephrotoxic and emetogenic effects in cats and dogs than Cisplatin.[24,25] In addition, Cisplatin (a first-generation platinum compound) induces acute pulmonary edema in cats which is often fatal. This is the reason for Cisplatin to be contra-indicated in cats.[25] An adverse effect of Carboplatin is myelosuppression , and anorexia is a very rare adverse effect that could also be seen. It is an effective chemotherapeutic agent in several types of tumors in dogs and cats, including some cases of feline ISS.[24,25] The antitumor working mechanism is by interacting with DNA strands to form crosslinks, which severely distort the DNA.[26]

Goal

General goal of the study

To determine if expression of DNA damage checkpoint and repair proteins correlates with chemosensitivity of feline injection site sarcomas.

Specific aims

- Aim 1.
  Determine if alterations in DNA damage checkpoint signaling predict the chemosensitivity of feline injection site sarcomas.
- Aim 2.
  Determine if alterations in DNA damage repair proteins predict the chemosensitivity of feline injection site sarcomas.

Hypothesis

General hypothesis of the study

Alterations in DNA damage response mechanisms modulate chemosensitivity of feline injection site sarcomas, explaining the inconsistent benefit of adjuvant chemotherapy and the low response rates noted for macroscopic disease.

Specific hypotheses

- Atm and p53
  - Low levels of Atm will allow damaged cells to persist and thus result in chemoresistance and p53 abnormalities will also be associated with chemoresistance.
- ERCC1
  - Due to the involvement of the nucleotide excision repair (NER) system in cell damage from Carboplatin, low ERCC1 will be associated with increased sensitivity of feline ISS to Carboplatin.
  - ERCC1 expression levels will not correlate with doxorubicin sensitivity, as NER is not involved in cell damage from doxorubicin.
- Rad51
  - Due to its role in homologous recombination, feline ISS with low Rad51 expression will be sensitive to both doxorubicin and Carboplatin chemotherapy.

Materials and methods

Tumor biopsy

Tumor biopsy specimens for evaluation of suspected ISS from clinical feline patients presented to Cornell University Hospital of Animals (CUHA) were acquired. The biopsy format comprised three to five 8 mm punch biopsies or an excisional or wedge biopsy. Each specimen was fixed in formalin and submitted to the Animal Health Diagnostic Laboratory, Cornell University to obtain a definitive histological diagnosis. Part of the biopsy specimens were embedded in paraffin. Two patients (cat 1 and cat 3) were presented with injection site sarcoma on the right hip and the right thoracic wall respectively, one patient (cat 2) had a recurrent non injection site sarcoma on the head.

Primary cell culture

Multiple punch biopsies of each tumor were kept in sterile tissue culture (TC) phosphate buffered saline (PBS) on ice and rinsed twice with sterile TC PBS. After soaking the biopsies in Trypsin for 5 minutes, each specimen was chopped into pieces and multiple explants were derived to grow multiple cell lines. The explants were placed into 12 well dishes to start with. As soon as a monolayer of cells was formed, the explant was removed from the dish. Primary cell culture was done in an incubator at 37°C.

Different media combinations were tried on the cell lines in culture. Culture of all cell lines was started in Minimum Essential Medium (MEM), and in trying to optimize media for growth Dulbecco's Modification of Eagle's Medium (DMEM) was used, or if needed MEM with 10% fetal bovine serum (FBS), DMEM with 10% FBS or DMEM with supplements added. These supplements consisted of 20% FBS, 1% L-glutamine, 1% non-essential aminoacids (NEAA) and 1% Penicillin/Streptomycin/Amphoterocin B solution. DMEM with all supplements eventually seemed to work best for all cell lines. Feeding of the cells on the plates was done every 3-4 days according to the following protocol.

Protocol for feeding sarcoma cells

1. Place all needed bottles of media in the water bath in the tissue culture room (37 °C).
2. Prepare hood (middle hood: Weiss lab for primary cultures). Sign up on sign up-sheet. Turn off UV light and turn on blower and light. Raise sash enough to work comfortably (hood will beep if sash is too high). Spray down surface with 70% ethanol and wipe with paper towel.
3. Wait at least 15 minutes for the media bottles to get the right temperature. Remove media bottles from water bath. Spray with 70% ethanol and rub to disinfect. Remove plates from lower incubator and carefully place in the hood.
4. Organize plates based on media type.
5. Loosen tops of media bottles.
6. Work with plates in stacks of 4.
7. Working from top to bottom of stack, aspirate ‘old’ media off of each plate (into waste bottle in the back of the hood). Tilt plate slightly toward back of the hood. Use separate sterile Pasteur pipette for each plate with a different label. Careful not to touch the bottom of the

plate (so as not to aspirate off cells). Change Pasteur pipette if any contamination occurs. When done, take top plate off of stack and start a new stack.

8. Working from bottom to top of the new stack, add back 10 ml of 'new' media on a 10 cm dish, 5 ml of media on a 6 cm dish, 2 ml of media on a 2 cm dish. Each plate labeled with appropriate type of media. Can use same serological pipette for multiple plates as long as tip does not touch anything other than the inner bottle of media. Change serological if contamination occurs. Apply the media to the plate using steady gentle drops or a gentle stream along the inner side of the tissue culture plate.
9. Repeat as needed until complete.
10. Return plates with 'new' media to lower incubator.
11. Tighten media bottles and return to tissue culture fridge.
12. Spray down surface of hood with 70% ethanol and wipe with paper towel. Turn off blower and light and close sash. Turn on UV light. Make sure there are enough Pasteur pipettes available for the next user. Empty waste bottle (deactivate with bleach before rinsing down the drain) if full.

It was microscopically determined with objective 2X and 10X if cells in the well or on the plate were ready to pass, with important criteria being high density of cells on the plate, confluency, cells piling up on each other, enough viable cells.

Passing of cells onto new plates was done according to the following protocol.

Protocol for passing sarcoma cells

1. Aspirate media off of the plate (into waste bottle in the back of the hood). Tilt plate slightly toward back of the hood. Use separate sterile Pasteur pipette for each plate. Careful not to touch the bottom of the plate (so as not to aspirate off cells).
2. Rinse with PBS (5 ml for a 10cm dish).
3. Put 2 ml trypsin on the plate and let it incubate in the lower incubator on the bottom shelf at the right for 5 minutes.
4. Rinse trypsin with suspended cells of the dish with 10 ml of the same media that was used to feed the cells until all cells are loose.
5. Put the suspension with the cell in a 10 ml tube (and label this tube accordingly). Pipet 6 times to make a single cell suspension.
6. Put 0,5 ml of the suspension in an Eppendorf tube to use for counting.
7. Repeat steps above for each plate that needs to be passed.
8. Spin the cells down from solution in the tube on 1,5 * 100 rpm for 5 minutes.
9. In the mean time, count cells using a hematocytometer under the microscope.
10. Count all four corners in the hematocytometer. Do a second count if the total number of cells counted is lower than 60. Calculate an average per corner based on all counted cells. Multiply this average with the volume in ml of cell-solution in the corresponding tube that is centrifuged, and then divide this by 100 to determine the volume of media (ml) that needs to be added to the cell pellet create a suspension of 1 million cells/ml.
11. Prepare new plates: put 5 ml TC gelatin in each dish, wait 5 minutes for it to adhere, then rinse off remaining gelatin with PBS and aspirate using a sterile Pasteur pipette. Label plates accordingly and put 9 ml of suitable media on the plate.

12. Aspirate media of the cell pellet in the tube using a sterile Pasteur Pipette. Careful not to touch the pellet of cells (so as not to aspirate off cells).
13. Put calculated volume of suitable media in the tube and suspend cell pellet until it forms a single cell suspension.
14. Pipette 1 ml of the suspension and distribute it careful and evenly onto the matching new plate. Carefully swivel the plate back and forth and sideways to distribute cells more evenly (keep the plate horizontal while doing this, don't tilt it to prevent cells from sticking at the sides of the dish).
15. Put all finished plates in the lower incubator.

Colony Forming Assays

Cell lines that were passing consistently underwent a pilot treatment with Carboplatin but first were used to determine optimum cell density to start the colony forming assays (CFAs) with. Cell densities of 500 cells/ml and 1,000 cells/ml for cell lines of cat 1 and cat 2 were used; 1,000 cells/ml and 10,000 cells/ml for cell lines of cat 3 were used. The pilot treatment consisted of positive control (untreated), 20 µg/ml Carboplatin and 40µg/l Carboplatin. Each treatment with a different dosage as well as the positive control was done in triplicate. Cells were plated onto 2 cm dishes according to the same protocol used for primary cell culture, however the media composition slightly differed. During the exposition to Carboplatin, the cells were kept in serum-free media to prevent Carboplatin to interact with this media. The cells were exposed to the Carboplatin-containing media for 48 hours and then switched to regular growth media.

The cells in the CFAs were fixed with methanol after 12 days (tumorcells of cat 1 and 2) and 21 days (tumorcells of cat 3) and stained with 0.1% crystal violet in 95% ethanol to visually assess the growth patterns. Colonies had to be in such a density that they could be microscopically distinguished from each other when determining the optimum cell density to start the CFAs with.

In a later stadium of this study, pilot and definitive CFAs will be done using Doxorubicin as well, and Cell Viability Assays (CVAs)will be performed in addition to the CFAs. The cells will be treated with Carboplatin and Doxorubicin in different experiments to determine the effect of different dosages in these CVAs. This however was beyond the extent of this research project.

Sectioning

Paraffin embedded tumor biopsies were sectioned using a microtome to make microscope slides for immunohistochemistry (IHC) staining. Of each tumor approximately 20 slides of were produced for IHC. From both patients with ISS, slides were also stained with hematoxylin and eosin (HE) staining according to standard protocol to study tumor morphology.

Immunohistochemistry (IHC)

Immunohistochemistry for γH2AX was done according to the following protocol.

Protocol for IHC for γH2AX

Start with fixed and paraffin-embedded tissues, sectioned.

1. Put microcope slides in glass basket 60°C oven for 40 min to de-wax and then dip in xylenes for 2 x 5 min.

2. Rehydrate with EtOH series:

100% EtOH 2 x 3 min
95% EtOH 2 x 3 min
70% EtOH 1 x 3 min
50% EtOH 1 x 3 min (in the mean time, warm EDTA in water bath)
H_2O de-ionized sterile 1 x 3 min
TBS 1X 1 x 5 min and then tap dry

3. Antigen retrieval with EDTA 0.25 mM 8.0 pH (50 ml 2.5 mM EDTA in 450 ml sterile water)
Warm EDTA buffer in coplin jars in microwave for 10 min (high) in water bath. If needed, put water in the second jar. Distribute slides evenly to both coplin jars. Use blank slides when you do not have an even number. Incubate slides in EDTA for 35 min at 95°: 3 x 10 min, 1 x 5 min microwave high, refill EDTA after each 10 min interval.

4. Leave at room temperature for 20 min to cool slides.

5. Wash slides 2 x 5 min in TBS 1X.

6. Peroxidase quenching solution: 1 to 9 H_2O_2 (30%) to CH_3OH (absolute methanol) mix well and submerge slides 1 x 12 min. 50 ml conical tube with 45 ml methanol and 5 ml H_2O_2 (light sensitive!) then add to slides in coplin jar.

7. Wash slides 2 x 5 min in distilled H_2O (d H_2O).

8. Block slides in humidified chamber with parafilm coverslips at room temperature for 30 min with 150 µl 1A per slide (from M/mouse kit).
Block mix for 2 slides: 1X TBS – 4% BSA – 0.02% Tween20. For 1 ml Ab mix: 40 mg BSA, 1 µl 20% Tween20, 1 ml 1X TBS. Transfer 200 µl to another vial. Add 1µl anti- γH2AX (mouse) to vial for 1:200 dilution.

9. Incubate slides overnight at 4C with anti- γH2AX diluted 1:200 (80-100 µl/slide with parafilm coverslips) in block mix in humidified chamber.

10. Wash slides 3 x 5 min in TBS 1X.

11. Incubate with 2nd antibody for 30 min (biotinylated 2nd Ab) RT in humidified chamber, mouse histostain kit – reagent 1B.

12. Wash with 1X TBS 3 x 2 min.

13. Linking antibody and enzyme: apply 1-2 drops enzyme conjugate (mouse histostain kit reagent 2) to each slide. Incubate 10 min in humidified chamber with parafilm coverslips.

14. Wash with 1X TBS 3 x2 min – prepare DAB.

15. Peroxidase substrate: for use with DAB peroxidase kit from Invitrogen (in box, small 4C): add 1 drop of reagent A to 1 ml dH_2O in microcentrifuge tube and mix. Add 2 drops of reagent B and mix. Add 1 drop of reagent C and mix. Wrap in foil (light sensitive).

16. Add 100 µl DAB per slide. Place on shaker for 4-5 min in humidified chamber (not closed) then put immediately in tap H_2O (from sink) for 4 x 1 min. (on shaker)

17. Wash slides in tap H_2O 1 x 5 min (extra). (on shaker)

18. Hematoxylin counterstaining: immerse slides in coplin jar of hematoxylin diluted 1:1 for 5 sec. Wash slides immediately in tap water (color turns from red to purple). Put slides in 1X PBS until sections turn blue (~30 sec). Rinse in dH_2O 1 x 2 min.

19. Dehydrate slides and clear with xylenes:

50% EtOH 1 x 1 min
70% EtOH 1 x 1 min
95% EtOH 2 x 1 min
100% EtOH 2 x 1 min
Xylenes 2 x 5 min → tap dry + lean on edge for 5 sec.

20. Mount in mounting media: apply 2 drops of histomount and glass coverslip. Leave to dry.

IHC for Atm was done according to the same protocol, but the primary antibody used was anti-Atm (Millipore, with cross species reactivity) diluted 0,5 µl in 500µl Ab mix.

IHC for p53 was done according to the same protocol using citrate buffer instead of EDTA for antigen retrieval. The primary antibody used was anti-p53 (Cell Signaling #2524) diluted 5 µl in 500µl Ab mix.

IHC for ERCC1 was done according to the same protocol using citrate buffer instead of EDTA for antigen retrieval. The primary antibody used was anti-ERCC1 (Cell Signaling, with cross species reactivity) diluted 5 µl in 500µl Ab mix.

IHC for Rad51 was done according to the same protocol. The primary antibody used was anti-Rad51 (Calbiochem, polyclonal) diluted 1µl in 800µl Ab mix.

Microscopic evaluation of IHC

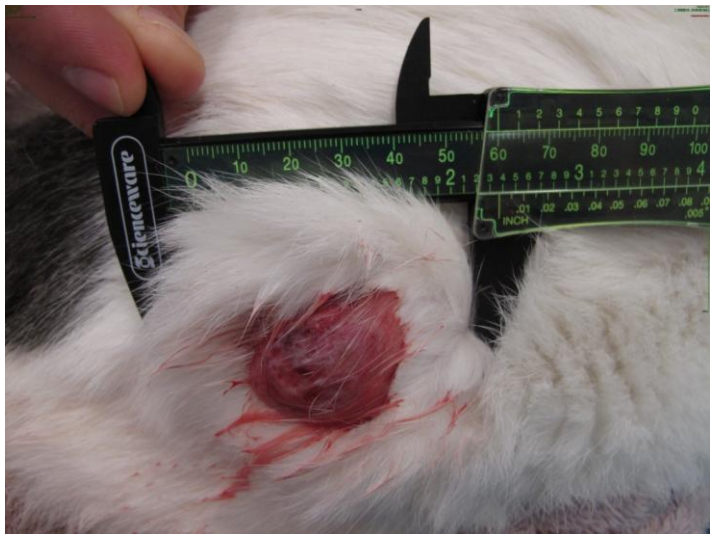
Assessment and scoring of the IHC staining, if applicable, was based on:

- Intensity of staining
    - Absent
    - Weak/moderate
    - Strong
- Location
    - Cytoplasmic
    - Nuclear
    - Both
- Percentage of tumor cells staining positive

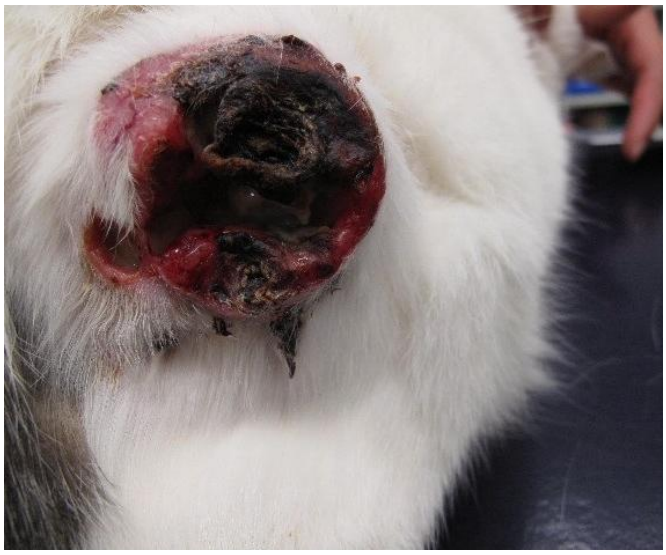
Results

Cats enrolled in study

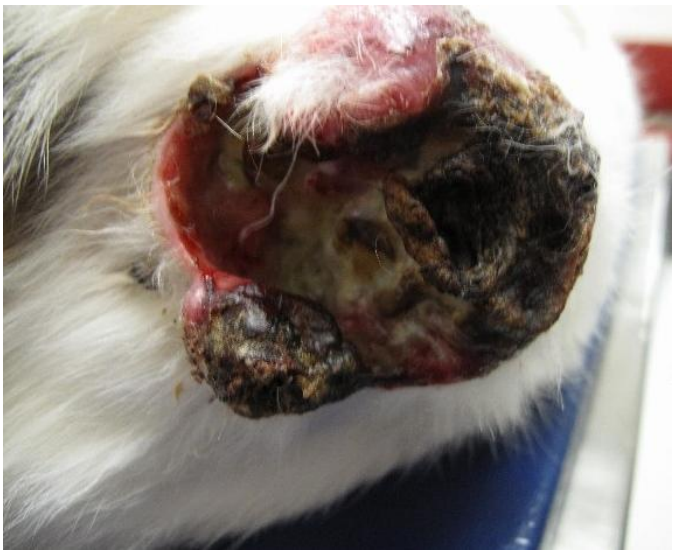
One patient (cat 1) was presented with an injection site sarcoma on the right hip and was presented to the clinic in January 2012. The tumor showed rapid growth in two months time, and the patient received two doses of Carboplatin with no response (see figure 3). The second patient (cat 2) had a recurrent non injection site sarcoma on the head. Even though it was not an ISS, the tumor exhibited similar characteristics as ISSs and was incorporated in the study for purposes of comparison. The third patient (cat 3) had a rapid growing injection site sarcoma on the right thoracic wall (see figure 4).



A.



B.



C.

Figure 3. Clinical presentation of a rapidly developing ISS on the right hip of cat 1. A. January 2012. **B**. March 6th 2012. **C.** March 14th 2012. The patient received two doses of Carboplatin with no response.

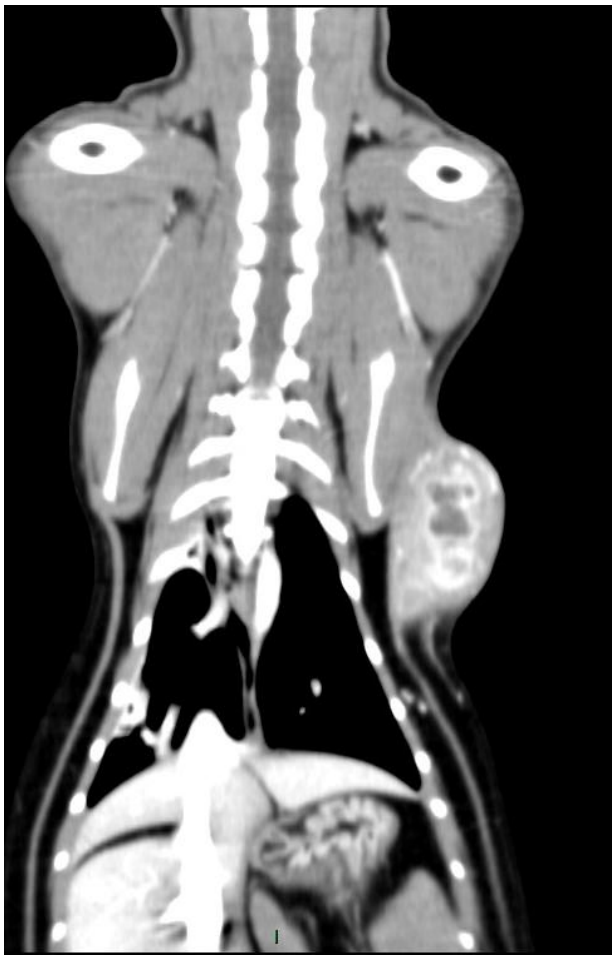


Figure 4. Computed tomography (CT) image of an ISS on the right thoracic wall of cat 3.

Primary cell culture

The cell culture medium that showed best growth in most cell lines was DMEM with 20% FBS, 1% L-glutamine, 1% NEAA and 1% pen/strep/ampB. Variability in growth pattern and speed was seen in explants (see figures 5, 6 and 7). The best growing cell lines were passed every 3-6 days and some slow growing cell lines every 15-18 days. Some cell lines did not survive primary cell culture, but from al cats several cell lines were derived from an explant and maintained in cell culture. The cell line showing best growth was at passage number 22 at the end of my participation in the study. This was a cell line derived from the tumor of cat 1. Cells that were growing well showed high density of cells on the plate, confluency, cells piling up on each other and enough viable cells to pass. Cells that deteriorated showed the following signs: granularity around the nucleus, cytoplasmic vacuolization and rounding up of the cells with detachment from the substrate in the dish.

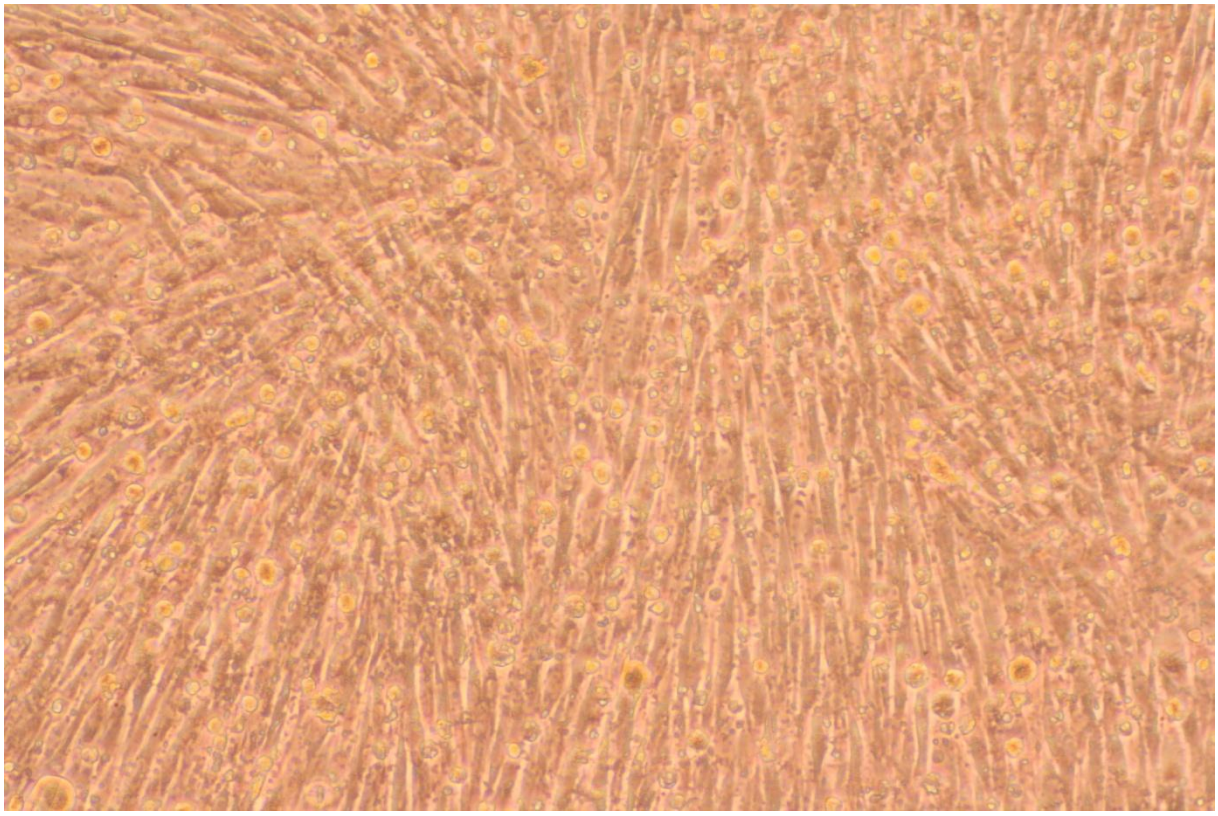


Figure 5. Microscopic picture of tumor cells in primary cell culture derived from cat 1, 10X objective. Intertwining spindle shaped cells are seen and cell density is very high in this culture with confluence of the cells.

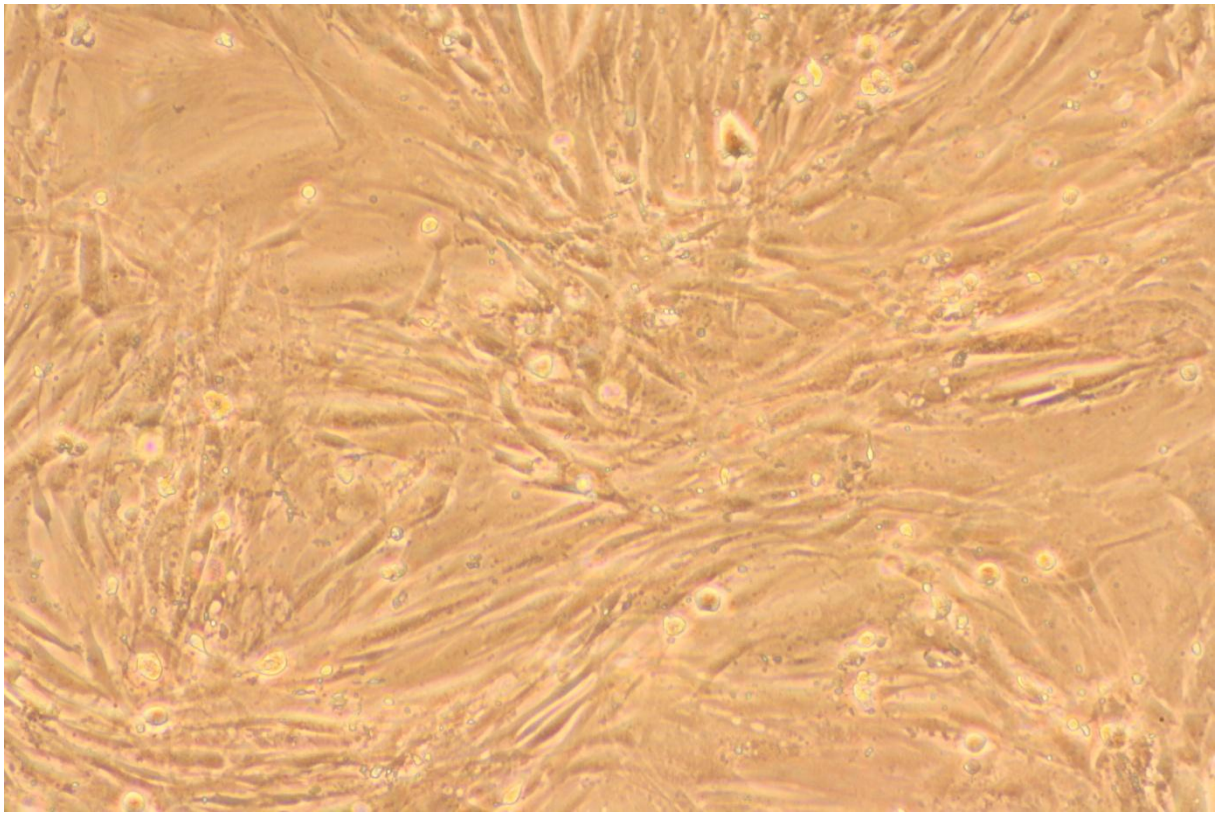


Figure 6. Microscopic picture of tumor cells in primary cell culture derived from cat 2, 10X objective. Intertwining spindle shaped cells are seen and cell density is mediocre to high in this culture with some confluence of the cells.

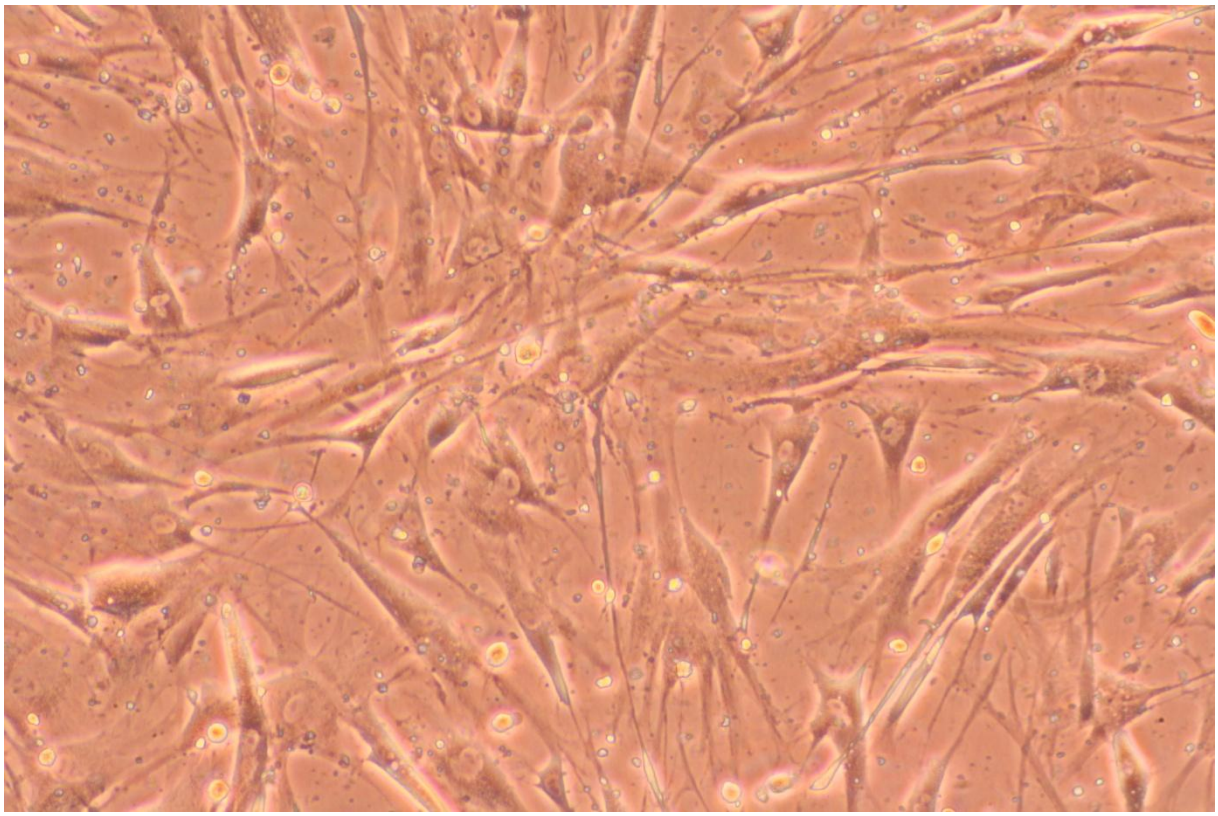


Figure 7. Microscopic picture of tumor cells in primary cell culture derived from cat 3, 10X objective. Intertwining spindle shaped cells are seen and cell density is mediocre in this culture with some confluence of the cells.

Colony Forming Assays

One most consistently passing cell line per cat was determined to use for the pilot CFAs. Cell densities of 500 cells/ml and 1,000 cells/ml for cell lines of cat 1 and cat 2 were used; 1,000 cells/ml and 10,000 cells/ml for cell lines of cat 3 were used. Based microscopic examination of the colony density, optimum cell density was determined to be 1,000 cells/ml for all cell lines to use in future CFAs (see figures 8-11). The pilot CFAs with Carboplatin consisted of positive control (untreated), 20 µg/ml Carboplatin and 40µg/l Carboplatin, each in triplicate. On microscopic evaluation colony forming was seen in positive controls. A very low number of cells was seen microscopically and no colonies were seen in dishes treated with either dosage of Carboplatin for cells of both cats (see figure 12).

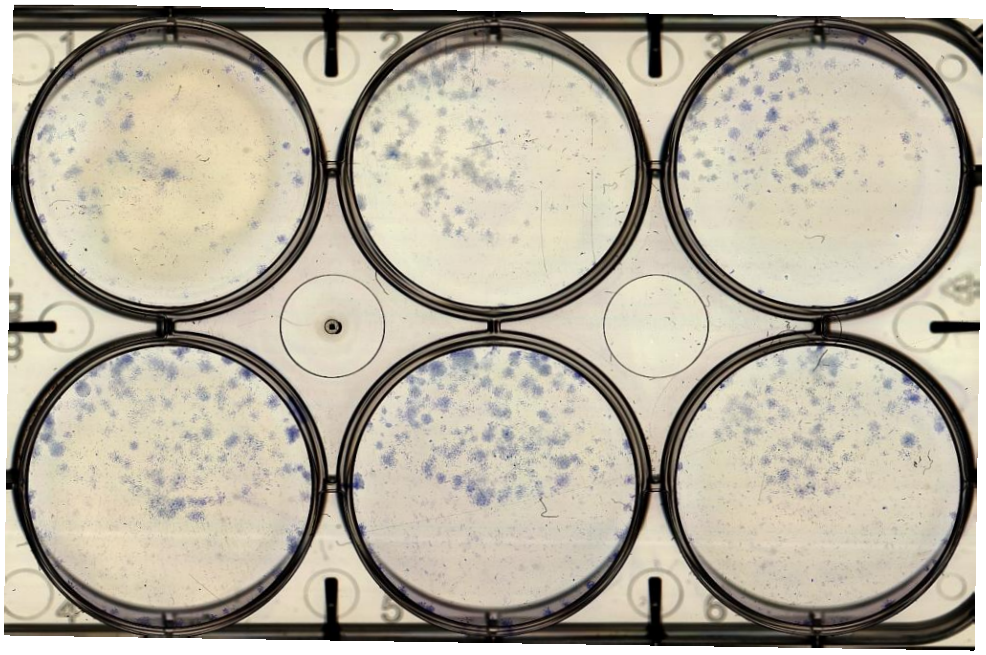


Figure 8. Colony forming assays of sarcomacells from cat 1 in passage 11 stained on day 12 of the experiment. Upper plates 500 cells/ml, lower plates 1,000 cells/ml. Different colonies could be distinguished at both densities. The halo effect in the dishes results from uneven distribution of cell suspension pipetted onto the dishes.

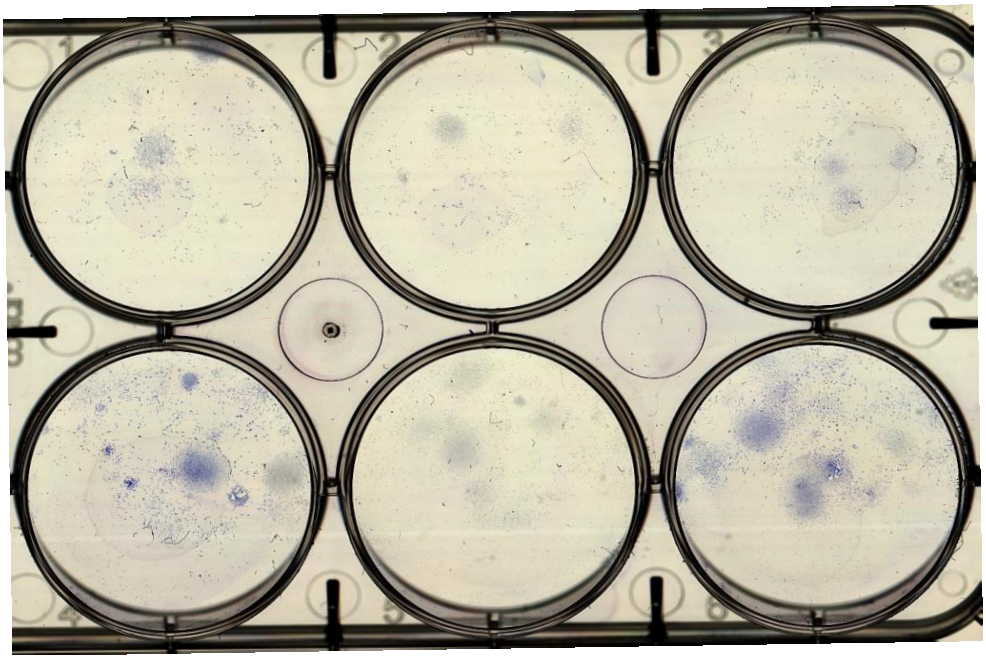


Figure 9. Colony forming assays of sarcomacells from cat 2 in passage 7 stained on day 12 of the experiment. Upper plates 500 cells/ml, lower plates 1,000 cells/ml. Different colonies could be distinguished at cell density of 1,000 cells/ml.

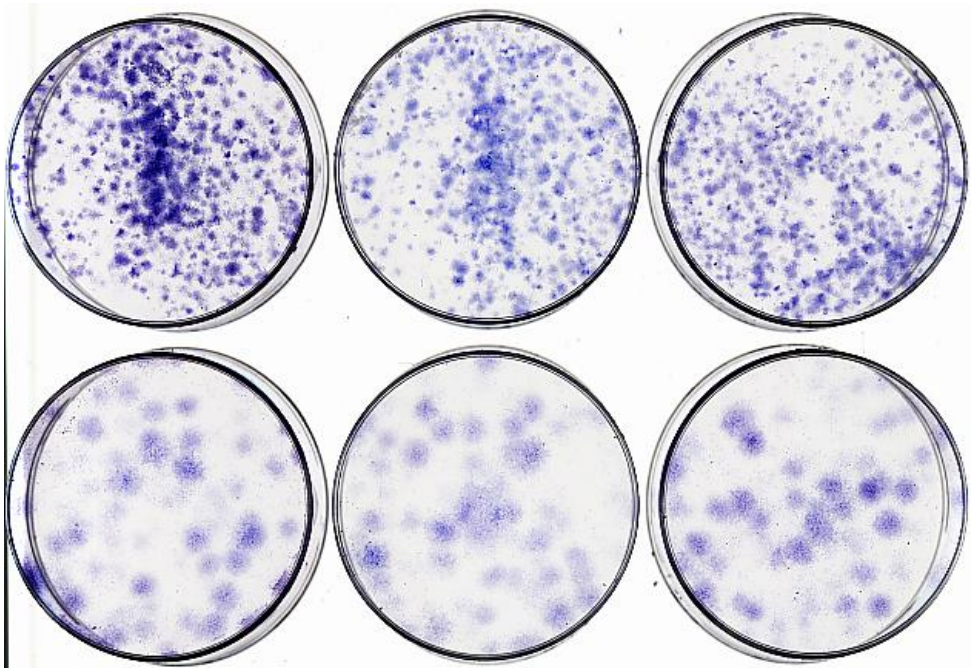


Figure 10. Upper plates: colony forming assays of sarcomacells from cat 1 in passage 14 stained on day 12 of the experiment, 1,000 cells/ml. Lower plates: colony forming assays of sarcomacells from cat 2 in passage 10 after 12 days of culture, 1,000 cells/ml. Different colonies could be distinguished in CFAs of both cats.

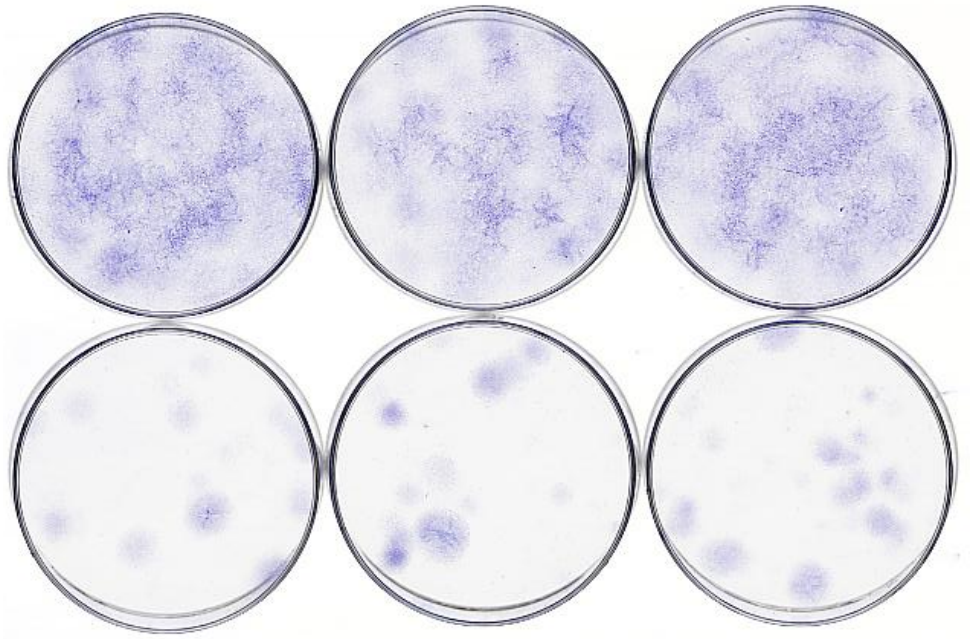


Figure 11. Colony forming assays of sarcomacells from cat 3 in passage 5 stained on day 20 of the experiment. Upper plates 10,000 cells/ml, lower plates 1,000 cells/ml. Different colonies could be distinguished at 1,000 cells/ml. At a density of 10,000 cells/ml different colonies could not easily be distinguished.

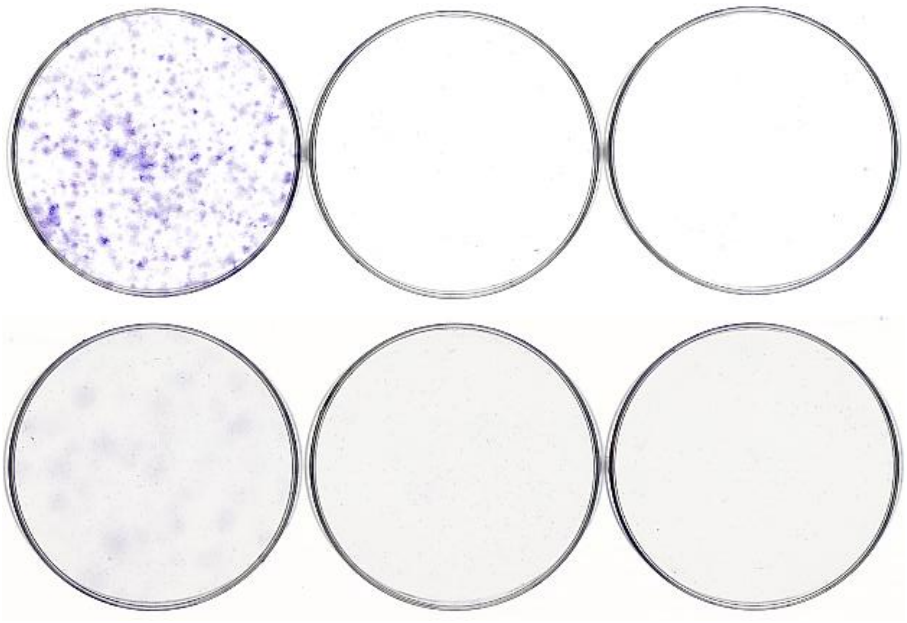


Figure 12. Pilot treatment with Carboplatin in colony forming assays of sarcomacells from cat 1 (upper plates) and cat 2 (lower plates). Left plates: positive control (untreated); colonies of cells are visible for both. Middle plates: 20 µg/ml Carboplatin. Right plates: 40µg/l Carboplatin. No colonies are visible in any Carboplatin treated dish.

Hematoxylin and Eosin (HE)

From both patients with ISS, some slides were also stained with hematoxylin and eosin (HE) staining to study tumor morphology without additional IHC staining. Both tumor cells of cat 1 and cat 3 show similar morphology (see figure 13 and 14). Intertwining spindle shaped cells are seen, most have one ovoid basophilic nucleus and pale eosinophilic cytoplasm.

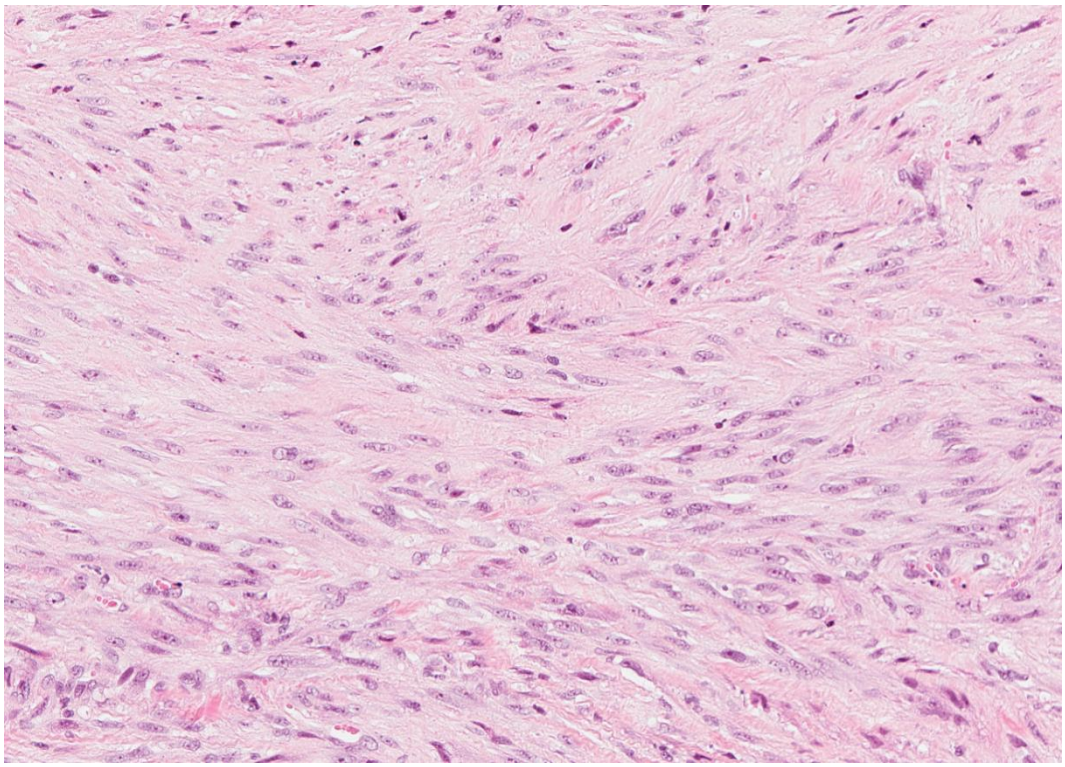


Figure 13. Hematoxylin and eosin (HE) staining of sectioned ISS tissue of cat 1, 20X objective. Intertwining spindle shaped cells are seen, most have one ovoid basophilic nucleus and pale eosinophilic cytoplasm.

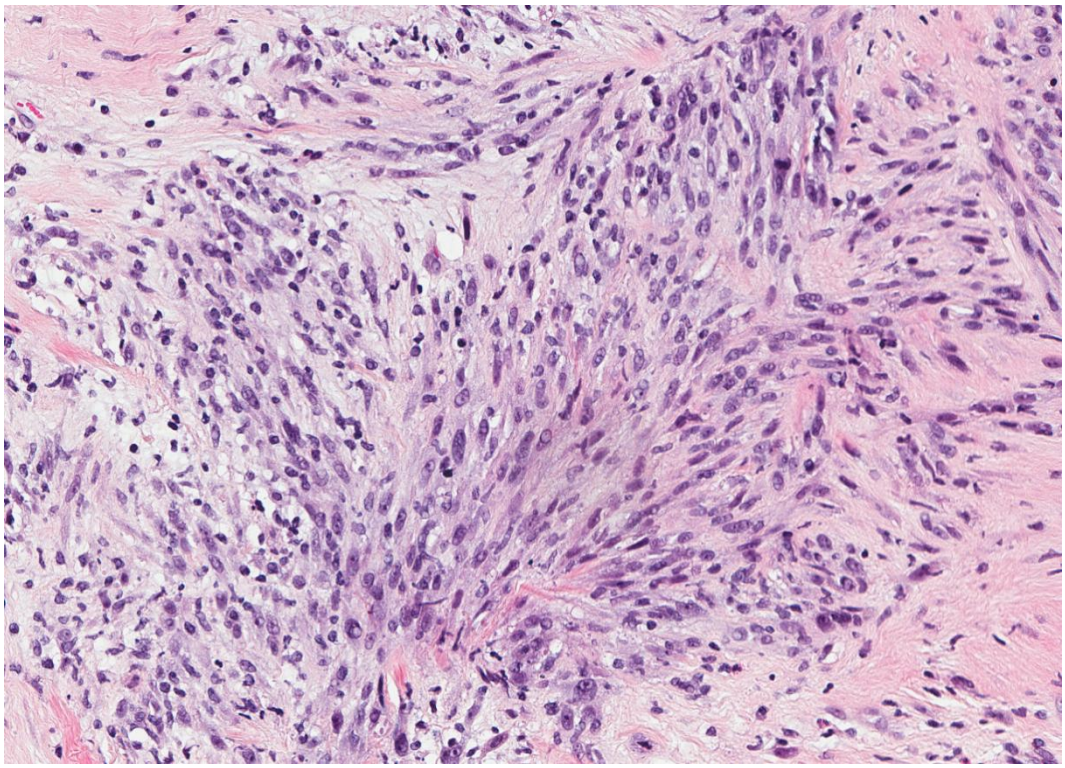


Figure 14. Hematoxylin and eosin (HE) staining of sectioned ISS tissue of cat 3 at, 20X objective. Intertwining spindle shaped cells are seen, most have one ovoid basophilic nucleus and pale eosinophilic cytoplasm.

Immunohistochemistry (IHC)

One pilot IHC staining round was done for each mentioned protein in order to test IHC methods and antibodies for feline sarcoma tissue. For both ɣH2AX and Atm slides with sarcoma tissue of cat 1 and 3 as well as a positive control slide (with irradiated mouse intestine) were stained. For p53, ERCC1, Rad51 with sarcoma tissue of cat 1 and 3 as well as a negative control. Positive control for the latter proteins was not available at the laboratory at the time of research. Time was limited for this research project so final IHC and counting of IHC positive cells in each slide was not possible. IHC staining was evaluated microscopically to determine if staining was positive and how pervasive staining was in each slide.

ɣH2AX

Sarcoma tissue of both patients shows positive nuclear staining in for ɣH2AX (see figures 14 and 15).

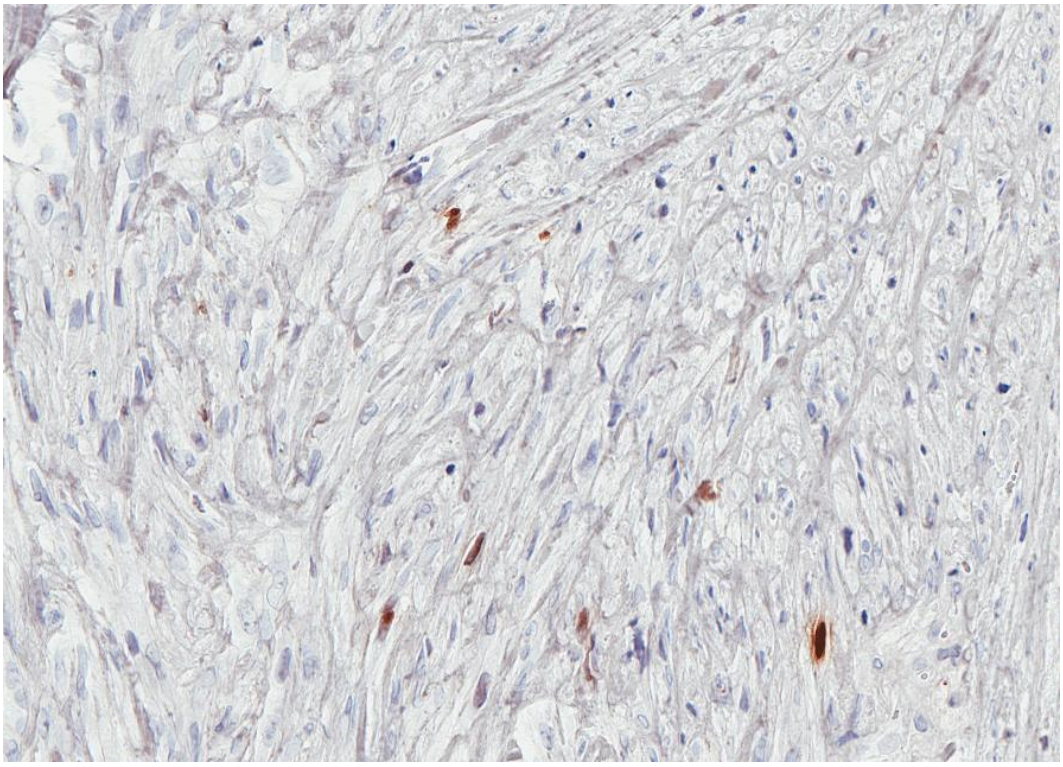


Figure 14. IHC for ɣH2AX of sectioned ISS tissue of cat 1, 20X objective. Positive nuclear staining is seen.

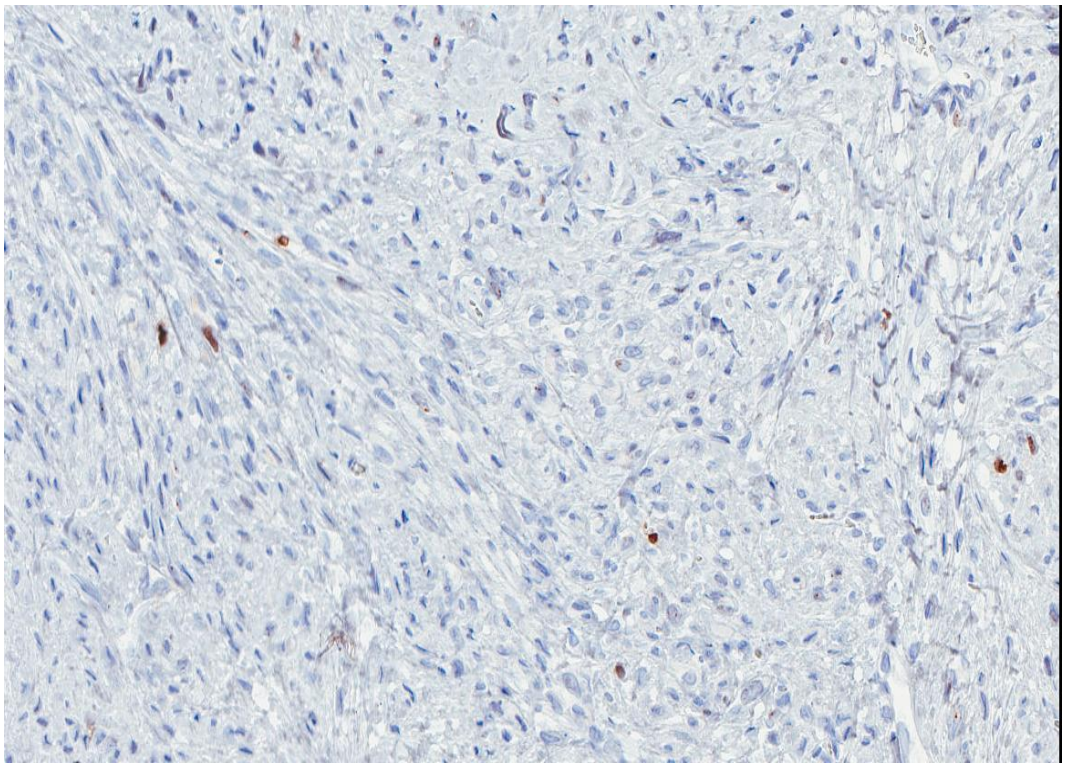


Figure 15. IHC for ɣH2AX of sectioned ISS tissue of cat 3, 20X objective. Positive nuclear staining is seen.

Atm

Sarcoma tissue of both patients shows pervasive positive staining for Atm (see figures 16 and 17).

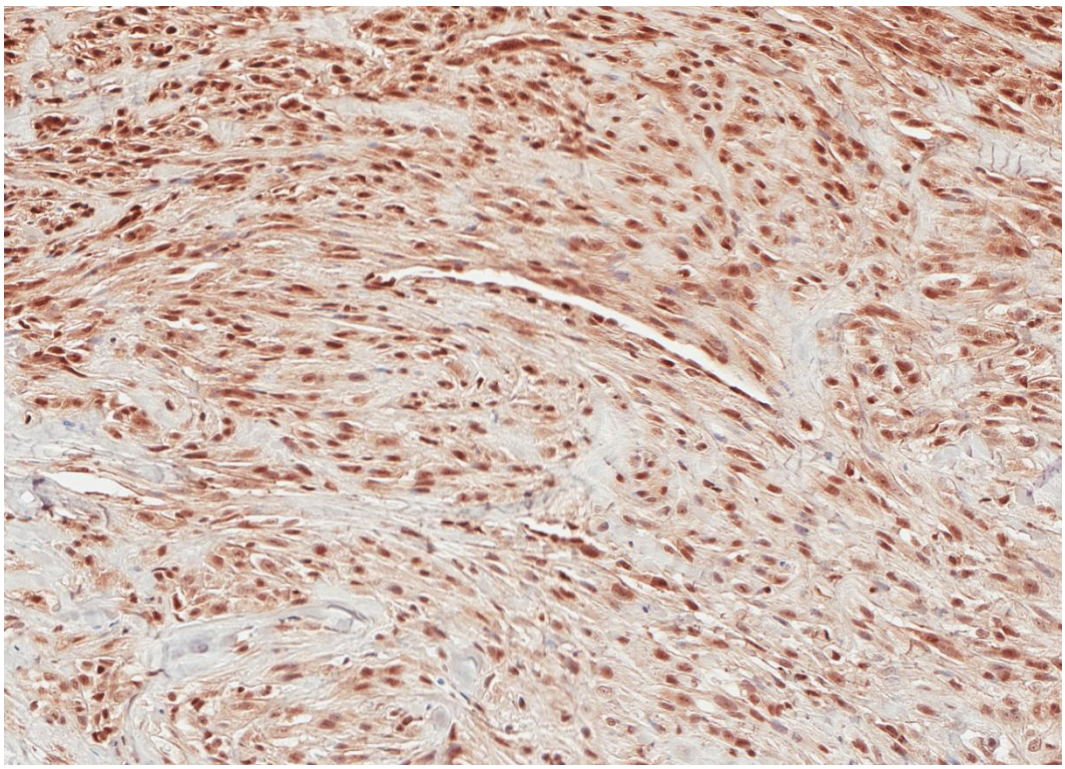


Figure 16. IHC for Atm of sectioned ISS tissue of cat 1, 20X objective. Pervasive positive staining is seen.

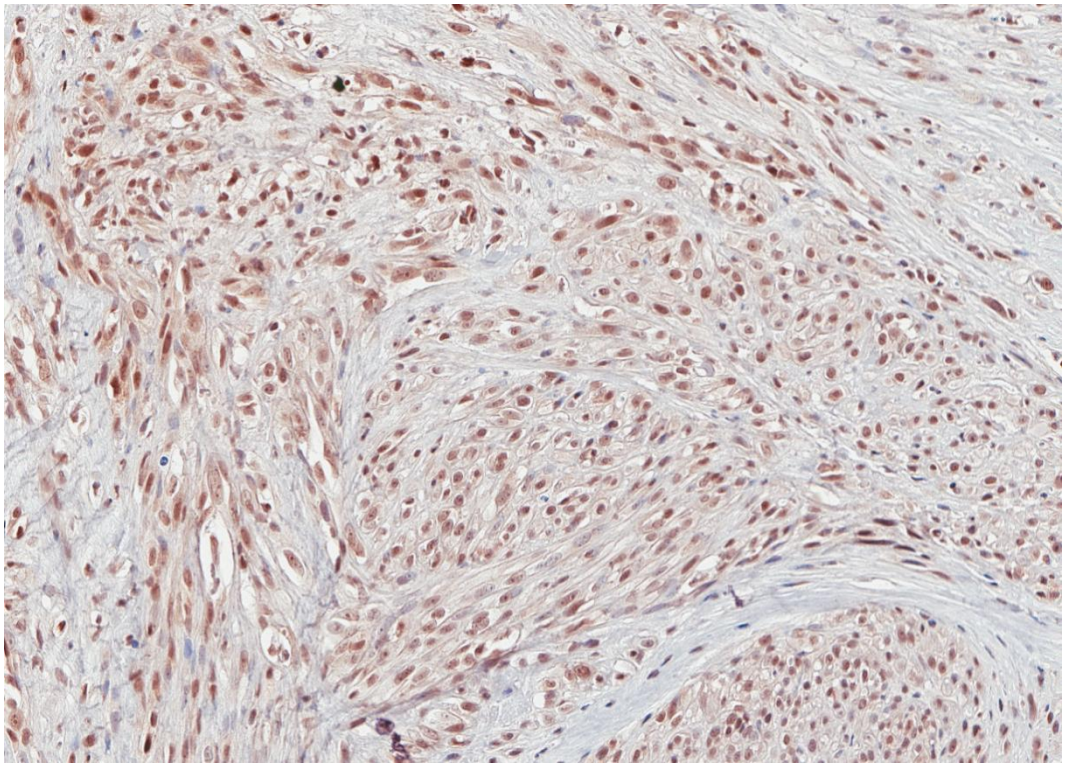


Figure 17. IHC for Atm of sectioned ISS tissue of cat 3, 20X objective. Pervasive positive staining is seen.

p53, ERCC1, Rad51

IHC for p53, ERCC1 and Rad51 did not stain positive in any of the slides.

Discussion

Samples from sarcomas on three different cats were used in this project, one of which (from cat 2) was not an injection site sarcoma but a fast growing recurrent sarcoma on the head with similar characteristics. The ISS from cat 1 and cat 3 were located on the right hip and the right thoracic wall respectively. In primary cell culture the cells derived from explants from different patients show variable growth patterns. This could be due to the individual characteristics of each tumor. All tumors microscopically showed intertwining spindle shaped cells in cell culture, indicating similar cell origin.

Colony forming assays using several cell densities were done to determine optimum cell density for further CFAs. We determined the cell density of 1,000 cells/ml to be optimal for CFAs due to the ability to form colonies for cells derived from all three sarcoma samples. These colonies were easily microscopically distinguishable from each other using this cell density. Pilot CFA's treated with Carboplatin at two different dosages were done using 1,000 cells/ml. Dosages of 20 µg/ml Carboplatin and 40µg/l Carboplatin were tested. Katamaya et al. described 20 µg/ml to be the IC_{50} (50% inhibitory concentration) for Carboplatin for feline ISS cells in vitro.[27] The other dose of 40 µg/ml was chosen to possibly inhibit a larger fraction of the sarcoma cells. Cells from all three cat sarcomas failed to grow colonies at 20µg/ml and 40µg/ml, however positive control did show colony formation. We determined the dosage of Carboplatin would need to be less than 20µg/ml in CFAs in order to detect a difference in chemosensitivity between sarcoma cells derived from different feline patients.

HE staining of both true ISSs was done to study tumor morphology without additional IHC staining. Both tumor cells of cat 1 and cat 3 showed similar morphology with intertwining spindle shaped cells, most of these had one ovoid basophilic nucleus and pale eosinophilic cytoplasm. This is conform what has been described on tumor morphology of feline ISS. [1,3,7-9]

Immunohistochemistry was performed on γH2AX , checkpoint proteins Atm an p53, and DNA damage proteins ERCC1 and Rad51. Due to limited time and extensive IHC procedures it was only possible to do IHC on slides from cat 1 and 3 plus a positive or negative control during this research project. A positive control slide which consisted of irradiated mouse intestine, known to have DNA damage and to show expression of γH2AX and Atm, was used for IHC for for γH2AX and Atm. No positive control was available in lab for p53, ERCC1 and Rad51 at the time of this research project, so negative control slides were used. IHC for γH2AX stained positive in the nucleus in both cat 1 and cat 3 slides, indicating presence of DNA damage in the sarcoma cells of the feline patients in this study.[5,6,18] IHC for Atm showed pervasive staining in cat 1 and cat 3 slides, however it is unknown whether there is upregulation of Atm or if optimalization of the IHC protocol is needed. IHC for p53, ERCC1 and Rad51 did not stain positive in any slide. A positive control was not used so the antibodies and method of IHC could not be confirmed to be effective in this type of tissue. Cat tissue was stained with anti-mouse antibodies for p53, but p53 IHC has been effective in other studies on feline ISS with polyclonal anti-human p53, monoclonal anti-mouse mutant p53 and polyclonal anti-human p53.[15,16] An ERCC1 antibody with cross species reactivity was used, expected to stain feline protein as well. For Rad51 IHC staining the same (murine) antibody was used that has been used in canine mammary carcinoma tissue.[28] Homology of Rad51 is extremely high for canine and murine protein, and similar results were expected for feline protein. Because no positive control was used for ERCC1 and Rad51, it is unknown if this is not correct or if IHC optimalization is needed.

Future research in this study will comprise several aspects. After determining the optimum IHC procedures and type of antibodies to be used for each protein, IHC for each protein in all slides from all three sarcoma samples will be performed also using positive and negative control slides. Assessment and scoring of IHC staining will be conducted according to the method described in this report. These IHC results will be compared to results of Colony Forming Assays and Cell Viability Assays (CVAs). The latter type of assay (also named MTT assays) will be done in addition to CFAs to validate results. CVA or MTT assay is a method in which viable cells reduce the yellow tetrazolium compound to insoluble purple crystals. These crystals are then solubilized and absorbance is compared by spectrophotometry between different cell lines or controls as a measure of cell viability. Optimal dosage of Carboplatin and Doxorubicin will have to be determined to use in both CFAs and CVAs. Then measurement of colony forming ability of the sarcoma cells following treatment with Doxorubicin and Carboplatin is possible. In addition to the assays, quantitative RT (reverse transcriptase) PCR analysis will be done on RNA from the sarcoma cells to verify IHC results. Evaluation of additional samples (more cats) is needed to draw conclusions from the results.

Conclusion

From the results of this research project, it could be concluded that sarcoma cells derived from different feline patients show variable in vitro growth patterns. DNA damage was detected in feline sarcomas, based on positive IHC staining of γH2AX in these cells. It is unknown whether there is upregulation of Atm or if optimalization of the IHC protocol is needed. Many methods of the study need to be optimalized in order to obtain useful results, and evaluation of additional samples is needed. Ultimately we hope expression of these proteins can be used to target patients to specific chemotherapy regimens.

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I want to thank Dr. Kelly Hume from Cornell University for the opportunity to do my research project under her supervision and participate in her very interesting study. She was a great mentor for me; I learned a lot and got to experience the research side of veterinary medicine.

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- Erin Daugherity
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- Timothy Pierpont
- Aria Hill
- David Karambizi
- Kelsey Poisson
- Sharon Guzman
- Yousuf Kharal
- Hilary Zhaoxu hu

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