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POTENTIAL PROGNOSTIC INDICATORS FOR CANINE HEMANGIOSARCOMA

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POTENTIAL PROGNOSTIC INDICATORS FOR CANINE HEMANGIOSARCOMA

By

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ABSTRACT

PROGNOSTIC INDICATORS FOR CANINE HEMANGIOSARCOMA

By

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Canine hemangiosarcoma is a common fatal disease. The goal of this study was to identify prognostic indicators for canine hemangiosarcoma patients treated with DAV. The focuses of this study were the *c-kit* receptor and the PI3k signaling pathway that have been associated with the pathology of many tumors. Medical records at Michigan State University Veterinary Teaching Hospital were reviewed for dogs with the diagnosis of hemangiosarcoma. Data was collected from sixteen dogs all treated with the same protocol. Immunohistochemistry was performed on the patients' tumor biopsies for p53, *c-kit*, PTEN, and caspase 3. The extraction of DNA was performed from paraffin embedded tissue biopsies to attempt to identify mutations in the *c-kit* gene. Amplification by polymerase chain reaction, and sequencing of exon 11 and exon 17 of the *c-kit* receptor were carried out. Immunohistochemistry identified 4 samples that stained positive for p53. There was no apparent PTEN staining in 5 samples, positive staining for PTEN in 9 samples, while 1 sample was not available for staining. The sequencing of the *c-kit* exon 11 and exon 17 did not reveal any mutations. Our results did not demonstrate a direct correlation between any of the markers studied and survival duration.

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1 INTRODUCTION

1.1 Research Problem:

Hemangiosarcoma is a common neoplasm in veterinary medicine and is a difficult disease to treat. The objectives of this project were to identify prognostic markers of tumor aggressiveness that might serve to inform therapeutic decisions and might further elucidate potential treatment targets.

1.2 Research Goals:

Our goal was to interrogate spontaneously arising canine hemangiosarcoma tumors using a panel of immunohistochemical markers. We sought to assess expression of members of the phosphatidyl inositol 3 kinase (PI3K) pathway, including mutational analysis of a prominent receptor tyrosine kinase of vascular endothelial cells, c-kit.

1.3 Research Hypothesis:

Our overarching hypothesis was that derangement of members of the PI3K pathway contribute to biologic behavior of canine hemangiosarcomas, thereby influencing duration of tumor response and patient survival in a cohort of dogs treated with a defined combination chemotherapy protocol.

1.4 Canine Hemangiosarcoma

Hemangiosarcoma (HSA) is an aggressive vascular endothelial tumor. In dogs hemangiosarcoma prevalence is 2 % of all tumors and accounts for 5-7% of visceral tumors(Aronsohn 1985; Spangler and Culbertson 1992; Wallace 2002). Hemangiosarcomas metastasize early in the course of tumorigenesis and 80 %

of patients with clinical signs will have evidence of metastasis on presentation(Withrow 2001). The mean age for the appearance of hemangiosarcoma is 10 years in dogs (Brown, Patnaik, and MacEwen 1985) younger individuals may be affected.

1.5 Epidemiology

Male dogs have been reported to have a higher probability for HSA development, but some reports suggest no gender predisposition. Large breeds are generally at higher risk with the German Shepherd dog the most common breed affected(Brown, Patnaik, and MacEwen 1985). Other breeds predisposed to HSA are the Golden Retriever, Pointer, Boxer, Labrador Retriever, Poodle, Great Dane, English Setter, and Siberian Husky(Brown, Patnaik, and MacEwen 1985; Srebernik and Appleby 1991). Lightly pigmented breeds are more likely to develop cutaneous and subcutaneous HSA (Blood Hound, Bulldog, English Pointer, Saluki, Dalmatian, and Whippet) (Wallace 2002).

Hemangiosarcoma is by far the most common tumor of the heart (Aronsohn 1985; Smith 2003; Ware WA 1995). Spayed females and neutered males had a reportedly greater risk to develop an atrial hemangiosarcoma as the primary tumor (Ware WA 1995). Liver prevalence for primary hemangiosarcoma is reportedly 5-6% of hemangiosarcoma cases. Concurrent cardiac and splenic tumors are seen in 25% of cases.

1.6 Biological Behavior

Hemangiosarcomas metastasize in the earliest stages of the disease through hematogenous routes, and metastases may be found in any organ. The most

common sites for metastasis are the liver, omentum, mesentery, and lungs. Hemangio-sarcomas metastasize to the brain, particularly to the cerebrum in 14% of the cases. Hemangiosarcoma is the most common tumor to metastasize to the brain in dogs(Waters and Hayden 1990; Waters, Hayden, and Walter 1989).

1.7 Etiology

Exposing dogs to ionizing radiation was reported to cause hemangiosarcoma in an investigative setting(Rebar et al. 1980). Subcutaneous and cutaneous lesions were observed commonly following radiation in an experimental model system. Bone and urinary bladder hemangiosarcomas were also observed to occur following experimental radiation(Nilsson, Morgan, and Book 1985; Rebar et al. 1980; Smith 2003).

Subcutaneous hemangiosarcomas account for 13-17% of hemangiosarcoma cases in dogs. Ultraviolet light injury seems to be the main etiological factor for cutaneous and subcutaneous hemangiosarcoma oncogenesis(Hargis et al. 1992). These lesions arise in areas of glabrous skin that lack pigmentation, but not in areas of haired skin(Hargis et al. 1992; Nikula et al. 1992).

During an experimental trial, dogs exposed to cesium144 or strontium90 absorbed large doses of radiation mainly to their lungs and perihilar lymph nodes. All the dogs exposed to this form of aerosol radiation developed pulmonary hemangiosarcoma. In addition, 40% of the exposed dogs developed osseous hemangiosarcoma (Benjamin 1975).

1.8 Clinical Presentation

On presentation, signs range from episodes of weakness, lethargy, and weight loss to acute collapse and death. In dogs affected with hemangiosarcoma, rupture of the tumor and bleeding into the abdominal cavity might present as a distended abdomen with signs of hypovolemic shock, pallor of mucus membranes, tachycardia and tachypnea(Kleine, Zook, and Munson 1970; Ng and Mills 1985). Patients have blood abnormalities with stage II or III hemangiosarcoma. Anemia, leucocytosis, and thrombocytopenia are the most common hematologic abnormalities(Kessler, Maurus, and Kostlin 1997).

Location of the hemangiosarcoma in the right atrium may result in bleeding followed by cardiac tamponade, jugular pulses, muffled heart sound and dyspnea(Aronsohn 1985; Berg 1984). Right heart failure signs may be observed. Seizures may accompany brain metastasis(Waters and Hayden 1990). Other clinical signs such as lameness, lower motor neurological deficit, or bloody urine may vary according to the tumor location (Barber 1973).

Cutaneous hemangiosarcoma lesions in the dog are usually nodules of 2 cm or less in diameter. The lesions are non-demarcated and have a red to blue-black discoloration(Goldschmidt 1992). When compared to hemangiomas, hemangiosarcomas tended to invade the subcutaneous (73%) tissue rather than being confined to the dermis (7%). Hemangiomas had the same rate of presentation in the subcutaneous and cutaneous dermal layers (Hargis et al. 1992). Subcutaneous lesions may be as large as 10 cm with poorly

circumscribed borders and dark red to blue-black discoloration. Alopecia, ulceration, and thickening of the skin are often seen associated with the lesion.

1.9 Diagnosis

1.9.1 Clinical Pathology

Complete blood counts performed on hemangiosarcoma patients will usually demonstrate a regenerative anemia with polychromasia and reticulocytosis (Kleine, Zook, and Munson 1970). In 50 % of canine patients, acanthocytes will be noticed on a blood smear and occasional schistocytes will be observed (Hammer, Couto, Swardson et al. 1991; Hirsch, Jacobsen, and Mills 1981). Maturation of the red blood cells takes place within the spleen, and as a result nucleated red blood cells are commonly seen in cases of splenic involvement or following splenectomy (Johnson et al. 1989). Nucleated red blood cells may be the result of bone marrow efforts to compensate for blood lost following ruptured hemangiosarcoma, due to acute hypoxemia, or may be due to extramedulary hematopoiesis (Kleine, Zook, and Munson 1970).

Thrombocytopenia can be seen in 75% of hemangiosarcoma patients. At least 50% of hemangiosarcoma patients will have coagulopathies on presentation, and 25 % of them will die due to this condition (Hammer, Couto, Swardson et al. 1991). Disseminated intravascular coagulation (DIC) was characterized in one study of solid neoplasms only by laboratory abnormalities: thrombocytopenia; prolongation of PT and APTT. Plasma antithrombin III activity and fibrinogen level is decreased in DIC and the fibrin degradation products (FDPs) are in most cases elevated. When three or more of the above

abnormalities are present, the coagulopathy is defined as constituting DIC. Following this criteria, 35% (n=20) of hemangiosarcoma patients are diagnosed with DIC (Maruyama et al. 2004).

Elevation of the neutrophil count may occur due to bone marrow compensation for blood loss, shock, stress response, and neutrophil demargination. In one study, 26 out of 34 canine patients presenting with hemangiosarcoma had leucocytosis, while 3% had leucopenia (Pintar et al. 2003). Eosinophilia was reported in 25% of the cases by one group (Bertazzolo et al. 2005).

Cytological samples of hemangiosarcoma show cells with a high-grade sarcomatous appearance and marked pleomorphism in 4 out of the 19 cases reported in a published paper. Most of the cases examined by the group had low-grade, spindle shaped, and monomorphic cells (Bertazzolo et al. 2005).

Pericardial effusion resulting from atrial hemangiosarcoma was compared to idiopathic pericardial effusion. The effusion PH was measured in both patient groups. Due to the overlaps in the results, the conclusion was that pH determination was neither sensitive nor specific (Fine 2003).

Cardiac troponin I (cTnI) and cardiac troponin T (cTnT) are specific markers for cardiac ischemia. When pericardial effusion resulted from hemangiosarcoma, the hemangiosarcoma group had significantly higher serum concentrations of cTnI but not cTnT when compared to the levels seen in idiopathic pericardial effusion cases (Shaw, Rozanski, and Rush 2004).

Urinary bladder hemangiosarcoma is rare and was reported to cause hematuria in one case and in another case induced hematuria, proteinuria and mild bilirubinuria (Liptak, Dernell, and Withrow 2004; Wang and Su 2001). Hemangiosarcoma of the urethra is not common but may cause oliguria, stanguria and hematuria due to partial or complete urinary obstruction (Tarvin, Patnaik, and Greene 1978). Urinalysis is among the diagnostic procedures recommended for staging and ruling out the presence of concurrent disease processes such as urinary tract infection.

1.9.2 Diagnostic Imaging

Three-view thoracic radiographs of HSA cases may reveal abnormalities such as metastatic or primary tumors in the lungs. The heart may show an abnormal oval shape in the case of pericardial effusion (Kleine, Zook, and Munson 1970). Soft tissue opacities are evident and evidence of peritoneal effusion such as hemoabdomen results in loss of visceral details which may be observed on abdominal radiographs.

An ultrasound may reveal a visceral location of hemangiosarcoma in the spleen, liver, omentum, mesentery, urinary bladder, urethra, or potentially in any other abdominal organ (Mellanby et al. 2004; Wrigley et al. 1988). An echocardiogram may demonstrate hemangiosarcoma in the heart or pericardium. The use of magnetic resonance imaging (MRI) proved to be a better diagnostic tool than ultrasound for diagnosis of visceral lesions such as those caused by hemangiosarcoma in the dog (Clifford et al. 2004). Computed tomography is efficient to demonstrate liver structure and may help in the diagnosis of soft

tissue abnormalities such as hemangiosarcoma (Winter, Kinney, and Kleine 2005). Electrocardiography may show pre-ventricular contractions associated mostly with tumors involving the spleen, and as a result of hypovolemia following tumor rupture (MacDonald et al. 1975).

Echocardiography is among the most accurate methods to diagnose cardiac abnormalities. The two dimensional echocardiograph can help detect soft tissue structures and space occupying fluids. Pericardial fluid will increase the contrast of the different media and will assist in obtaining better image (Thompson 1995).

1.10 Staging

The TNM staging system developed by the World Health Organization (WHO) was adopted to better characterize canine hemangiosarcoma patients.

Primary Tumor

T0:	no tumor evident
T1:	tumor less then 5 cm in diameter, confined to one organ, and does not invade beyond the dermis.
T2:	tumor greater than or equal to 5 cm in diameter or ruptured or invasive to the subcutaneous tissues.
T3:	tumor invades adjacent structures

Regional lymph nodes

N0:	no regional node involvement.
N1:	regional node involvement
N2:	distant node involvement

Distant metastasis

M0: no distant metastasis
M1: distant metastasis confirmed

Staging system

Stage I:	T0 or T1, N0, M0
Stage II:	T1 or T2, N0 or N1, M0
Stage III:	T2 or T3, N0, N1, or N2, M1

1.11 Treatment

Table1: Treatment of canine hemangiosarcoma

Tumor site	Treatment	No. Patients'	Median survival (days)	reference
Spleen	Surgery alone	59	19	Prymak et al
	Surgery alone	21	65	Brown et al
	Surgery alone	19	56	Johnson et al
	Stage I	4	91	Johnson et al
	Stage II	1	168	Johnson et al
	Stage III	14	56	Johnson et al
	Surgery alone	32	86	Wood et al
	Surgery +MBV	10	91	Brown et al
	Surgery + MBV+VMC	10	117	Brown et al
	Surgery +VAC +Cholrambucil	6	145	Hammer et al
	Surgery + VAC	3	140	Johnson et al
	Surgery + AC	16	141	Vail et al
	Surgery + AC +L-MTP-PE	16	273	Vail et al
Right atrium	VAC (alone)	1	140	De Madron

1.12 Comparative Aspects

1.12.1 Human Angiosarcoma

Hemangiosarcoma is considered to be a reasonable comparative model for human angiogenic tumors. Similarities are found between the biological behavior of the splenic form of human angiosarcoma and canine hemangiosarcoma (Fosmire 2004). Angiosarcoma is a high-grade aggressive sarcoma that carries a poor prognosis. Disease free intervals of 44% at 2 years and 24 % at 5 years were reported to be the overall human prognosis (Mark et al. 1996).

1.12.2 Epidemiology

In humans, angiosarcomas account for up to 1-2% of all the soft tissue sarcomas. Most angiosarcomas are cutaneous and are observed in Caucasians between the ages of 56-92. The ratio of disease between men and women is 2:1 (Naka et al. 1995). Dermal lesions are largely actinic in origin and the most common location for dermal HSA is the scalp and the upper face (Holden, Spittle, and Jones 1987; Morrison et al. 1995).

Angiosarcomas are subdivided to several groups: (1) cutaneous angiosarcoma (2) angiosarcoma of the breast (3) angiosarcoma of deep soft tissue, (4) angiosarcoma affecting the parenchymal organs (Fedok et al. 1999; Pollock 2005).

Stewart-Treve syndrome was reported as a consequence of radiation therapy for mammary carcinoma. The clinical presentation of this angiosarcoma syndrome occurs following radical node dissection for breast cancer with

resultant chronic lymphedema. Angiosarcoma arises as a cutaneous lesion after radiation of the breast (Billings et al. 2004). Angiosarcoma is the most common sarcoma of the liver and accounts for 2% of all liver tumors (Forbes et al. 1987; Maluf et al. 2005).

1.12.3 Etiology

Visceral angiosarcoma etiology is related to occupational exposures. Vinyl chloride is a known etiological cause for hemangiosarcoma in humans. The K-ras-2 oncogene is point mutated, causing T transition in the second nucleotide at codon 13 which results a glycine substitution by aspartic acid in the p21 protein (Marion, Froment, and Trepo 1991). Angiosarcoma of the liver was associated with exposure to vinyl chloride, arsenic, and Thorotrast®, a now-banned radiographic contrast agent (Maluf et al. 2005). In 25% of the reported cases there was an involvement of synthetic vessel grafts, heritable conditions, or prior trauma or surgery (Meis-Kindblom and Kindblom 1998).

Kaposi's sarcoma presents on histology as multicentric endothelial cell growth and many times may be indistinguishable from angiosarcoma pathology (Dictor, Ferno, and Baldetorp 1991). Human Herpes Virus-8 (HHV-8) is necessary for the development for Kaposi's sarcoma, by an unknown pathogenic route. Direct tumor cell transmission by sexual contact was suggested to occur in Kaposi's sarcoma as the disease is often present in immune-deficient patients (Peterman, Jaffe, and Beral 1993) Human Herpes Virus-8 was detected in all the cases of Kaposi's sarcoma but was not detected in most other types of angiosarcoma (Schmid and Zietz 2005).

Kaposiform hemangioendothelioma has been reported once in the dog. The case report described a 10 year old dog that developed 1-3 mm red circumscribed papules on his left ventromedial posterior limb. On histology, the lesion was a multinodular focal infiltrated tumor; the cells in the deeper dermis had a spindloid shape forming vascular slits (Vincek, Zaulyanov, and Mirzabeigi 2004).

1.12.4 Biological Behavior

After treatment, 81% of human hemangiosarcoma patients have local failure and 50% of these patients are reported to have distant metastasis (Mark et al. 1996). The most common metastatic sites for angiosarcoma are lungs, lymph nodes, soft tissues, bone, liver, and other sites (Meis-Kindblom and Kindblom 1998). In one paper most of the prognostic factors for angiosarcoma were independent, with only mitotic index significant as a prognostic indicator (Naka et al. 1996). Tumors of the head and neck exceeding 7 cm in diameter had a less favorable prognosis when compared to smaller tumors (Aust et al. 1997).

Angiosarcoma of the spleen metastasizes in 80% of human patients, with decreased survival when hemoabdomen is present (Hsu et al. 2005).

1.12.5 Clinical Presentation

The clinical presentation varies with the location of the disease. Angiosarcoma of the scalp and face usually presents as bruise-like lesions, dusky plaques, chronic edema or cellulitis, ulcerated nodules, and pyoderma, (Holden, Spittle, and Jones 1987). Spleen angiosarcoma presents as

splenomegaly in 50% of the cases, and 30 % will have a ruptured spleen on presentation (Hsu et al. 2005).

1.12.6 Treatment

Surgery is the treatment of choice in human angiosarcoma, followed by radiation when the margins are not completely excised or in cases when the tumor size is large, with deep extension or multi-centric distribution (Lydiatt 1994). Paclitaxel and pegylated-liposomal doxorubicin (PLD) are highly effective in Kaposi's sarcoma (KS) and angiosarcoma as well as in other soft tissue sarcomas in human medicine. In a phase II trial, administration of Paclitaxel as a single agent resulted in complete response in four of nine patients and partial response in four other patients (Fata et al. 1999). Interferon Alfa-2a and 13-cis-Retinoic Acid were reported to produce remission when injected subcutaneously in a small number of cases (Romano et al. 2004; Spieth, Gille, and Kaufmann 1999).

1.13 C-kit

KIT was originally isolated from Hardy-Zuckerman Feline sarcoma retrovirus. (Besmer 1986). The virus was extracted from a cat's soft tissue sarcoma but was found in normal cat tissues as well (Besmer 1986). In 1993 the KIT protein was assigned the cluster of differentiation number CD-117.

1.13.1 *c-kit* Gene

A cellular homolog of the KIT protein is encoded by the *c-kit* gene, which is made of 21 exons. The *c-kit* gene structure resembles the CSF-1R gene (*c-fms*)

and the PDGF gene, both of which are part of the receptor tyrosine kinase family (Vandenbark et al. 1992).

In humans, the *c-kit* gene was mapped at position 4q12 in the pericentromeric location of the long arm of chromosome 4. KIT is the product of the (W) locus or white spotting locus on chromosome 5 of mice (Chabot et al. 1988). The murine *c-kit* gene is made of 21 exons of 100-200 base pairs each (Gokkel et al. 1992).

1.13.2 KIT Ligand and Receptor Structure

The KIT ligand is also known as stem cell factor (SCF). This ligand may exist in a soluble or transmembrane form. The ligand corresponds to the mouse steel locus factor (SLF) (Williams et al. 1990). The first 165 amino acids of the 189 amino acid extracellular domain encode for the soluble SCF (Arakawa et al. 1991). The soluble form of the KIT ligand results from the cleavage of the transmembrane precursor (Huang et al. 1992). There are three regions essential for ligand function in SCF, which are located between the amino terminus and amino acid G35, between amino acid L79 and amino acid N97, and between amino acid R121 and amino acid D128 (Matous, Langley, and Kaushansky 1996).

Normal KIT ligand and normal receptor function is essential for proper hematopoesis, gametogenesis, and melanogensis. Growth, differentiation, and adhesion of mast cells are also KIT dependent (Heinrich et al. 2000; Migliaccio et al. 1991). Stem cell factor expressed during embryogenesis is seen in cells associated with migration of melanoblasts, germ cells and hematopoietic stem cells (Mattsson et al. 1977). KIT signaling is necessary but not sufficient to

stimulate bone marrow colony forming units. The KIT ligand effect is potentiated by erythropoetin, granulocytic colony stimulating factor, and interleukins (IL-1, IL-3, IL-6, IL-7, and IL-12) (Matous, Langley, and Kaushansky 1996; McNiece, Langley, and Zsebo 1991; Molineux et al. 1991).

The KIT tyrosine kinase receptor consists of four domains. The extracellular domain is coded by exons 1-9 and is made of five immunoglobulin-like loops. Exon 10 encodes the transmembrane domain and exon 11 encodes the juxtamembrane domain. The tyrosine kinase domain is made of two domains: TK1 and TK2. The tyrosine kinase domain is encoded by exons 13-21 (Miettinen, Sarlomo-Rikala, and Lasota 2000). The KIT protein is 145 kd in weight and is part of the subclass III PDGF and CSF family of receptors {Heinrich, 2000; Yarden, 1987}. The subclass members of this receptors family share similar structural features including sequence homology and distribution of cysteine residues in the extracellular domain (Yarden et al. 1987). As is common to other members of the subclass III PDGF group, the endoplasmic domain of the kit receptor has a long hydrophilic chain (Yarden et al. 1987).

Many normal cells express KIT receptors. Cajal cells in the gastrointestinal tract carry KIT. Cajal cells may have an important role in gastrointestinal pacemaking, and their normal function seems to be KIT-dependent (Huizinga et al. 1995). Nocka et al proved that KIT receptors are present on normal melanocytes and that these cells are dependent on normally functional KIT receptor. The absence of KIT activity in melanocytes results in piebaldism in knock-out mice (Nocka et al. 1989). KIT was demonstrated to be important in

mast cells and in erythropoetic cells of fetal and adult tissues as well (Matsui, Zsebo, and Hogan 1990; Nocka et al. 1989). Some germ cells carry KIT, and the presence of the receptor and its importance for cell function has been well investigated. Ovocytes in the primary follicle are dependent on kit for normal oogenesis. In testicular tissue, some spermatogonial stages are KIT dependent (Manova et al. 1990; Nocka et al. 1989).

1.13.3 KIT Mutations

A mutation in the W locus in mice induces a loss of pigmentation due to melanocyte dependence on functional KIT. Macrocytic anemia and sterility in mice were demonstrated in a KIT deficient knock-out model, as well a coat color changes (Chabot et al. 1988).

Naturally arising mutations of KIT can be divided into two main categories: the 'regulatory type mutations', and the 'enzymatic pocket type' mutations. 'Regulatory type' mutations are in the juxtamembrane domain and impact regulation of the kinase activity. The 'enzymatic pocket type' mutations cause alteration in the amino acid sequence of the enzymatic site (Longley, Reguera, and Ma 2001).

Mutation in exon 11 between the amino acids 550-560 of the inhibitory alpha helix was found to be common in 74% of gastrointestinal stromal tumors (GIST). This mutation causes missense sequence, in-frame deletions, and in-frame duplications. Mutations in exon 9 and 13 were noted in 13% and 2.4% of GIST's, respectively (Lasota et al. 1999; Rubin et al. 2001). A point mutation at codon 816 causes a substitution of valine for aspartate in the activation loop of the

enzymatic pocket. This mutation is present in all adult mast cells tumors and in small number of pediatric mastocytosis cases in humans (Longley et al. 1999). In human acute myelogenous leukemia (AML), exon 8 mutation was found to be common to one third of cases. Exon 8 mutations result in the loss or replacement of the codon for Asp 419 in the extracellular domain of the KIT receptor (Gari et al. 1999).

The roll of KIT mutation in melanomas is controversial. Human uveal melanoma was reported to express KIT mutations in more than 64% of cases and the same molecular lesions are also present in metastasis of this tumor (All-Ericsson et al. 2004; Guerriere-Kovach et al. 2004; Mouriaux et al. 2003). Some reports show that the expression of the KIT receptor is lost in melanoma (Montone et al. 1997). Other reports support KIT receptor expression in melanoma, associated with exon 11 mutations. However, most invasive metastatic human melanomas contain wild type KIT protein (Willmore-Payne et al. 2005).

Angiosarcoma tissues from 50 patients were tested for the presence of the KIT receptor with 56% found positive. The CD-117 marker was not present on benign tumors such as angiomas. Following the previous results the same group examined exon 11 and 17 for presence of mutation. Only five of the samples had mutation in any of the investigated sites, and were suggested to follow a fetal capillary endothelial cell pattern (Miettinen, Sarlomo-Rikala, and Lasota 2000).

The presence of KIT receptor was associated with seven out of the seven-endothelial angiosarcoma cases studied and was suggested to be associated with the higher-grade tumors.

1.13.4 KIT in Canine Tumors

Canine *c-kit* mutations have been found in mast cell tumors (MCT). When the three different grades of mast cell histologies were evaluated, 13.6% of 88 samples showed KIT receptor mutations (Zemke, Yamini, and Yuzbasiyan-Gurkan 2002). The grade I mast cell tumors did not express any *c-kit* mutations. Grade II tumors had less then 10% KIT mutations. In the 58 samples of grade II MCT evaluated by PCR, 4 samples were identified to have duplication mutations in the juxtamembrane region and 4 samples had deletions in this domain. Four of 6 tumors of grade III histology had KIT mutation in this study, all of which were duplication mutations (Zemke, Yamini, and Yuzbasiyan-Gurkan 2002). Tandem duplication was found to be a common *c-kit* mutation in canine mast cell tumor. Exon 11 in the juxtamembrane domain is believed to be the KIT receptor negative control region. In one study, exon 11 had five tandem duplications at the 3' region of the exon adjacent to the ATP binding region of the intracellular kinase. The fifth tandem duplication was located at the 5' end of exon 12. Tandem duplication of exon 11 may be extensive, through intron 11 extending to exon 12. When the C2 canine mast cell tumor cell line was examined, a deletion of 4 amino acids was found on exon 9 of the extracellular binding domain. This C2 cell line showed autophosphorylation in the absence of the SCF ligand (London et al. 1999).

Canine hemangiosarcoma was found to express KIT receptor by one group of investigators. The tyrosine kinase receptor KIT was expressed in 8 canine cell lines that were produced from hemangiosarcoma lesions (Fosmire 2004). To our knowledge KIT mutation has not been investigated in spontaneous canine hemangiosarcoma lesions.

1.14 Apoptosis and the Caspases

Apoptosis, or programmed cell death, is an essential process for development and maintenance of organism homeostasis (Thompson 1995). Different insults may cause irreparable damage to the cell's DNA, thereby initiating apoptosis through the intrinsic pathway. The first description of the physiology of apoptosis was produced in 1972 (Kerr, Wyllie, and Currie 1972). Apoptosis requires the cell to invest energy to accomplish its own destruction. Cells going through apoptosis have a unique morphology. The cytoplasm appears shrunken and has membrane alterations including blebbing. In apoptotic cells, the nucleus is condensed and then cleaved to mono or oligo fragments of nuclear DNA. Ultimately, the cells are destroyed, with the production of smaller nuclear bodies that are recognized microscopically as nuclear pyknosis (Leist and Jaattela 2001).

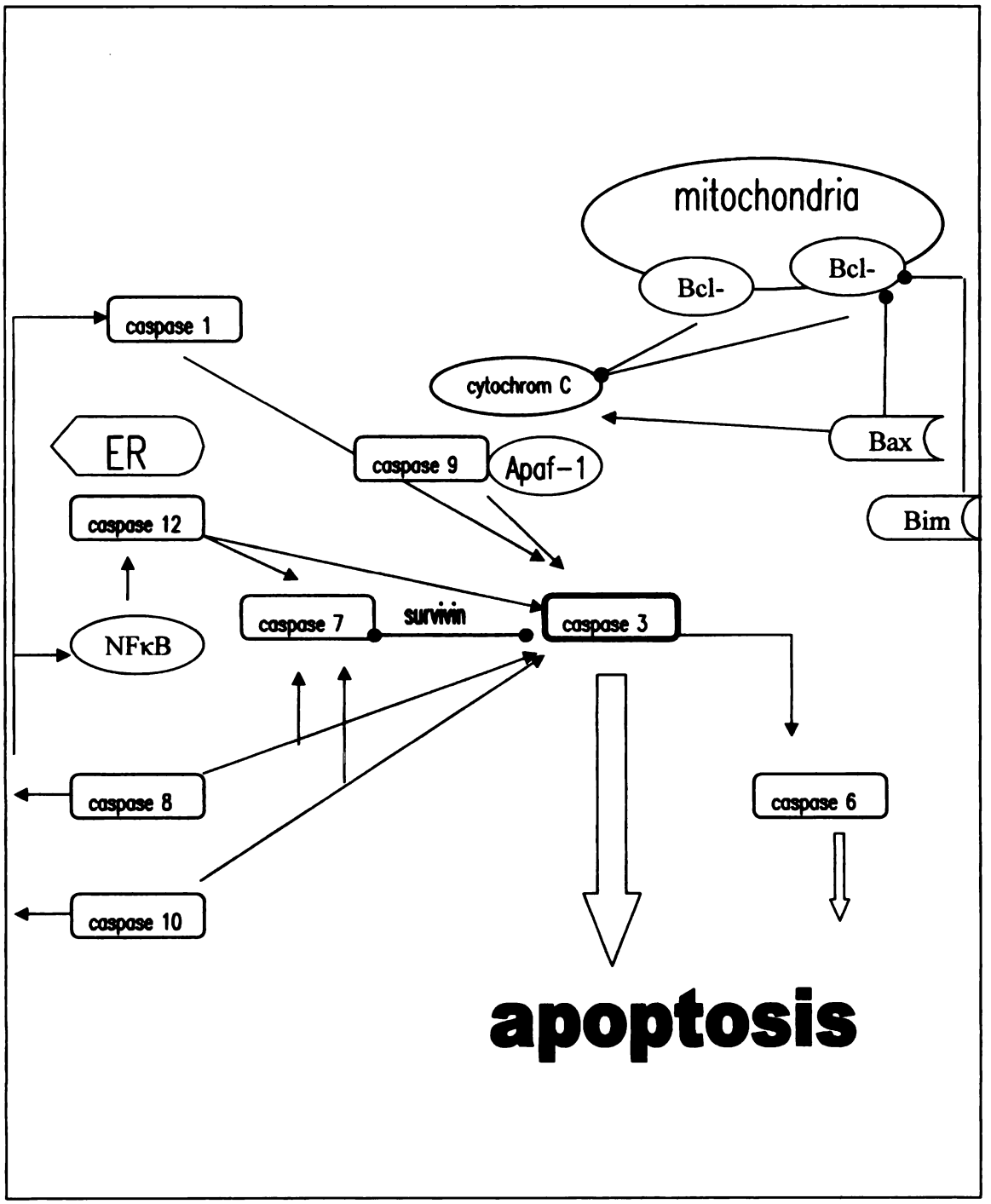
Apoptosis is a caspase-dependent process. Caspases demonstrated in *Caenorhabditis elegans* were proven to be similar to human and murine interleukin-1 beta-converting enzyme (Yuan et al. 1993). There are two main groups of caspases called initiator and effector caspases. The initiators include caspases 2, 8, 9, and 10 while the effectors include caspases 3, 6, and 7. The

caspases are produced as zymogens that are inactive. When cleaved by converting enzymes, the caspases become a tetrameric enzyme with two active sites (Mittl et al. 1997; Walker et al. 1994). Caspases have a prodomain on the amino-terminal side followed by a large 20 kDa (p20) subunit, and by small 10kDa (p10) subunits (Yan et al. 2006).

The initiator caspases can activate themselves and subsequently activate the effector caspases. The activity of the initiators is tightly regulated, and may be dependent upon complex formation (Benedict et al. 2000). Caspases 8 and 10 have death effector domains (DED) in the prodomain region. Caspases 1, 2, 4, and 9 contain the caspase recruitment domain (CARD) in the same location, instead of the DED (Hofmann, Bucher, and Tschopp 1997; Tartaglia 1993).

The active site of all the caspases has affinity for electrophilic carbonyl and aspartyl moities. This site is the most investigated target to inhibit caspase activity (Howard et al. 1991; Okamoto et al. 1999). Within the active site, there is an allosteric region that contains cysteine. When cysteine is bound by a disulfide bond to the 2-(2,4-Dichlorophenoxy)-N- (2-mercapto-ethyl)-acetamide (DICA) and 5-Fluoro-1H-indole-2-carboxylic acid (2-mercapto-ethyl)-amide (FICA) compound, caspase 3 and 7 activity is inhibited (Hardy et al. 2004).

Figure 1: The caspases and apoptosis



1.14.1 Caspase 9

Activation of caspase 9 is complex dependent. Apaf-1 regulates the activation of caspase 9. A knock-out murine model with Apaf-1 deficiency showed brain tissue growth abnormalities. The caspase 9 pathway is activated by cytochrome c that is released from the mitochondria and by the Apaf-1 complex. When the cytoplasmic reticulum is insulted, interleukin 12 activation of caspase 9 is initiated (Morishima et al. 2002). Caspase 9 activation activates downstream effectors caspase 3 and caspase 7 (Srinivasula et al. 1998).

1.14.2 Caspase 8

Caspase 8 is an initiator caspase that can start a downstream apoptotic pathway mediated through caspase 3 and caspase 6 (Hirata et al. 1998; Takahashi et al. 1997). Caspase 8 may also initiate Bid cleavage to a fragment that includes *cytochrome-c*, which then will bind to Apaf-1 and caspase 9. This pathway may be blocked by Bcl-2 (Krippner et al. 1996; Scaffidi et al. 1998).

1.14.3 Caspase 3

Caspase 3 was first described in 1994 when cloned from human Jurkat T-lymphocytes. Similar to other caspases, caspase 3 has two subunits: a large 20 kDa unit and a small 10 kDa unit. The N-terminus of caspase 3 is short, as is true for other effector caspases such as caspase 7. Caspase 3 is highly expressed in tumor cells of hematopoietic origin such as lymphocytes and promyelocytes. Brain glioblastoma and fetal tissues also demonstrate high baseline expression of caspase 3 (Fernandes-Alnemri, Litwack, and Alnemri 1994).

In the absence of caspase 3, knock-out mice (caspase 3-/-) were born at a lower rate than the expected mendelian frequency (9%). The newborn caspase 3 deficient mice were smaller than their wildtype counterparts and succumbed at 1-3 weeks of age. These knock-out mice developed brain tissue abnormalities. Phenotypically these lesions present as variety of hyperplasias of brain tissue along with disorganization of brain tissue architecture. The mutated mice had a variety of neurological abnormalities and had visible masses on their heads. Lack of caspase 3 did not affect immature T and B-lymphocytes' ability to undergo apoptosis or maturation in this setting. (Kuida et al. 1996; Woo et al. 1998).

Canine caspase 3 is homologous with human caspase 3. The dog caspase 3 amino acid sequence was compared to those of human, pig, mouse, chicken and zebra fish. Caspase 3 of the dog was found to be homologous at rates of 88.4%, 88.0%, 85.9%, 65.9%, and 60.6% respectively at the amino acid level. The active catalytic site of caspase 3 in the dog is identical to that of human caspase 3 (QACRG;Gln-Ala-Cyst-Arg-Gly). The expression of caspase 3 in normal dog tissue was found to be strong in the skin, lymph node, and bone marrow, while expression in the lung was weak (Sano et al. 2004).

To my knowledge there are no published studies research concerning the role of caspase 3 in canine hemangiosarcoma.

1.15 PTEN

The "phosphatase and tensin homolog deleted on chromosome 10" (PTEN) was first described and linked to carcinogenesis by Li et al in 1997. The same

group mapped the location of PTEN to chromosome 10q23 (Li and Sun 1997). The gene is known to be mutated or deleted in several human tumor types.

The PTEN gene is located on chromosome 10q23 and comprises 9 exons. The gene encodes 403-amino acids, which make up the PTEN protein. The N-terminal region of this protein shares high homology to other protein phosphatases and to tensin (Li and Sun 1997; Steck PA 1997).

The PTEN protein functions as a phosphatase to phosphatidylinositol 3, 4, 5 - trisphosphate (PIP3). The PIP3 protein is an important factor in cell growth and is produced by phosphoinositide 3-kinase (PI3K) when stimulated by tyrosine kinase receptors bound to a ligand or mutated tyrosine kinases that autophosphorylate PI3K. The PTEN protein has the capacity to dephosphorylate PIP3, thus preventing downstream signaling through the PI3K pathway. When PTEN is mutated it loses the ability to stop the PI3K signaling pathway (Maehama and Dixon 1998).

The effect of PTEN regulation on cell migration and invasion was suggested to be mediated by the inhibition of cofactors FAK and Shc that support migration, invasion, and growth (Tamura et al. 1999).

A mutation in position 319 of PTEN is the most common observed. This mutation creates a stop codon that blocks the normal production of PTEN protein. This type of mutation is common to several tumors, including: glioblastoma; endometrial cancer; and epithelial ovarian tumor (Duerr, 1998; Kurose, 2002; Kurose, 1998; Obata, 1998). In gliomas and glioneuronal tumors, an in-frame deletion in PTEN changes the recognition of codon 319 (Duerr,

1998). The carboxyl region of the PTEN protein regulates its stability and enzyme activity. A mutation in codon 342 or 342 results in a stop codon (Georgescu MM 1999; Li and Sun 1997). Deletion in the PTEN-351 position leads to a mutation in intrinsic phosphatase activity that is affected by the conformation of the C-terminal structure. This mutation will disturb the normal function of the protein as a catalase (Georgescu MM 1999).

1.16 P53

1.16.1 p53 Gene

The p53 gene was first identified in the late seventies. The p53 protein was described as a phosphoprotein that bound to the SV40 DNA virus (Harris et al. 1986). The 54 kd size protein was produced after murine and hamster cells were stimulated by the tumor-inducing SV 40 virus (Harris et al. 1986).

In humans, the p53 gene is located on the short arm of chromosome 17p13 (Miller et al. 1986). The human gene is larger than the mouse gene, but otherwise shares the same organization. The size of the gene in humans is 20 kb compared with 12kb in the mouse (Bienz et al. 1984; Lamb and Crawford 1986). As seen in mice, the human gene is made of 11 exons and 10 introns, so the differences in overall gene size are due to differences in intron size (Lamb and Crawford 1986). The first and the second exons in both species are separated by a 10kb intron (Lamb and Crawford 1986). Exons 2, 4, 5, 7, and 8 encode for the conserved domains I-IV. The conserved domains earned their name by being similar in different species and being conserved through evolution (Prokocimer and Rotter 1994; Soussi et al. 1987).

1.16.2 Gene Polymorphism

The human p53 gene contains several polymorphic sites including a single nucleotide polymorphism (SNP). These polymorphic restriction sites exist in specific locations along the gene (Prokocimer and Rotter 1994). The polymorphic sites are located at codon 21, codon 36, codon 72, codon 213, codon 47, codon 49, codon 189, intron 1, intron 2 intron 3 intron 6, intron 7, intron 10, exon 11 (Ahuja, Testa, and Cline 1990; Felley-Bosco et al. 1993; Harris et al. 1986; Matlashewski 1987; Prokocimer and Rotter 1994; www/p53.free.fr).

A common polymorphism is characterized on codon 72 where arginine is replaced to proline. Codon 72 polymorphism seems to be more common in populations that are geographically close to the equator (Sjalander et al. 1995). Recent studies demonstrate that polymorphism at codon 72 is associated with an increase in incidence and a less favorable prognosis for several cancers (Perez et al. 2006; Takeuchi et al. 2005). Serine 47 polymorphism changes the p53 residues from proline to serine. This SNP is not observed in the Caucasian population and is rare (less then 5%) in African-Americans (Felley-Bosco et al. 1993). This SNP causes impaired activation of target genes such as PUMA. Thus the apoptotic function of p53 is decreased by 5 fold, but the protein's capacity to bind DNA is not affected (Li 2005). To date, there is no information connecting the S47 SNP to increased cancer susceptibility.

An Intron 3 polymorphism is characterized by a 16 base pair duplication. In vitro, this intron 3 polymorphism decreased the apoptotic ability of lymphoblastoid cell lines (Wu et al. 2002). Intron 3 polymorphism was suggested to be

associated with increased risk for lung cancer (Wu et al. 2002), colorectal cancer (Gemignani et al. 2004), and ovarian cancer (Wang-Gohrke et al. 1999). Intron 6 polymorphism is a SNP that causes change of guanine to a cysteine base. This polymorphism is associated with decreased apoptosis in cell lines of individuals with familial breast cancer (Lehman et al. 2000).

1.16.3 P53 Protein

The p53 protein is a phosphoprotein consisting of 393 amino acids. The p53 NH2 terminal domain is acidic, and is made of 160 amino acids that play a crucial role in transcription (O'Rourke et al. 1990). Transcription of p53 up-regulates genes that are crucial to cell cycle regulation (O'Rourke et al. 1990).

Within the middle section of the p53 protein, a proline rich domain is located between amino acids 80-150 which has a hydrophobic character. This domain takes part in p53 and DNA binding (Levine and Momand 1990).

The C-terminus of p53 is made of 233 amino acids and has a basic character. The C-terminus has an oligomerization and transformation function (Wang et al. 1994). Oligomerization has a role in DNA binding and the two domains essential for this task are located from amino acids 80 to 290. In mice, This region binds DNA specifically, while the other domain located between amino acid 280 to 390 (or in human 283 to 293) forms a stable tetramer that binds DNA in a nonspecific way (Pavletich, Chambers, and Pabo 1993). As many as 47 amino acids at the C-terminus side have the main function of p53 protein binding capacity (Foord 1991). Mutated p53 protein lacking these 47 amino acids had impaired binding capacity as compared to wild type p53 (Foord 1991).

1.16.4 p53 Protein Subcellular Location and Function

The p53 wild type protein changes location during the cell cycle. During G1 phase, the p53 protein is located in the cellular cytoplasm and accumulates there (Shaulsky et al. 1990). The direction of the protein into the nucleus is mediated by nuclear localization signals (NLS I, NLS II, and NLS III) that are located within the p53 gene C-terminus (Shaulsky et al. 1990). The NLS I region is located between amino acids 313 to 322 and is conserved in humans, mice, and rats (Soussi et al. 1988; Zakut-Houri et al. 1983). The NLS I is the most important cofactor in mediating transportation to the nucleus while NLS II and NLS III seem to be less crucial for this process (Shaulsky et al. 1990). During S phase, the p53 wild type protein accumulates in the nucleus while mutated p53 tends to accumulate in the cytoplasm (Ginsberg et al. 1991).

The p53 protein accumulates in the cell following DNA damage and induces apoptosis when the damage is too extensive to repair. In case of repairable damage, the cell cycle arrests at the G1 phase restriction point and repair will be initiated (Fritsche, Haessler, and Brandner 1993). This function of wild type p53 was demonstrated in vitro when p53 mutant leukemic cells were transfected by adding p53 wild type protein and apoptosis was observed (Yonish-Rouach 1991). When normal skin is exposed to ultraviolet radiation, the p53 concentration in damaged fibroblasts can be detected at the superficial layer two hours after exposure. This demonstrates p53 efforts to repair the damaged DNA (Hall et al. 1993). In cancerous cells, a p53 mutation may prevent apoptosis; when wild type p53 protein was added to colon cancer cells in vitro, apoptosis was detected in

the neoplastic cells (Shaw et al. 1992). Knock-down mice, with a homozygous null allele genotype, have a normal phenotypic appearance but are much more susceptible to lymphoid origin tumors, with most mice developing neoplasm by 6 month of age (Donehower et al. 1992).

1.16.5 Mdm2

Murine double-minute 2 (Mdm2) protein has a regulatory function on the p53 protein. The mdm2 protein has a regulatory function on the p53 protein. By binding to p53, Mdm2 mediates the ubiquitination and proteosomal degradation of p53 protein (Haupt et al. 1997). A mono-ubiquitinition will not cause p53 degradation, but will be sufficient to prevent the protein from being transported into the nucleus (Haupt et al. 1997). Murine double-minute 2 is upregulated at the gene level by the p53 protein, but the formation of p53-Mdm2 complex will prevent further upregulation by the p53 protein (Wu et al. 1993).

The Mdm2 protein has the ability to reverse the cell cycle arrest induced by p53 following a DNA insult, thereby promoting the cell cycle from G1 phase to S phase (Chen et al. 1996). An overexpression of Mdm2 is observed in several tumor types. Excess Mdm2 inhibits the apoptotic abilities of wild type p53 and contributes to cellular immortality (Finlay 1993).

1.16.6 p53 Mutations

Mutations in p53 are common in 50% of all human cancers. Out of all the cases of human lung cancer diagnosed each year, 70% are p53 mutated. About 60% of colon cancers have p53 mutations, and human stomach cancer is mutated in 45% of the cases (www/p53.free.fr)

Mutations of the p53 protein can be divided to two subclasses according to the three-dimension protein structure and according to the assay of detection. Class one mutations are mutations that affect the p53 protein and DNA binding. The mutations seen in the first class group have a similar conformation to the wild type protein. The mutated proteins bind to conformational monoclonal antibodies (for example, pab240) and do not bind non-specifically to hsp70 (Ory et al. 1994; Soussi and Lozano 2005). Mutations of the class two type involve an irreversible phenotypic change in the protein. Addition of normal protein will not re-establish the original p53 structure by addition of peptides, suggesting a dominant-negative mutation (Selivanova et al. 1997; Soussi and Lozano 2005).

Most p53 mutations are missense mutations and result in amino acid substitutions. These point mutations are located mostly in the hydrophobic region of the protein. Other missense mutations exist with lower frequency (Greenblatt et al. 1994; www/p53.free.fr). The most common mutation in human p53 was located at codon 175, where a G>A variant exists that results in an arginine to histidine substitution. This mutation accounts for 5% of all p53 mutations and is found commonly in colon cancer. Of all the p53 mutations, 51% are G: C>A:T and out of those, 60 % effect the CpG dinucleotide (Beroud and Soussi 2003; www/p53.free.fr).

1.16.7 P53, PTEN, and PI3K

The PTEN gene may be upregulated directly by the p53 protein. P53 increases transcription of PTEN, which functions as a suppressor to the PI3K pathway (Stambolic V 2001). The p53 protein has a specific binding site at the

PTEN promoter between bases 1190 and 1157. This region contains two half regions identical to the p53 binding domain area. The regions are separated by 14 bp. By activation of PTEN, a diphosphorylation of PIP3 will take place and prevent the formation of AKT.

Mutations in p53 have not been found to exist concurrently with elevation in the PIK3C oncogene in the same tumor (Stambolic et al. 2001). The fact that tumors have mutation in one but not both of these pathways illustrates that each mutation can independently damage the cell's ability for apoptosis. Stambolic at el. suggest that p53 induction of apoptosis and the simultaneous inhibition of the PI3K/AKT pathway are important to control cells that are PTEN mutated.

The p53 protein may mediate regulation of the PI3K pathway. The PIK3CA gene is located at chromosome 3q26 and encodes the p110α catalytic subunit of PI3K that was identified to be an oncogene. The p53 protein has the ability to inhibit PI3K formation directly or by the induction of PTEN (Singh et al. 2002). Mayo et al. showed the ability of PTEN to prevent the ubiquination of p53 by Mdm2, thereby protecting p53 and increasing the cell's sensitivity to chemotherapy-induced apoptosis (Mayo et al. 2002).

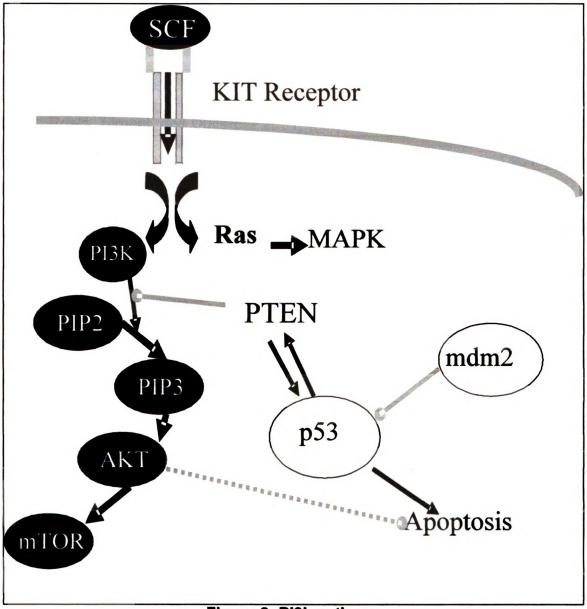


Figure 2: PI3k pathway
The tyrosine kinase receptors initiate the PI3k pathway. The activation of the whole cascade may be hindered by PTEN. The activation of the whole cascade will increase cell survival, resistance to apoptosis, metastasis and angiogenesis.

1.16.8 p53 in Dogs

Mutations of p53 protein were reported in the following canine tumors:

lymphoma, osteosarcoma, mammary tumors, transmissible venereal tumors

(TVT), mast cells tumors, melanoma, intestinal carcinoma, and hemangiosarcoma (Choi and Kim 2002; Koenig et al. 2002; Lee 2004; Loukopoulos et al. 2004; Mayr and Reifinger 2002; Mayr et al. 2002; Ozaki et al. 2002; Roels, Tilmant, and Ducatelle 2001; Sokolowska, Cywinska, and Malicka 2005). In TVT's, the p53 protein was found to be mutated at nucleotide 964 (T>C) resulting in a change of amino acids (Phe>Ser) (Choi and Kim 2002). In cecal carcinoma, p53 protein was found to be mutated at codon 249 where CGG>TGG changes caused an amino acid substitution (arginine>tryptophan) (Mayr and Reifinger 2002). In dogs p53 defective mammary carcinoma was recognized in 3 out of 10 patients in one study. The mutations were variable within the different patients and were located at exons 2 and 5; a deletion of exon 3 to 7 was also evident. A SNP polymorphism was detected on position 69 (CCC>CTC) of exon 4 that was similar to a common human breast cancer codon polymorphism. In one case of HSA, a p53 protein mutation was found to be a 24 bp deletion in exon 5 (Mayr et al. 2002). Regardless of tumor type, mutation of p53 is associated a less favorable prognosis for survival (Lee 2004).

1.17 Doxorubicin

Doxorubicin is an antitumor antibiotic that was isolated from *Streptomyces S. peucetius var. caesius.* The chemical formula of doxorubicin 8S,10S)-10-(4-amino-5-hydroxy-6-methyl-tetrahydro-2H-pyran-2-yloxy)-6,8,11-trihydroxy-8-(2-hydroxyacetyl)-1methoxy7,8,9,10-tetrahydrotetracene-5,12-dione.This anthracycline was reportedly isolated in the late sixties, and since then has been broadly used as a single agent and as a key member in multidrug combination

therapies (Arcamone F 1969). The common trade names for doxorubicin are Adriamycin and Hydroxydaunorubicin.

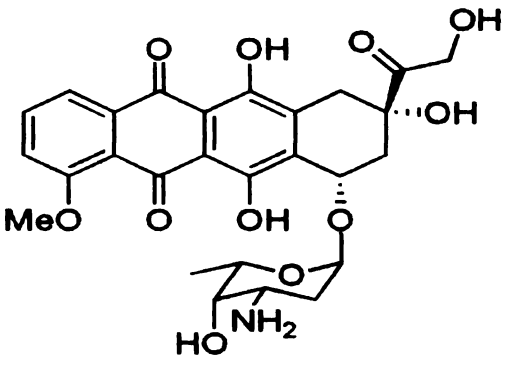


Figure 3: Doxorubicin chemical structure

Doxorubicin is indicated in human medicine for treatment of acute lymphoblastic leukemia, acute myeloblastic leukemia, Wilm's tumor, neuroblastoma, soft tissue and bone sarcomas, breast carcinoma, ovarian carcinoma, transitional cell bladder carcinoma, thyroid carcinoma, gastric carcinoma, Hodgkins disease, malignant lymphoma, and bronchogenic carcinoma (Beattie 1975; Case, Young, and Lee 1977; Hagin and Jain 1985; Mattsson et al. 1977; Shimaoka 1980).

1.17.1 Pharmacodynamics

Doxorubicin intercalates within cellular DNA, specifically in 5'-TCA nucleotide sequence regions. Cell death following intercalation was originally assumed to result from polymerase inhibition, though the anthracycline concentration needed for enzyme inhibition of polymerase can't be achieved by in vivo administration. Therfore, DNA intercalation as a crucial factor in cellular

cytotoxicity from doxorubicin therapy is no longer the favored mechanism (Siegfried 1983). Following the intercalation into DNA the aggregation of doxorubicin, DNA and topoisomerase II α form a unit called the "cleavable structure". DNA cleavage and breakdown is mediated by the topoisomerase IIα enzyme activity within the complex. Scission DNA by Topisomarase II α then induces cellular apoptosis by the intrinsic pathway (Ross, Glaubiger, and Kohn 1979). The incorporation of anthracyclines in DNA forms irreversible structures that inhibit the activity of helicases. By disruption of helicase activity, DNA strand unwinding is prevented and cellular replication is hindered (Bachur et al. 1992). Furthermore, doxorubicin also inhibits the RNA helicase substrate, thus inhibiting RNA polymerase activity and protein production in a cell cycle phase non-specific maner (Zhu et al. 1999). Doxorubicin produces oxygen free radicials through NADPH mediation of quinone and hydroxyquinone side chains. This produces free radical damage to DNA and results in strand cleavage with cellular apoptosis (Mizutani et al. 2005). Free radical damage trigers apoptosis independent of p53 activity and therefore enables cell apoptosis independent in p53 mutated cells as well as in p53 wild type tumors (Tsang et al. 2003).

1.17.2 Mechanism of Drug Resistance

The glutathione S-transferase (GST) enzyme plays a significant role in neoplastic cell resistance to doxorubicin. The GST enzyme is found in the cytoplasm and also in the nucleus (Goto et al. 2001). The free radical effect on DNA may be prevented by doxorubicin interaction with the GST enzyme, which has the capacity to protect cellular lipids and DNA from oxidizing effects (Baez et

al. 1997; Berhane 1994). The level of nuclear GST was found to be elevated in cancer cells expressing doxorubicin resistance (Goto et al. 2001).

Cellular cytotoxicity of doxorubicin may be reduced tremendously by expression of P-glycoprotein pumps that rapidly export doxorubicin and other large ring xenobiotic compounds from the cytoplasm of the cell. Drug resistance was demonstrated to be directly correlated with the presence of these transmembrane protein pumps (Epstein 1989). Several tumor cell lines have also demonstrated doxorubicin resistance resulting from inappropriate function of the topisomerase II α enzyme. The resistance mechanisms induced by topoisomerase alteration are postulated to be due to several factors. First, mutation in the topoisomerase gene in codons adjacent to the DNA binding site will lead to alteration in the formation of the "cleavable structure" (Patel et al. 1997). Decreased topoisomerase gene copy number results in a decrease in mRNA and protein production, but also causes a lower number of cleavable structures (Withoff et al. 1996). Transcriptional down-regulation of topoisomerase gene expression causes the same end result as decrease gene number (Wang et al. 1997).

The tumor suppressor gene p53 plays a crucial role in cellular DNA repair mechanisms and apoptosis. Cellular mutations in suppressor genes such as p53 and *BCL2* are broadly common in tumors and may alter the cytotoxic effect of doxorubicin by a global anti-apoptotic mechanism (Cinti C 2000; Kuhl JS 1997). Apoptosis may be accommodated in p53 mutant cells through initiation of alternate apoptotic mechanisms.

1.17.3 Clinical Pharmacology

The binding of doxorubicin to plasma protein is greater then 70% and is independent of plasma drug concentration (Doxorubicin HCL drug insert 2002). Doxorubicin is poorly penetrant through the blood brain barrier but has a wide volume of distribution in other tissues (Siegal et al. 1987). In humans, the main doxorubicin metabolite (doxorubicinol) demonstrates a two-phase drug distribution pattern with peak plasma values observed in 2 to 12 hours (Reich et al. 1979). Doxorubicin's initial half life is short and lasts for 5 minutes, probably due to rapid tissue uptake; the terminal half life is long and lasts for 20 to 48 hours (Doxorubicin insert package). The elimination of doxorubicin is acomplished mostly by biliary excretion (40%), with a smaller amount excreted by the kidneys (10 to 12%)((Doxorubicin insert package).

1.17.4 Adverse Effect of Doxorubicin

Dermal adverse effects following drug administration include alopecia; skin hyperpigmentation of nail beds and dermal creases. Gastrointestinal adverse effect may include: acute nausea and vomiting; mucositis; ulceration necrosis of the colon that may lead to fatal infections; anorexia; and diarrhea. Phlebosclerosis may occur following repeated use of the same vein for drug administration. Facial flushing was reported with rapid administration. Extravasation of doxorubicin may result in cellulitis; and tissue necrosis. Occurrence of secondary acute myeloid leukemia was reported in a patient previously treated with doxorubicin. Hypersensitivity will be expressed as fever, chills, urticaria, and anaphylaxis. Ocular conjunctivitis and lacrimation reported to

be rare in adult patients. Cardiotoxicity without early electrocardiographic signs may appear due to accumulative damage of doxorubicin (Doxorubicin insert package). The primary acute dose limiting adverse effects of doxorubicin are myelosuppression and gastrointestinal toxicity.

1.17.5 Doxorubicin in Veterinary Medicine

Doxorubicin is a widely used drug in veterinary oncology. Doxorubicin is administed as a single agent or as part of combined chemotherapy protocols. Doxorubicin was proved to be efficacious towards a wide variety of tumors such as lymphoma, carcinoma, osteosarcoma, hemangiosarcoma and other soft tissue sarcomas(Garrett 2002; Kent 2004; Selting 2005; Sorenmo et al. 2004). The drug is administered by intravascular injection at a standard dose of 30mg/m2, over 20 minutes. Small dogs should have dose reduction to avoid toxicity, to a dose of 1 mg/kg. The most common dose limiting factor in canine doxorubicin administration is bone marrow suppression that may be expressed 7 to 10 days following drug administration. Acute toxicity following doxorubicin administration in the dog may present as gastrointestinal signs include vomiting, diarrhea, colitis, and anorexia (Ogilvie et al. 1989). The accumulation of a total doxorubicin dose that exceeds 240 mg/m2 may cause cardiotoxicity in dogs and should be avoided. In one study 32 dogs (n=175) showed arrhythmia in association with doxorubicin administration. In the same study 9 dogs developed a fatal congestive heart failure (Mauldin et al. 1992).

1.18 Dacarbazine

5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (dacarbazine) is a chemotherapeutic agent known by several trade names: DIC, DTIC-Dome, Dacarbazine, and Imidazole Carboxamide. This drug is an alkylating agent that induces non-specific cell cycle arrest. Dacarbazine is a pro-drug. Activation depends on cytochrome P450 enzymes, particularly CYP1A1, CYP1A2, and CYP2E1 (Reid et al. 1999). The location of CYP1A1 protein is primarily extrahepatic, and the enzyme is believed to activate the drug in sites other than the liver. Several tumors have been demonstrated to contain CYP1A1 activity In the liver, the most significant activition of dacarbazine is mediated by CYP1A2, and to a lesser extent by CYP2E1 (Reid et al. 1999).

Following activation of dacarbazine, methylation of the O6 position of guanine in cellular DNA and RNA results in an insult sufficient to cause cell cycle arrest. Following administration of O6-methylguanine agents such as dacarbazine, and the new analog temozolomide, a mismatch formation of the DNA sequence will result in cell cycle arrest or apoptosis in cells that contain appropriate mismatch repair mechanism (D'Atri et al. 1998). Cells with alterations in mismatch repair mechanism are extremely resistant to alkylating agents (Koi M 1994). The magnitude of accumulation of methylated guanine residues is dose dependent. These adducts can be detected in the DNA of different organs (Kyrtopoulos et al. 1993; Meer et al. 1986).

The cytotoxic adduct formation effect, as detected by 7-[14C]methylguanine labeling, was found in the DNA and RNA of different organs of rats, including the

lungs, kidneys, and liver (Meer et al. 1986). The highest level of adducts following dacarbazine administration to rats was measured in the liver (35 μ moles 7-methylguanine/mole guanine), while the lowest level was seen in the brain (1 μ mole/mole guanine) (Meer et al. 1986). Following dacarbazine administration to people, a two-compartment model was found to best describe the plasma distribution of the drug. The half time of the α compartment had a range of 0.1-0.26 h with a mean time of 0.17 hours, while the β compartment half-time range was measured to be between 1.5 to 2.5 hours with a mean of 2 hours (Buesa 1991). Dacarbazine administered to rats is excreted mostly by the kidneys (54%) in an unchanged form. Twenty-four hours after administration of 850–1,980mg/m2 of dacarbazine to people, 11% to 63% of the delivered dose was detected in the urine. The activated metabolite of dacarbazin is aminoimidazole carboxamide which was found to follow a monophasic dissipation pattern with a mean half time of 3.5 hours. Dogs that received 100 mg/kg dacarbazine had a tissue perfusion range of 70 to 400 micrograms/ml without evidence of formation of metabolites (Aigner et al. 1983).

Drug resistance to dacarbazine is substantially due to the activity of the cellular enzyme alkylguanine DNA alkyltransferase (AGT) that provides a repair mechanism for DNA and RNA alkylation. This enzyme activity is described as a "suicide repair" mechanism, because repair of one DNA adduct residue results in degradation of the repairing AGT molecule. Thus the AGT repair mechanism may be depleted by exposure to any agent causing O6 DNA adducts. The O6-methylguanine-DNA-alkyltransferase (MGMT) gene encodes the AGT enzyme,

which may be upregulated by alkalyting agent exposure. (Brent O6-Alkylguanine-DNA alkyltransferase activity correlates with the therapeutic response of human rhabdomyosarcoma xenografts to 1-(2-chloroethyl)-3-(trans-4-methylcyclohexyl)-1-nitrosourea).

A phase II trial of 39 human patients with recurrent gliomas demonstrated that grade I-II nausea was the most common toxicity following dacarbazine administration. Other toxicities reported in this study were lethargy in 28% of the patients, diarrhea (15%), alopecia (15%), and grade 3 neutropenia (8%) (Rajkumar et al. 2000). When a dose of 750-mg/m2 IV once every 28 days was administrated to a group of patients, four patients were reported to have an intravascular hemolysis (Rajkumar et al. 2000). Group B patients in this study received dacarbazine at 200 mg/m2 IV on days 1-5 every 28 days. These patients experienced less toxicity than those receiving continuous IV infusions (Rajkumar et al. 2000).

When dacarbazine was administered at 250 mg/m2/day daily for 5 days on a 28-day cycle, emesis, leukopenia, and thrombocytopenia were reported to be the most common adverse effects (Costanzi 1976). Levy et al reported hypersensitivity reaction in as many as 20% (n=20) of patients treated with dacarbazine for metastatic melanoma. The hypersensitivity hallmarks were fever, elevation of eosinophil blood count (> 500/mm3), with or without liver dysfunction as demonstrated by more than double pre-therapeutic liver enzymes values. Two of the patients in this study were reported to have delayed bone marrow aplasia and liver dysfunction that was evident as cholestasis and hepatocellular necrosis

(Levy et al. 2006). A high dose infusion of dacarbazine (1.2 g/m2 infused over 20 minutes, repeated every 21 days) given to adult humans (n=50) was followed by grade 3 or higher leukopenia in 36% of patients, nausea and vomiting in 90%, a flu-like syndrome in 49%, and thrombocytopenia in 26% of patients. Hypotensive episodes were experienced by 4% of the patients in this study (Buesa 1991). Vascular pain in the vein used for drug administration was seen in as many as 28% of the patients in several studies (Buesa 1991; Klener and Donner 1977).

The use of dacarbazine as a chemotherapeutic drug is extensive, as a single agent or as part of multidrug protocols. The response rate for metastatic melanoma patients treated with dacarbazine as a single agent was reported to be 19%, while for soft tissue sarcomas patients and Hodgkin's disease patients the response rate was 22% (Costanzi 1976). Doxorubicin, bleomycin, vinblastine and dacarbazine (ABVD) are part of the standard treatment for advanced Hodgkin's lymphoma. Stage I and II Hodgkin's lymphoma patients were treated with six ABVD cycles. A complete response was observed in 89 patients (94%) and six patients (6%) showed a partial response. Progression-free survival rates at 7 years were 96% for the stage I patients and 84% for the stage II patients (Rueda Dominguez et al. 2004). When the Southwest Oncology Group evaluated dacarbazine as a therapy for sarcoma, an overall response rate of 17 % was reported(Costanzi 1976).

In dogs; dacarbazine was given in combination with doxorubicin as a rescue treatment for lymphoma patients that failed therapy with single agent doxorubicin. Out of the fifteen dogs, five had a complete response following the first treatment,

and three had partial responses. From eight dogs that received a second cycle, two had complete responses, while one dog had a partial response (Van Vechten, Helfand, and Jeglum 1990). Dacarbazine has also been used as a rescue drug for dogs (n=16) that had failed previous treatment for hemangiosarcoma in a combination protocol of cyclophosphamide and doxorubicin (Sorenmo, Jeglum, and Helfand 1993). Dacarbazine was given as 200mg/m2 IV daily for five days, which was repeated every three weeks. The median survival time was reported to be 250 days with a mean survival of 403 days (Sorenmo, Jeglum, and Helfand 1993). In the DAV protocol studied here, the recommended dacarbazine dose is 800mg/m2 given IV as an 8 hours infusion, in combination with doxorubicin and vincristine.

1.19 Vincristine Sulfate

Vincristine was originally extracted from *Catharanthus roseus* (previously called *Vinca roseus*) and was used in folk medicine to treat diabetes mellitus. Vincristine itself is the salt of the vinca alkaloid. Commercial names for vincristine include Oncosar PFS, Oncovin, Vincasar, and Vincrex. Vincristine is a white powder soluble in methanol and water. The chemical formula of vincristine is $C_{46}H_{56}N_4O_{10} \cdot H_2SO_4$ (Vincasar PFS drug insert).

Common adverse effects encountered after administration of vincristine to people include: cellulitis due to extravasation; cough or hoarseness; flu-like symptoms; pinpoint red spots on the skin; unusual bleeding or bruising; constipation. Acute tumor lysis syndrome may be seen following rapid apoptosis of lymphocytes in non-Hodgkin's lymphomas. This tumor lysis syndrome can

result in hyperuricemia or uric acid nephropathy, which may cause joint pain and lower back pain with blood in urine or stool. Because of the dependence on microtubule function for axonal transport in neurons, neurotoxicity can develop as demonstrated by weakness, digital paresthesia, pain in testicles, blurred or double vision, drooping eyelids, and headache. Less common human side effects are expressed as agitation, confusion, seizures, decrease or increase in urination, orthostatic hypotension, hallucinations, anhydrosis, anorexia, mental depression, painful or difficult urination, unconsciousness, and the syndrome of inappropriate antidiuretic hormone release (SIADH) (Oncovin package insert US).

Vincristine should be administered intravenously. Following extravasation, injection of the drug should be stopped immediately. Extravasation results in tissue necrosis that can be minimized by applying moderate heat to the damaged area and by local injection of hyaluronidase (Oncovin package insert US).

Vincristine is considered a mitotic spindle poison, or anti-mitotic agent. Vincristine specifically binds the β-tubulin subunit of the microtubules, preventing α and β tubulin assembly required to form the mitotic spindle apparatus. Microtubule inhibitors promote a specific cell cycle arrest. Thus, vincristine leads to cell arrest at the metaphase-to-anaphase transition, where the microtubules play a crucial role in cell cycle regulation. Vincristine induces cell death during the G2 and M phases of the cell cycle. (Schrek 1975).

Resistance to vinca alkaloids is mediated in part by P-glycoprotein cell membrane pumps that rapidly eliminate large negatively charged ringed

molecules from the cytoplasm. These pumps include the originally described Pgp or MDR gene product. P-glycoprotein (or gp 170) is part of the ATP binding cassette family of drug resistance proteins that were discovered to confer plieotrophic drug resistance. The Pgp pumps decrease the concentration of cytotoxic xenobiotic drugs within neoplastic cells, thereby decreasing drug efficacy (Huang et al. 2006). Another possible mechanism of drug resistance includes downregulation of cellular topoiosmerase activity, which leads to delay in G2 phase and an early prolonged onset of the M phase which can increase the cell's resistance to vincristine (Skladanowski et al. 2005). A mutation in the α and β tubulin subunits may also occur due to genetic modification or posttranslational alteration. These structural alterations in tubulin subunits effect avidity of vinca binding, and result in formation of spiral-like strong spindles (Donoso, Haskins, and Himes 1979; Sirotnak 2000).

Intravenous injection of vincristine follows a triphasic elimination pattern. The half-life of the initial phase is 5 minutes, the intermediate elimination phase is 2.3 hours with the final phase as long as 85 hours. The major route of metabolism involves liver conjugation followed by biliary excretion. The CYP3A subfamily of P450 enzymes is located in the liver and plays a central role in drug metabolism. About 80% of the injected drug is excreted through the feces and 10-20% is eliminated in urine (Vincasar drug insert 2003)

Vincristine binds to plasma proteins such as albumin and globulins, which limits drug penetration to the brain (Greig 1990) Vincristine has strong binding affinity to platelets, due to the abundance of tubulin in megakaryocyte and

platelet cytoplasm. Due to the strong affinity of vincristine for negatively charged proteins, the drug tends to accumulate in internal organs tissues. It was reported to accumulate particularly in the dog and rat spleen, but drug concentration in brain was very low in both species (Castle, Margileth, and Oliverio 1976).

In veterinary medicine, vincristine is administered as a single agent for the treatment of transmitted venereal tumor (TVT). In one study of dogs with TVT (n=38), 31 showed a complete remission followed vincristine is administered (Nak D 2005). More commonly, vincristine is used as part of combination protocols involving drugs such as doxorubicin and cyclophosphamide. The VAC protocol consists of vincristine, doxorubicin, and cyclophosphamide. The VAC is applied to hemangiosarcoma patients at different stages of the disease. Efficiency of the VAC protocol was evaluated in 15 dogs in varying stages of hemangiosarcoma progression and showed a median survival time of 172 days while mean survival time was 316 days (Hammer, Couto, Filppi et al. 1991). Vincristine is part of many protocols used for the treatment of lymphoma. The COPLA protocol for example, combines five different drugs (cyclophosphamide, vincristine, prednisone, L-asparginase, and doxorubicin). Dogs with lymphosarcoma treated with the COPLA protocol achieved remission with a median duration of 36 weeks (Boyce and Kitchell 2000). Mast cells tumors in dogs have also been successfully treated with cyclophosphamide, vincristine and prednisone in one protocol (Naganobu et al. 2000).

Table 2: Most common protocols utilizing vincristine in human medicine

Protocol acronym	Drugs in protocol	Clinical indication	Citation
CHOP	Cyclophosphamide, doxorubicin,vincristine , prednisone	Non-Hodgkin's lymphoma,	(Burton et al. 2006)
CMFVP	Cyclophosphamide, methotrexate, fluoroucil, vincristine, prednisone	Breast cancer	(Rivkin et al. 1996)
COP or CVP	Cyclophosphamide, vincristine, prednisone	Mycosis fungoides, Non-Hodgkin's	(Molin et al. 1980) (Klener, Donner, and Roth 1976)
CyVADIC	Cyclophosphamide, vincristine, prednisone, dacarbazine	Sarcomas	(Kakizaki, Takagi, and Hosaka 1997). (Odunsi et al. 2004)
MOPP	mechlorethamine, vincristine, procarbazine, prednisone	Hodgkin's lymphoma	(Jacobs 1976)
VAC	Vincristine, dactinomycin, cyclophosphamide	Soft tissue sarcomas, Small cell lung carcinoma	(Wilbur 1975) (McIlmurray et al. 1989)

1.20 DAV protocol

The initial protocol used to treat all patients in this study was a novel combination of three different drugs: doxorubicin, dacarbazine, and vincristine (DAV). A tenet of chemotherapy is to combine drugs that have documented single agent efficacy against the target tumor, that achieve these anti-tumor

effects by differing molecular mechanisms of action to achieve maximum tumor response. This combination chemotherapy theory posits that combining agents that work at different sites in the cell's machinery will optimize tumor response by overcoming intrinsic and acquired mechanisms of individual drug resistance.

One of the main mechanisms of cancer cells drug resistance to doxorubicin is the increased expression of glutathione S-transferase (GST). The GST enzyme is localized in the cytoplasm and also in the nucleus (Goto et al. 2001). The free radical effect on DNA may be prevented by doxorubicin interaction with the GST enzyme, which has the capacity to protect cellular lipids and DNA from oxidizing effects (Baez et al. 1997; Berhane 1994). The level of nuclear GST was found to be elevated in cancer cells expressing doxorubicin resistance (Goto et al. 2001). Administration of dacarbazine over several hours depletes the GST enzyme. Application of both drugs on the same day would be predicted to result in a synergistic effect to increase efficacy and overcome this potential source of doxorubicin resistance. Further, dacarbazine is a potent non-traditional alkylating agent with known efficacy in the setting of human and veterinary sarcoma therapy. Therefore, co-administration of these agents would be anticipated to improve tumor response over that achieved through the use of either agent separately. Vincristine causes its effect on the malignant cells by targeting the alpha and beta tubulin subunits necessary to create microtubular structures that comprise the mitotic spindles. Antimitotic agents such as vincristine have modest single agent efficacy against human and veterinary sarcomas.

In the DAV protocol, doxorubicin is given by intravenous infusion over 20 minutes in a dose that equals 30mg/m2. Dacarbazine is given intravenously over 8 hours at a dose of 800mg/m2. Vincristine is given as an intravenous bolus injection of 0.5mg/m2. On day 1, the patient receives doxorubicin followed by dacabazine. The administration of vincristine follows on day 8 and day 15. Due to the potential for doxorubuicin cumulative cardiotoxicity, a maximum of 6 cycles are administered.

2 MATERIALS AND METHOD

2.1 Sample Selection

The hemangiosarcoma tissue samples were collected from patients presented to the Veterinary Teaching Hospital at Michigan State University. All hemangiosarcoma patients in our study were treated with the same protocol as a base for comparison (DAV). No other exclusion or inclusion criteria were used for case selection.

2.2 Isolation from paraffin blocks

Tumor biopsies embedded in paraffin blocks where collected from all the patients participating in this project. By using a scalpel blade, a 0.5-2mm thick tissue was extracted from each of the paraffin blocks embedded tissues. The samples where placed in 1.5 ml plastic tubes with 400 μl of lysis buffer (50 nMTris-HCL, pH 8.5: 1 nM EDTA and 0.5% Tween®20). After sealing with parafilm the tubes were placed on a 95°C heat block for 5 minutes. Throughout the heating, the samples were vortexed intermittently to promote the suspension of the tissue by the solution. The tubes were heated one by one in a microwave oven three times for 30 seconds on high power (700W). After the samples were cooled to room temperature, 5μl of a 15-mg/ml-proteinase K solution were added to each tube. The tubes were incubated over night at 42°C and then at 95-100°C for 10 more minutes to inactivate residual protease. The tubes were centrifuged for 10 minutes at 12,000 rpm. An amount of 5μl of this preparation was used as a template for the PCR reaction.

2.3 Amplification of Juxtamemebrane Region of *c-kit* (exon 11)

The first six samples consisted of undiluted DNA, dilutions of 1:50, 1:100, and 1:200 used as templates for polymerase chain reaction (PCR). The rest of the PCR templates use in this study were in dilutions of 1:50. The target of amplification was a region of exon 11 that encodes the regulatory juxtamembrane region of the *c-kit* receptor. The reactions had a total volume of 25µl each and included 5 micromol (mmol) of each primer, 0.1 U of *taq* polymerase (Gibco BRL), 1 mM of dNTP, and 50 mmol of MgCl2. The primers used to amplify exon 11 were forward: 5'-GTT CCC TAA AGT CAT TGT TAC ACG-3'; reverse: 3'-CAT TTG TTC TCT ACC CTA AGT GCT-5'.The PCR reaction included conditions had an initial denaturation step at 94°c for 1 minute, followed by a step lasting for 1.5 minutes at 55°c. A final step was conducted at 72°c for 2 minutes length. Each cycle was repeated 41 times and had an expected product size of 270 base pair.

2.4 Amplification of the Tyrosine Kinase Domain of *c-kit* Receptor

Exon 17 of the c-kit receptor encodes the tyrosine kinase domain. Amplification followed a protocol similar to that used for exon 11 amplification. The forward primer: - ATA GCA GCA TTC TCG TGT TG-; and the backward primer: - AAC TAA AAT CCT TCA CTG GAC TG-, were used to amplify the desired domain. The total volume used for the PCR reaction was 25 µl each and included 5 mmol of each primer, 0.1 U of *taq* polymerase (Gibco BRL), 1 mM of dNTP, and 50 mmol of MgCl2. The conditions of the PCR reaction included an initial step at a temperature of 94°c for 4, minutes followed by 41 cycles of 94°c

for 1 minute, 60°c for 1 minute and 72°c for 1 minute. The last step lasted for 5 minutes at 72°c.

2.5 Purification of the Amplified Product

The separation of the PCR product of exon 11 and exon 17 amplification was performed in a 2% agarose gel suspended in Tris-acetate EDTA buffer. Following the separation of the individual bands of exon 11, the appropriately sized bands were dissected and purified by a silica particle-based DNA purification method (QIAEX II kit-Qiagen) following the manufacturer's instructions (QIAEX II kit-Qiagen).

2.6 Sequencing of Purified Products

The purified PCR products were sent to the Michigan State University research technology support facility for sequencing. From each DNA product, 4µl were placed in two different tubes. The forward and reverse primers were placed separately with each primer placed in a different tube for a total concentration of 730 pmol. Distilled water was added to create a total concentration of 12 µl in each tube. A bidirectional analysis was performed on all products. An automated fluorescent DNA sequencer and a walking primer technique were performed by the *ABI 3730 Genetic Analyzer or ABI Prism 3700 DNA Analyzer.*

2.7 Immunohistochemistry

Immunohistochemistry for p53, caspase-3, PTEN and KIT was performed on sections of formalin fixed paraffin embedded tumors using different automated staining systems. Briefly, deparaffinization, antigen retrieval and immunostaining

for p53 and caspase-3 were performed on the Bench Mark Automated Staining System (Ventana Medical Systems, Inc.) using the Enhanced V-Red Detection (Alk. Phos. Red) Detection System (Ventana Medical Systems, Inc.) and a rabbit polyclonal antibody against p53 (Signet Laboratories) at a dilution of 1:100 or a rabbit polyclonal antibody against activated caspase-3 (RDI) at a dilution of 1:100, respectively. Antigen retrieval was achieved using the Ventana Medical Systems Retrieval Solution CC1 (Ventana Medical Systems) for 60 min. Sections were counterstained with haematoxylin. Positive immunohistochemical controls included a canine soft tissue sarcoma with strong p53 expression, a canine malignant lymphoma with strong expression of caspase-3 and normal canine lymphoid tissue to which the appropriate antisera were added. For negative controls the primary antibodies were replaced with homologous non-immune sera. Only nuclear staining was evaluated as positive staining for p53 caspase-3. For PTEN and KIT immunostaining sections of neoplastic tissue were deparaffinized in xylene, rehydrated in graded ethanol and rinsed in distilled water. Endogenous peroxidases were neutralized with 3% hydrogen peroxide for 5 minutes followed by rinsing for 5 minutes in distilled water. Antigen retrieval was achieved by incubating slides in antigen retrieval solution in a steamer (Black & Decker) for 20 min. Non-specific immunoglobulin binding was blocked by incubation of slides for 10 min with a protein-blocking agent (Dako, Carpinteria, CA) prior to application of the primary antibody. Using the Dako autostainer, (Dako, Carpenteria, CA) slides were incubated for 30 minutes with a monoclonal mouse anti-PTEN antibody (Santa Cruz) at a dilution of 1:50 or a

polyclonal rabbit anti-KIT antibody (Dako, Carpenteria, CA) at a dilution of 1:100. A streptavidin-immunoperoxidase staining procedure, LSAB2 (Dako, Carpinteria, CA), was used for immunolabeling. The immunoreaction was "visualized" with 3,3'-diaminobenzidine substrate (Dako, Carpinteria, CA). Sections were counterstained with Mayer's haematoxylin. Positive immunohistochemical controls included canine osteosarcomas with cytoplasmic PTEN staining and canine mast cell tumors with characteristic membrane-associated staining of neoplastic mast cells for KIT. For negative controls the primary antibody were replaced with homologous non-immune sera.

2.8 Statistics

Overall survival was calculated by the Kaplan Meyer product limit method. Log Rank tests were used to examine the prognostic value of potential risk factors (p53, PTEN, *c-kit*, caspase-3, visceral or non-visceral tumor location). Multivariate analysis, using a Cox proportional model, with forward data entry method, was performed to identify potential prognostic markers. Chi square analysis to detect potential association between risk factors was used. A p value of <0.05 was considered statistically significant. Commercial software was used for all statistical analyses (Medcalc for Windows, version 10.2.0.0, MedCalc Software, Mariakerke, Belgium).

3 RESULTS

Our study includes 15 hemangiosarcoma patients referred to the Veterinary Teaching Hospital of Michigan State University. The presence of hemangiosarcoma in all the patients was confirmed by histologic biopsies performed and analyzed at the pathobiology laboratory of Michigan State University. A total of 6 males and 9 females where enrolled, with an age range of 5.5 to 13 years. The average age of these dogs was 9.5 years. The weight range was 6.8-41.8 kg, with the average weight was 29 kg.

Four out of the 15 dogs were Golden Retrievers, 3 were German Shepherds, 3 were of mixed breeds, and 2 were Labrador retrievers. The rest of the patients included one Shitzu, one English setter and one Beagle. Staging was based on the WHO classification system; fourteen patients were classified as stage III patients. One patient was classified as being in stage II. Thus the majority of dogs in this study presented with overt metastasis. In ten patients, the tumor involved the spleen as the primary site. The remaining 5 patients had primary tumor of the mediastinum, lungs, rectum, and subcutaneous.

Table 3: Immunohistochemistry results versus survival

Patient ID No.	Organ	PTEN	p53	Ckit	cas3	Survival (d)
1	spleen	N/A	positive (weak)	positive (weak)	positive	101
2	spleen	positive	neg	neg	neg	407
3	spleen	positive (weak)	neg	positive	neg	119
4	mediastinum	neg	neg	positive	neg	228
5	spleen+liver	neg	neg	neg	neg	236
6	spleen	neg	positive	positive (weak)	neg	213
7	N/A	neg	positive	positive	neg	193
8	lungs	neg	positive	positive	positive	335
9	liver	positive (weak)	neg	neg	positive	61
10	rectal	positive	neg	neg	neg	217
11	subcutan	positive	neg	neg	neg	590
12	spleen	positive	neg	positive	N/A	102
13	liver	positive	neg	positive	neg	96
14	spleen	positive	neg	positive	neg	260
15	subcutan	positive	neg	neg	neg	173

All 10 cases involving the spleen had metastasis to other sites such as liver, omentum or concurrently in the right atrium. In eight patients, the tumor involved both the liver and spleen. In three patients, the tumor was present in the lung at diagnosis with one additional patient having hemangiosarcoma confined to the lung. A single case in our study had hemangiosarcoma located subcutaneously and evidence of metastasis at the time of diagnosis. In one patient, the tumor was a solitary mass confined to the mediastinum.

All the patients in this study were treated with the same protocol. The DAV protocol comprises a combination of three drugs: dacarbazine, doxorubicin, and vincristine. Each DAV chemotherapy cycle lasted three weeks with a maximum of 6 planned cycles for the total treatment. Three patients completed 6 cycles. Four patients completed 5 cycles, 3 patients completed 4 cycles and two patients completed 2 cycles. One patient received 3 cycles, wit treatment delays due to toxicity. Two patients received less than a single complete cycle, in both cases protocols were stopped due to toxicity.

In addition to the protocol, rescue therapy was attempted in 15 patients. Five patients had a rescue therapy that included treatment with ifosfamide. Toxicity following DAV chemotherapy was reported in 10 of 15 cases. Gastrointestinal toxicity of grade 3 or 4 was predominant in 6 cases. Grade three neutropenia was reported in two patients and one additional patient had grade three toxicity and grade four in successive cycles. Grade four neutropenia was reported as a single event in two patients. Grade three lymphopenia was reported in one patient. Grade four thrombocytopenia was reported in one patient.

Splenectomy was the most common surgical procedure and was performed on 9 patients. One patient had a treatment that included splenectomy and right auricular resection.

The survival duration for this patient cohort ranged from 41 to 407 days. The mean survival time was 204 days with a median of 193 days. All the dogs in this study excluding one were euthanized due to disease. One dog was still alive with

a survival time of 590 days at the time the study was completed.

Immunohistochemistry Results

The paraffin blocks were obtained from tumors in all 15 patients. Samples were obtained from the following organs: 7 from spleen, 2 from subcutaneous tissue, 2 from liver, 1 from skeletal muscle, 1 from lung, 1 from rectum, and 1 from vagina. The blocks were cut in 3μ sections and stained with the described immunohistochemistry technique to identify potential mutations of p53, PTEN, and caspase 3. The immunohistostaining for the c-kit receptor identified the presence of the receptor, but c-kit mutation could not be identified by this technique.

As many as 9 patients had positive staining for PTEN while 5 samples were negative and one sample failed to stain. Most samples were negative for abnormal p53 staining pattern. Of the 15 patients, 11 were negative and 4 were positive. The c-kit staining assay showed presence of the receptor in 9 tumor samples while 6 samples were negative. Only 14 samples were available for activated caspase 3 immunohistostaining. The presence of an activated caspase 3 was prominent in 3 cases while 12 samples were negative.

The immunohistostaining demonstrates that PTEN and p53 were never concurrently positive (zero frequency) and rarely were both negative (14% frequency). The concordance of PTEN positivity with p53 negativity was the most common observation (64% frequency). The observation of PTEN positive with c-kit negative immunohistochemistry staining appeared very rarely (7% frequency).

3.1 *C-kit* sequencing

The DNA from 18 samples was extracted from paraffin embedded blocks. Sequencing of exon 11 was successful in 16 of these samples. The sequencing of exon 17 was successful in 15 samples. The exon 11 did not show any duplication mutations when examined by gel electrophoresis.

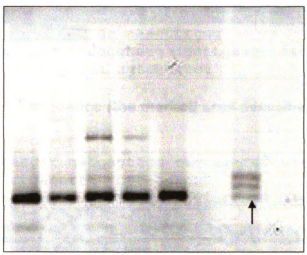


Figure 4: Exon 11 electrophoresis (1% acrylamide gel). Following *c-kit*, exon 11 amplification, electrophoresis of the product did not reveal any visible mutations. The arrow marks the positive control which demonstartes tandem duplication.

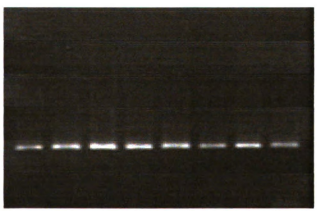


Figure 5: Exon 17 electrophoresis (1% acrylamide gel). Following amplification of the *c-kit* receptor exon 17, electrophoresis of the product was performed revealing no visible mutations.

Electrophoresis of the exon 17 amplicon did not reveal any obvious mutation. Exon 17 sequencing was required to identify the existence of point mutations in this domain.

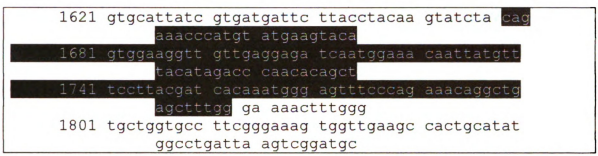


Figure 6: Exon 11 sequence (the marked area describes exon 11 of the *c-kit* receptor)

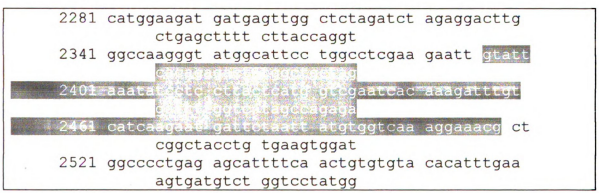


Figure 7: Exon 17 sequence. The marked area describes exon 17 of the *c-kit* receptor

4 DISCUSSION

Hemangiosarcoma is a common, aggressive disease that carries a poor prognosis in dogs. Current therapeutic modalities are unsatisfying and new approaches to the hemangiosarcoma patient need to be explored (Withrow 2001).

We looked at the DAV protocol in a limited cohort of dogs in a clinical phase I and II pilot study. In conjunction with this clinical investigation, we sought to uncover underlying carcinogenic events that might be associated with poor outcome. The methods that we used in investigating the PI3K pathway included:

1) Immunohistochemistry based on retrospective sample availability.

2) Sequencing common genes deranged in other canine cancers.

Receptor tyrosine kinases (RTK'S) are located on the cell membrane and undergo autophosphorylation following an extracellular signal through ligand binding, or also through homoreceptor dimerization. Activation of the receptor is accomplished through appropriate binding of a specific ligand such as a stimulating growth factor. If the receptor is capable of autophosphorylation, a cascade will follow which promotes cell proliferation, growth, and metabolism.

There are several ways that tyrosine kinases may be involved in malignancy. First, a mutation in the RTK may lead to illegitimate continuous signaling without an appropriate ligand mediator. Continuous signaling may occur because of autocrine production of RTK ligand that is secreted by the cancer cell itself, thereby activating the receptor of the malignant cell as well as other neighboring cells in paracrine manner. Finally, over-expression of RTK's on the cell

membrane may also lead to an increase intensity of signaling, either through spontaneous receptor dimerization or by decreasing the threshold for ligand triggering.

Previous reports describe the role of the receptor tyrosine kinase c-kit in some malignancies. This induced speculation about the role this receptor might play in malignant endothelial cell transformation and in hemangiosarcoma. A signal from *c-kit* activates the conversion of PI2 to PI3 through PI3k. A functional PTEN protein may block the conversion. The cascade will continue with the activation of mTOR by AKT. A mutation in the receptor tyrosine kinase, has been demonstrated to result in continuous mitogenic signaling through both the PI3k and RAS pathways. Excess stimulation of these pathways may increase cell survival, angiogenesis, and metastatic ability of the neoplastic cells.

In our study, we evaluated the potential for c-kit mutation at two sites frequently found mutant in other tumor systems. Exon 11, which encodes the juxtamembrane regulatory area of the receptor, did not reveal any mutation to cause continuous signaling. Exon 17 was reported in several tumors to have point mutations; however, in our study sequencing of this site did not reveal any such mutation. Other sites encoding the receptor were not examined. The immunohistochemistry positivity but absence of mutation in the exons evaluated suggests that the receptor is present in hemangiosarcoma but does not exhibit common mutations. Further studies need to be done in order to confirm a complete absence of mutation. The potential for over-expression of the c-kit

receptor, or for continuous activation following autocrine ligand looping, were not examined in this study.

Mutation in PTEN was demonstrated previously in canine hemangiosarcoma. The presence of mutations, commonly at exons 7 and 8, causes error in the C terminus region (Dickerson et al. 2005). In human cancer these mutations were shown to associate with malignancy in a variety of tumors (Kurose et al. 1998). In canine hemangiosarcoma, the common mutation at the C-terminus of PTEN presents as a deletion mutation, resulting in a stop codon that will prevent the formation of intact functional PTEN. Positive immunohistochemistry staining for PTEN illustrates the presence of PTEN protein, however information concerning PTEN's proper function cannot be gleaned by an immunohistochemistry approach. In the absence of PTEN activation, the PI3k pathway stimulated by RTK'S will not be regulated. An over stimulation of the PI3k pathway in a PTEN mutated cell may thus lead to neoplastic transformation.

The functional consequence of a PTEN mutation depends on initiation of a signal upstream to the protein. A mutated *c-kit* receptor will increase the impact of a PTEN mutation by allowing a continuous signal to the cascade(Dickerson 2005). It is logical to assume that a combination of an upstream RTK mutation that coexists with a PTEN mutation would be associated with significant decreased in survival time. An upstream mutation in c-kit was not found in this study, and thus a definite conclusion concerning the significance of PTEN as a survival predictor could not be drawn in our study.

The mutation of p53 is known to be common and highly significant for carcinogenesis. The p53 gene serves as a cellular "gate keeper". When a mutation occurs in DNA a correction of the mutation is dependent on a functional p53 gene. When the mutation is too extensive to repair, the p53 protein is responsible for initiation of cell death through the intrinsic apoptotic pathway. The mutation of the p53 gene results in an accumulation of mutated p53 protein in the cell. This protein accumulation may be detected by immunohistochemistry. The short half-life of wild type p53 protein means that excess immunohistochemistry staining of p53 within the cell may be due to mutation accumulation. Alternatively but less probably, functional p53 may be detected in the process of cell death initiation. The existence of p53 mutations in many human tumors has been shown previously to be significant in prediction of tumor aggressiveness and as a result patient survival. In our study there were 4 samples with strongly positive immunohistochemistry staining, suggestive of p53 mutation. A correlation between p53 mutated cells and patient survival was not found to be significant when survival times were compared. The presence of activated caspase 3 staining reflects the existence of an active apoptotic pathway. The fact that p53 mutations were found to coexist with caspase 3 positivity suggests the existence of a parallel tumor-suppressor pathway responsible for the initiation of cell death in hemangiosarcomas. However, only a small number of tumors stained positive for caspase 3 in this study.

5 CONCLUSION

Our study represents an exploration of potential predictors of biologic behavior and treatment outcome in dogs treated with by a single protocol for hemangiosarcoma, the targets we chose to evaluate failed to reveal any contribution to patient outcome. Provocative findings included the concordance of PTEN and p53 derangements in our model system. A larger study with more patients treated by the DAV protocol would be expected to prove or disapprove the observation made here. While the RTK *c-kit* was found to be expressed in canine hemangiosarcoma, mutations were not observed in the examined domains. Interestingly a mutation of *c-kit* in hemangiosarcoma was not found in the common sites that were investigated in this study. Due to limited amount of patients a further investigation is needed to evaluate the existence of such pathology. The results of the immunohistostostaining could explain in some measure related survival times. A bigger study is required to identify the potential of these factors as a prognostic indicator. Validation of the immunohistochemistry results by sequencing the area of interest is needed for further investigations. Survival of patients treated with the DAV protocol was similar to previous reports of hemangiosarcoma patients treated with other protocols.

6 APPENDICES

Table: Clinical features and therapeutic responses of 15 dogs with hemangiosarcoma treated with doxorubicin, dacarbazine, and vincristine (DAV)

Name	pathology No.	breed	age	gender	location	stage
1	3023331	Golden ret	5.8	FS	spleen+liver mets	Stage 3
2	2900281	English set	9.9	MC	right atrium+spleen	Stage 3
3	2907765	Mixed breed	12.4	MC	spleen+liver mets	Stage 3
4	3016819	Shitzu	10.7	FS	mediastinum	Stage 3
5	3040180	German Shep	10	MC	spleen+liver mets	Stage 3
6	3097703	Mixed breed	5.5	FS	spleen +mets to omentum	Stage 3
7	3042063	Golden ret	11.1	FS	vagina	Stage 3
8	2940094	Labrador ret	10	FS	lungs	Stage 1
9	3022128	German shep	8.1	FS	spleen,pulmonary and hepatic mets	Stage 3
10	2995798	Mixed breed	8.2	FS	rectal colonic hemangiosarc	Stage 3
11	431591	Golden Ret	10	FS	Mass on left abdomen+sub lumb LN	Stage 3
12	sp-6002588	Golden ret	9.9	MC	spleen+liver mets+mets to lungs	Stage 3
13	3042384	Beagle	13.1	FS	spleen+liver+skin mets	Stage 3
14	5091404	Labrador ret	6	FS	spleen	Stage 3
15	3141963	German Shep	10.5	MC	spleen +mets to liver omentum and mesentary	Stage 3

Name	procedure	DTIC dose	Adria dose mg/m2	vinc dose	#cycles
1	splenectomy	800(*5)	30	0.5	5.5
2	removal of mass from atrium+splenectomy	800(*1)&600	30	0.5	4 DAVE
3	splenectomy	800(*4)&600(*2)	30	0.5	5.5
4	non resectable	500(*1)&400(*2)	30(1)=>1 mg/kg		4DAVE
5	splenectomy+ resection of liver mass	800(*5)&600(*1)	30(*5) 22.5(*1)	0.5	6
6	splnectomy	750(*2) 650(*1) 600(1) 6(*1)=dose red	30	0.5	4DAVE 1 actino/DAVE
7	N/A	800(*3)&600(*1)	30	0.5	4
8	N/A	700	30	0.5	5 cycles
9	splenectomy resection of liver mass	600	25	0.5	2 cycles
10	splenectomy	600&480*4	30	0.5	5
11		700	30	N/A	only one week
12	splenectomy	800	30		only one week
13	splenectomy	600(*1)&500(*1)	30	0.5	2.5
14	splenectomy	700/m2	25/m2	0.5	6 DAVE+DCP for maintanans
15	splenectomy	4*600/4*800	30	0.5	6cycles DAV/1actino,doxo,1Temodar

name	toxicity	response	rescue	survival month	comments
1	no	N/A	N/A	3.5	Chemo started at 21/12/04
2	neutropenia grade 2 dose reduction. Diarrhegrade 3(*2)+ vomiting grade 3(*1)	CR	N/A	13.5	first two cycles only Adria. From 10/7/2004 actino and temazolamide. Tx stoped 5/5/2005
3	lymp grade 2(*3)		ifosf	4	
4	WBC grade 4 GI grade4	CR	ifosf	7.5	
5	plt grade 4 Neut grade3 diarrhea 1-2 (*3)	CR	N/A	7.8	Chemo started at 2/11/05 at 2/9/905 reopend sergical site =>gran tissue
6	GI grade 3 diarhea (*3)	CR	N/A	7.2	four cycles of DAVE and then actino/DTIC
7	decreased dose of DTIC due to neutropenia grade IV	CR	N/A	6.4	
8	no	CR	N/A	11	
9	no	CR	ifosf	2	
10	no	CR	N/A	7	
11	N/A	N/A	N/A	19.6	previous similar lession was removed from leg
12	GI tox 3	progr	N/A	3.4	

Name	toxicity	response to tx	rescue	survival	comments
13	neutropenia grade 3	progressive	ifosfamide(*2)	2.5	
14	N/A	CR	N/A	N/A	
15	Neutropenia grade3 and 4	CR	no	5.5	

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