OVARIAN CARCINOMA IN JAGUARS (*PANTHERA ONCA*): INVESTIGATING THE GENETIC MECHANISMS OF TUMORIGENESIS

By

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ABSTRACT

OVARIAN CARCINOMA IN JAGUARS (*PANTHERA ONCA*):
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Jaguars are near threatened throughout their entire geographic range and endangered in the United States. Wild jaguars are threatened by habitat loss and other human impacts, and maintaining a healthy breeding population in zoological institutions is important for survival.

We performed a retrospective analysis of reproductive tract and mammary gland lesions from 56 jaguars housed in North American zoological institutions and found a high prevalence (26 of 49 jaguars; 53%) of ovarian papillary cystadenocarcinomas (OC) in aged jaguars (mean 17 years; range 8-27 years), including some that were of reproductive age. Neoplasms were bilateral, had stromal invasion, and were histologically similar to high-grade serous OC in humans. OC is a significant cause of mortality in jaguars due to metastatic implantation throughout the peritoneal cavity. This neoplasm is very rare in domestic cats, and has not been reported in other zoo or wild felids. Other neoplasms in zoo felids, including jaguars, such as endometrial carcinoma and mammary carcinoma, are associated with the use of exogenous progestin contraceptives like melengestrol acetate (MGA). In contrast, we found that OC in jaguars were not associated with exogenous progestin exposure. Additionally, pedigree analysis of affected jaguars was suggestive of an autosomal dominant pattern of inheritance, similar to hereditary breast and ovarian cancer in humans. In line with these findings, the central hypothesis of this dissertation is that the high prevalence of ovarian carcinomas in jaguars is associated with a germline mutation in BRCA1, BRCA2, or other tumor suppressor gene known to be involved in human hereditary breast and ovarian cancer.

To elucidate the genetic mechanisms of ovarian carcinoma in jaguars, we first assessed the genetic diversity of the North American zoo population overall compared to reported wild jaguar populations. Using novel universal primer sequences, we created a full multiplex of twelve tetra- or pentanucleotide microsatellite markers that have not previously been described for use in the jaguar. We found that genetic diversity has been maintained in the North American zoo population, and that population heterozygosity and allelic diversity were comparable to reported wild jaguar populations in Central and South America. This set of microsatellite markers also has cross-species utility in other felid species.

To evaluate candidate cancer genes for germline mutations associated with ovarian carcinoma, we used a targeted next generation sequencing approach to sequence the coding regions of 276 cancer genes in paired ovarian carcinoma and normal jaguar tissues. We found a missense germline variant in BRCA2 exon 11 resulting in a nonconservative amino acid change (c.3732C>G_p.Ser1248Arg) that was present in all jaguars with ovarian carcinoma, as well as some jaguars in the unaffected zoo population. This variant has also been reported in humans with hereditary breast and ovarian cancer, and is of unknown functional significance. We tested the functional significance of this variant using a CRISPR/CAS strategy to knock-in the S1248R substitution into human embryonic kidney (HEK293T) cells. We also generated BRCA2 -/- cells, and evaluated relative DNA repair ability by measuring percentage of cell viability when exposed to cisplatin, a DNA-damaging agent. We found that cells harboring the S1248R variant had intermediate cisplatin sensitivity at high concentrations compared to wild type and BRCA2 -/- cells. Jaguars represent a valuable natural model of ovarian carcinoma. Evaluating jaguar OC-associated variants in genes such as BRCA2 contributes to the understanding of BRCA2 function overall, as well as the pathogenesis of OC in both jaguars and humans.

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KEY TO ABBREVIATIONS

AE1/AE3: Broad cytokeratin antibody

AI: Allelic imbalance

ATM: Ataxia telangiectasia mutated

AZA: Association of Zoos & Aquariums

BP: Base pair

BRCA1: Breast cancer 1

BRCA2: Breast cancer 2

BRIP1: BRCA1 interacting protein 1

CA-125 cancer antigen 125

Cas9: CRISPR-associated protein 9

CHEK2: Checkpoint kinase 2

CK7: Cytokeratin 7

CRISPR: Clustered regularly interspaced short palindromic repeats

DSB: DNA double strand break

EGF: Epidermal growth factor

ERα: Estrogen receptor alpha

FFPE: Formalin fixed paraffin embedded

H_e: Expected heterozygosity

HDR: Homology directed repair

HR: Homologous recombination

HE: Hematoxylin & Eosin

HEK 293T: Human embryonic kidney (strain 29T) cells

H_o: Observed heterozygosity

HPF: High-power field

HWE: Hardy-Weinberg equilibrium

IHC: Immunohistochemistry

LOH: Loss of heterozygosity

Mb: Megabase

MGA: Melengestrol acetate

MLH1: MutLhomolog 1

MS: Microsatellite

MSH2: MutS homolog 2

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NA: North American

NGS: Next generation sequencing

OCCR: Ovarian cancer cluster region

OSE: Ovarian surface epithelium

PALB2: Partner and localizer of BRCA2

PDX: Patient-derived xenograft

PI: Probability of identity

PIsibs: Probability of identity of siblings

PR: Progesterone receptor

PTEN: Phosphatase and tensin homolog

RAD51C: RAD51 homolog C

RB: Retinoblastoma

RHSP: Reproductive Health Surveillance Program

RMC: Reproductive Management Center

SD: Standard Deviation

SE: Standard Error

STK11/LKB1: Serine-threonine kinase 11/liver kinase B1

TP53: Tumor protein p53

VUS: Variants of uncertain significance

CHAPTER 1

Introduction and Literature Review

Ovarian Cancer in Humans

Overview, Classification and Pathogenesis

Ovarian cancer is the most lethal gynecologic malignancy in women, with approximately 239,000 new cases per year worldwide. In the United States, there are 22,000 new cases and 14,000 deaths per year. Ovarian cancer is most commonly diagnosed in postmenopausal women between the ages of 55-64 years, and overall lifetime risk of developing ovarian cancer is 1.3%. The majority of women (59%) have distant metastases at the time of diagnosis, with a 5-year survival rate of 29% (SEER Cancer Stat Facts: Ovarian Cancer. National Cancer Institute. Bethesda, MD; Accessed 01 June 2018; https://seer.cancer.gov; Reid et al. 2017).

Neoplasms of the ovary in humans are separated into major morphologic categories of epithelial, mesenchymal, sex cord-stromal, and germ cell tumors (Kurman et al. 2014; Prat and Jarboe 2014). Epithelial tumors are further characterized into major categories of benign or malignant serous, mucinous, endometrioid, or clear cell tumors based on histologic features and cell of origin (Kurman et al. 2014).

The vast majority of ovarian tumors in women are of epithelial origin (90%), and the malignant serous subtype is most common (Bell 2005; Kurman et al. 2014). Ovarian serous carcinomas are further characterized into low-grade (type I) or high-grade (type II) based on morphologic and molecular characteristics (Shih and Kurman 2004).

Low-grade serous carcinomas account for 5% of all serous carcinomas and develop from a distinctly different pathway than high-grade serous carcinomas (Shih and Kurman 2004; Kurman et al. 2014). Low-grade carcinomas arise from serous cystadenomas and serous borderline tumors from the ovarian surface epithelium or inclusion cysts, and many (60%) have somatic activating mutations in KRAS and BRAF oncogenes (Ho et al. 2004; Shih and Kurman

2004; Lim and Oliva 2013). KRAS and BRAF have key functions in the mitogen activated protein kinase (MAPK) pathway involved in cell proliferation and differentiation (Oikonomou et al. 2014).

High-grade serous epithelial carcinomas are the most common type of ovarian neoplasia in women (Gilks 2004; Kurman et al. 2014). These neoplasms are usually bilateral and frequently metastasize by implantation throughout the peritoneal cavity. Histologic features include cystic, solid, and papillary regions, necrosis, cellular pleomorphism, and high mitotic count (Kurman et al. 2014; Prat and Jarboe 2014). Somatic p53 mutations are observed in most high-grade carcinomas (Buller et al. 2001; Bell et al. 2011). The site of origin of high-grade serous carcinoma was unknown until recently, and was presumed to originate from the ovarian surface epithelium or epithelial inclusion cysts. However, the fallopian tube is now considered the site of origin of high-grade serous carcinomas. Precursor lesions (foci of p53 overexpression and serous tubal intraepithelial carcinomas) were found in the fallopian tube of patients with high-grade serous carcinoma, and identical p53 mutations were later discovered by whole exome sequencing (Lee et al. 2007; Labidi-Gali et al. 2017). Fallopian tube epithelium also had similar gene expression profiles as high-grade serous carcinomas (Tone et al. 2008). Reported p53 mutations associated with high-grade serous carcinoma are numerous, and include indels and SNPs resulting in loss of protein function (Bell et al. 2011; Kanchi et al. 2013). Interestingly, tubal ligation and salpingectomy both reduce the risk of ovarian cancer in women (Sieh et al. 2013; Daly et al. 2015).

Hormone receptor protein expression profiles in ovarian carcinomas and their significance in prognosis and treatment are not as well characterized as in breast cancer (Lakhani et al. 2012; Voutsadakis 2016). Most high-grade serous carcinomas have estrogen-receptor α

expression, and less than 50% have progesterone receptor expression (Kurman et al. 2014; Prat and Jarboe 2014). Loss of progesterone receptor expression and association with poor prognosis has been reported but progesterone receptor is not routinely used as a prognostic marker (Lenhard et al. 2012).

Inherited susceptibility to ovarian cancer

Family history of ovarian or breast cancer is the most important and well-characterized risk factor for ovarian cancer (Ramus and Gayther 2009). All ovarian carcinomas associated with breast cancer 1 (BRCA1), breast cancer 2 (BRCA2), and other familial syndromes are of the high-grade (type II) serous carcinoma subtype (Kurman et al. 2014; George and Shaw 2014). Approximately 15-20% of high-grade serous carcinomas are due to a hereditary predisposition (Ramus and Gayther 2009; George and Shaw 2014; Kurman et al. 2014). BRCA1 mutation carriers have greater than 50% lifetime risk of developing ovarian cancer, and BRCA2 mutation carriers have a 20-30% risk (George and Shaw 2014). Most hereditary ovarian cancers are caused by a mutation in tumor suppressor genes involved in DNA repair in the homologous recombination pathway, such as BRCA1 and BRCA2 (Petrucelli et al. 2010).
Numerous mutations have been identified in these genes, commonly small insertions and deletions resulting in complete loss of protein function (Takahashi et al. 1995; Gayther et al. 1999). Certain populations have an even higher risk of breast and ovarian cancer, such as the Ashkenazi Jewish population, which has three common founder mutations (BRCA1*185delAG, BRCA1 *5382insC, and BRCA2*6174delT) (Robles-Díaz L et al. 2004). BRCA1 and BRCA2 germline mutations are also associated with breast cancer in males, as well as pancreatic and prostate cancer (van Asperen et al. 2005). Most germline mutations are monoallelic, with locus-

specific loss of heterozygosity in the tumor tissue, resulting in loss of DNA repair function (Maxwell et al. 2017). Additional somatic mutations in p53 result in disruption of cell cycle checkpoint control (Maxwell et al. 2017). Heterozygous mutation carriers do not have a known disease phenotype, however alterations in DNA repair at the cellular level have been found in-vitro in cells with heterozygous mutations in BRCA genes (Warren et al. 2003; Vaclová et al. 2015). In mouse models, homozygous knockout of BRCA1 or BRCA2 is embryonic lethal, and mice that are germline heterozygous for well-known deleterious BRCA1 and BRCA2 mutations do not have increased frequency of ovarian or mammary cancer (Hakem et al. 1998). Mouse models of high grade serous carcinoma originating from the fallopian tube have been developed by conditional inactivation of BRCA1, BRCA2, PTEN, and p53 (Hasan et al. 2015).

Biallelic loss of function mutations in BRCA2, also known as FANCD1 gene, are associated with Fanconi anemia, an autosomal recessive syndrome consisting of congenital abnormalities, early-onset cancers such as acute myeloid leukemia, bone marrow failure, and increased sensitivity to DNA damaging agents like cisplatin (Howlett et al. 2002; D'Andrea and Grompe 2002; Nalepa and Clapp 2018). More recently, biallelic mutations have also been found in BRCA1 and other DNA repair genes (RAD51C, PALB2, BRIP1) associated with similar Fanconi anemia syndromes (Sawyer et al. 2015; Kennedy and D'Andrea 2018).

The Fanconi anemia-associated proteins interact directly with BRCA1 and BRCA2 in homologous recombination functions. The Fanconi anemia-associated proteins form a complex that plays a role in DNA damage repair by recruiting effector proteins to sites of DNA damage (Nalepa and Clapp 2018). Specifically, a heterodimer composed of FANCD2-FANC1 undergoes monoubiquitination by a larger protein complex that initiates the DNA damage response by recruiting effector proteins such as BRCA2 (Nalepa and Clapp 2018).

BRCA1

In 1990, chromosome 17q21 was identified by linkage analysis as the likely site of an inherited susceptibility gene in families with a high incidence of breast cancer, that was later identified as BRCA1, and numerous mutations were found (Hall et al. 1990; Miki et al. 1994). The same chromosomal location and BRCA1 gene along with regional loss of heterozygosity were identified in families with ovarian cancer shortly thereafter (Narod et al. 1991; Futreal et al. 1994). The most important and highly conserved domains of the BRCA1 protein are the N-terminal RING domain, the BRCT domain, and the region encoded by exons 11-13, which are also the most common locations of germline loss of function mutations (Clark et al. 2012). The RING domain has E3 ubiquitin ligase function and binds BRCA1 associated ring domain 1 (BARD1) protein to activate cell cycle checkpoint controls (Clark et al. 2012; Zhao et al. 2017). Exons 11-13 encode the majority of the BRCA1 protein and contain nuclear localization sequences and binding sites for important tumor suppressor proteins such as retinoblastoma (RB), involved in cell cycle regulation, and PALB2, another essential protein involved in homologous recombination (Aprelikova et al. 1999; Li and Greenberg 2012). This region is also phosphorylated by ataxia telangiectasia mutated (ATM) and checkpoint kinase 2 (CHEK2) (Clark et al. 2012). Exon 11 contains numerous germline mutations associated with ovarian cancer (Shattuck-Eidens et al. 1997; Rebbeck et al. 2015). The BRCT domain is composed of two tandem amino acid repeats that recognizes phosphoproteins (Clark et al. 2012). After phosphorylation, BRCA1 forms several complexes with other proteins at the site of DNA damage and function in homologous recombination (Roy et al. 2012). One of these complexes also includes BRCA2 and RAD51C, which acts as a signal mediator to promote RAD51 strand invasion at the site of DNA double- strand breaks (DSB) (Roy et al. 2012).

BRCA2

After the discovery of BRCA1 as a tumor suppressor gene and association of germline mutations with breast and ovarian cancer, BRCA2 was identified in families with breast cancer at chromosome 13q12-13 (Wooster et al. 1994). BRCA2 is an essential effector protein in DNA repair of DSBs by homologous recombination (Roy et al. 2012). The main highly conserved regions of the BRCA2 protein are eight amino acid (BRC) repeats, a DNA binding domain, and a PALB2 binding site at the N terminus (Yoshida and Miki 2004). The eight highly conserved BRC repeats are the sites required for RAD51 binding, and each repeat binds to RAD51 with varying affinity (Bork et al. 1996; Wong et al. 1997; Carreira and Kowalczykowski 2011). The BRCA2-RAD51 complex is required for the initiation of homologous recombination by binding of RAD51 to single-stranded DNA (Shahid et al. 2014). The BRC repeats are encoded by a region of the BRCA2 gene that contains many mutations associated with ovarian cancer (ovarian cancer cluster region) that interfere with BRCA2-RAD51 interaction (Tal et al. 2009; Rebbeck et al. 2015). The N terminus region contains the binding site for PALB2, which links the BRCA1 and BRCA2 proteins, and is essential for both BRCA1 and BRCA2 function (Roy et al. 2012).

Other genes associated with inherited ovarian cancer

BRCA1 and BRCA2 functions are tied to many other genes associated with DNA repair, DNA damage recognition, and cell cycle control, such as checkpoint kinase 2 (CHEK2), partner and localizer of BRCA2 (PALB2), BRCA1 interacting protein 1 (BRIP1) and RAD51C, which also have germline mutations associated with hereditary breast and ovarian cancer (Roy et al. 2012). Other major genes with germline mutations associated with moderate to high penetrance of breast and ovarian cancer, major functions, and associated human syndromes are summarized in Table 1. These genes are classified as tumor suppressors involved in DNA damage recognition and repair, all of which interact with BRCA1 or BRCA2 genes in overlapping pathways (Meindl et al. 2011). In addition to these genes, with the more common use of high-throughput sequencing in cancer diagnostics, additional tumor suppressor genes with germline mutations associated with ovarian cancer continue to be discovered (Permuth-Wey J et al. 2013; Phelan et al. 2017).

Table 1.1: Major functions of tumor suppressor genes with high to moderate penetrance associated with hereditary syndromes that predispose to ovarian, breast, and other cancers.

Gene	Main functions	Associated human syndrome	References
BRCA1	Homologous recombination: dsDNA break repair	Hereditary breast and ovarian cancer syndrome	Petrucelli et al. 2010 Roy et al. 2012
BRCA2	Homologous recombination, dsDNA break repair, recruits RAD51 to DNA damage sites, Cell cycle regulation	Hereditary breast and ovarian cancer syndrome	Petrucelli et al. 2010 Roy et al. 2012
RAD51C	Homologous recombination, localization and stability of BRCA2, binds to ssDNA at site of DNA damage	Hereditary breast and ovarian cancer	Meindl et al. 2011 Pelttari et al. 2011 Roy et al. 2012
PALB2	Homologous recombination, Forms BRCA1-PALB2-BRCA2 complex, Promotes localization and stability of BRCA2	Hereditary breast and ovarian cancer	Zhang et al. 2009 Walsh et al. 2011 Antoniou et al. 2014
PTEN	Phosphatase, inhibits cell proliferation, Inactivates PI3 kinase pathway	Cowden syndrome, including breast cancer	Liaw et al. 1997 Martins et al. 2014
STK11/LKB1	Serine threonine kinase, DNA damage response	Peutz-Jeghers syndrome, including ovarian and breast cancer	Hearle et al. 2006
TP53	Cell cycle checkpoint regulator, DNA repair, apoptosis, regulates BRCA1 transcription	Li-Fraumeni syndrome, including breast cancer Inherited ovarian cancer	Xu et al. 2001 Walsh et al. 2011 Malkin et al. 2011
CHEK2	DNA damage recognition, Cell cycle checkpoint regulator, Mediates interactions between BRCA1-PALB2	Hereditary breast and ovarian cancer	Nevanlinna et al. 2006 Lawrenson et al. 2015
BRIP1	Binds BRCA1, DNA repair by homologous recombination, S phase cell cycle checkpoint	Hereditary breast and ovarian cancer	Rafnar et al. 2011 Roy et al. 2012
ATM	Serine threonine kinase, DNA damage recognition, dsDNA break repair	Ataxia-telangiectasia, breast and ovarian cancer	Thorstenson et al. 2003 Roy et al. 2012
MLH1	DNA mismatch repair, DNA damage recognition	Lynch syndrome, including ovarian cancer	Bonadona et al. 2011 Roy et al. 2012
MSH2	DNA mismatch repair, DNA damage recognition	Lynch syndrome, including ovarian cancer	Bonadona et al. 2011 Roy et al. 2012

The role of endogenous and exogenous hormones in ovarian cancer

Exposure to endogenous estrogen is a risk factor for both breast and ovarian cancer, although the exact mechanisms by which estrogen influences the development of ovarian cancer is not well-characterized (Lakhani et al. 2012; Kurman et al. 2014). Estrogens have mitogenic properties that stimulate cellular proliferation of the ovarian surface epithelium, which is the site of origin of benign and low-grade ovarian carcinomas (Lukanova and Kaaks 2005). Estrogen stimulates cell proliferation by activation of growth factors, such as insulin-like growth factor and epidermal growth factor, which can act as oncogenes (Skandalis et al. 2014). Any event that increases the lifetime exposure to endogenous estrogen, such as early age at menarche, late age at menopause, and nulliparity all increase the risk of ovarian cancer (Lukanova and Kaaks 2005; Brown and Hankinson 2015). Exogenous estrogens, most commonly in the form of postmenopausal hormone replacement therapy, also increase the risk of ovarian cancer (Ho 2003). In contrast, oral contraceptives (combined estrogen and progesterone) decrease the risk of ovarian cancer, and is primarily thought to decrease risk by suppressing ovulation (Havrilesky et al. 2013). Progesterone may have a protective effect on ovarian cancer development, and has been found to induce apoptosis in the ovarian surface epithelium and ovarian cancer cells (Ho 2003; Hee Han et al. 2013). In line with the protective effects of progesterone, parity decreases the risk of ovarian cancer, and the protective effects increase with each live birth (Hee Han et al. 2013).

Diagnosis and Treatment

Reliable screening methods for asymptomatic individuals with stage I ovarian cancer (tumor confined to the ovaries) are lacking. Presenting symptoms are usually nonspecific and related to general abdominal discomfort (Karst and Drapkin 2010).

The most common screening tools are transvaginal ultrasonography and cancer antigen-125 (CA-125) serum biomarker testing (Karst and Drapkin 2010; Jelovac and Armstrong 2011). CA-125 is a mucin glycoprotein (also known as MUC16) expressed by normal epithelial cells, including bronchial, endometrial, ovarian, and corneal epithelial cells (Felder et al. 2014). CA-125 is mainly used as a prognostic marker in patients currently undergoing chemotherapy for ovarian cancer, and is an indicator of disease recurrence (Jelovac and Armstrong 2011). However, this marker is less useful as a general screening tool, as less than 50% of women have elevated CA-125 with early stage ovarian cancer (Nguyen et al. 2013). Additionally, a recent meta-analysis involving the most common screening methodologies (ultrasound and CA-125 testing) found no improvement in ovarian cancer mortality in asymptomatic women with average ovarian cancer risk that had early screening tests compared to those without screening (Henderson et al. 2018). There is still a need for better screening tools for early ovarian cancer detection, and numerous additional biomarkers have been evaluated but are not yet widely used (Nguyen et al. 2013).

After surgical debulking of the primary neoplasm and metastasis to peritoneal surfaces, most high-grade serous carcinomas are treated with cytoreductive chemotherapeutics. The volume of remaining tumor burden after primary surgical debulking of advanced stage high-grade serous ovarian carcinoma is a strong prognostic indicator (Jelovac and Armstrong 2011; Coleman et al. 2013). In patients with advanced high-grade serous carcinoma, prognosis is

associated with the amount of remaining grossly visible tumor tissue after initial surgical debulking (Coleman et al. 2013). Chemotherapy consists of platinum-based drugs such as carboplatin and cisplatin, via intravenous and less commonly intraperitoneal routes, in combination with paclitaxel (Jelovac and Armstrong 2011; Coleman et al. 2013). Cisplatin and carboplatin directly damage DNA by binding to purine residues, resulting in intrastrand cross-linking, ultimately leading to cessation of cell division and apoptosis (Dasari and Tchounwou, 2014). Paclitaxel is a taxane that binds microtubules and halts cell cycle progression, inhibits cellular proliferation, and is effective against some ovarian carcinomas that are resistant to platinum-based chemotherapeutics (Kampan et al. 2015). High-grade serous ovarian carcinomas associated with germline BRCA1 and BRCA2 loss of function mutations and dysfunction in DNA repair are highly sensitive to DNA damaging agents such as cisplatin, and remission is often achieved at initial treatment (Bowtell et al. 2015). However, the majority of patients will have recurrent disease and long term survival is ultimately poor (Hoppenot et al. 2018). Resistance to platinum-based chemotherapeutics in high-grade serous carcinomas occurs commonly, and contributes to the very low 5-year survival rate overall for high-grade ovarian carcinomas (Coleman et al. 2013). Absence of locus-specific loss of heterozygosity of BRCA1 or BRCA2 in breast and ovarian cancers of germline BRCA mutation carriers has been proposed as an important mechanism of resistance to platinum-based chemotherapeutics and PARP-inhibitors (Maxwell et al. 2017). Additionally, tumor gain of function mutations that restore DNA repair function have been proposed as potential mechanisms of resistance to DNA damaging agents (Maxwell et al. 2017).

Newer treatments include anti-angiogenic antibodies, such as bevacizumab, an anti-vascular endothelial growth factor (VEGF) antibody, and poly (ADP-ribose) polymerase (PARP)

inhibitors (Monk et al. 2016; Dziadkowiec et al. 2016). Hormone-based therapies such as antiestrogens (tamoxifen) have shown some success in clinical trials but are not widely used in the treatment of ovarian cancer, as the role of hormones in ovarian cancer pathogenesis are not well understood (Jelovac and Armstrong 2011).

Ovarian Cancer in Animals

Overview, classification, risk factors, and pathogenesis

Neoplasms of the ovary in animals are separated into four broad classifications: Sex cord-stromal (granulosa cell tumor, thecal cell tumor, interstitial cell tumor, or any combination of these cell types), germ cell (dysgerminoma, teratoma, embryonal carcinoma), epithelial (adenoma and adenocarcinoma), and mesenchymal (leiomyoma, hemangioma, and their malignant counterparts) (Kennedy et al. 1998). Epithelial neoplasms are further characterized by main histologic features, most commonly papillary and cystic patterns (Kennedy et al. 1998).

In domestic animals, ovarian adenoma and carcinoma are common only in the dog and occur without a breed predisposition (Patnaik and Greenlee 1987; Dobson 2013). Risk factors for the development of ovarian carcinoma in domestic animals are not well-characterized. Reports of canine ovarian carcinoma in the literature are rare, likely because ovariohysterectomy is commonly performed at a young age as part of routine preventative care. Ovarian carcinomas in dogs are often bilateral and metastasize by peritoneal implantation (Patnaik and Greenlee 1987). Epithelial neoplasms in dogs are most similar to the serous subtype of ovarian carcinoma in women, and other subtypes (endometrioid, mucinous, clear cell) have not been described (Agnew and MacLachlan 2017).

Other than the dog, spontaneous epithelial ovarian neoplasia in other animals has rarely been reported. Sex cord-stromal neoplasms are the most common ovarian neoplasm in domestic cats, and ovarian carcinoma is very rare (Norris et al. 1968; Gelberg and McEntee 1985). Ovarian adenocarcinoma is common in the laying hen, has a similar histotype as humans, and has been used as a model for human ovarian carcinoma in a few studies (Vanderhyden et al. 2003; Barua et al. 2009; Hawkridge et al. 2014). In a study of aged female Sprague Dawley rats, spontaneously developing ovarian epithelial neoplasms classified as tubular adenoma and adenocarcinomas were reported (Gregson et al. 1984). Ovarian epithelial neoplasia is also rare in nonhuman primates. In a retrospective study of ovarian lesions in 458 rhesus macaques from one primate research facility, two epithelial neoplasms were detected (one cystadenoma and one cystadenocarcinoma (Marr-Belvin et al. 2010). In a study of twelve baboons at another research facility, two had ovarian carcinomas (Moore et al. 2002).

In dogs, ovarian carcinomas are histologically similar to the serous subtype of ovarian carcinoma in humans, and arise spontaneously from the ovarian surface epithelium or subsurface epithelial structures (Kennedy et al. 1998). Other than metastasis, criteria for malignancy include size (extension beyond the ovarian bursa), stromal or vascular invasion, cytoplasmic and nuclear pleomorphism, and high mitotic count (Kennedy et al. 1998; Agnew and MacLachlan. 2017). Similar to humans, protein expression in canine ovarian carcinomas include broad cytokeratin markers (AE1/AE3) and cytokeratin 7 (Akihara et al. 2007; Riccardi et al. 2007). Canine ovarian epithelial neoplasms express Hector Battifora mesothelial epitope-1 (HBME-1), and ovarian carcinomas in humans have variable HBME-1 expression (Yaziji et al. 2006; Banco B, et al. 2011). HBME-1 can be used to differentiate epithelial ovarian neoplasms from sex cord-stromal neoplasms in dogs (Banco B et al. 2011). HBME-1 is not specific for ovarian carcinoma and is

not routinely used to confirm the diagnosis in humans, due to immunoreactivity with other neoplasms such as peritoneal mesothelioma (Yaziji et al. 2006; Mittal et al. 2008). Some canine ovarian carcinomas also have positive immunoreactivity for cyclooxygenase -2 (COX-2) (Borzacchiello et al. 2006). In humans, COX-2 expression in ovarian carcinoma is associated with poor prognosis (Sun et al. 2017).

Diagnosis and Treatment

Similar to humans, dogs usually present late in the disease process with abdominal distension as one of the initial clinical signs, and can be diagnosed based on cytology of abdominal effusion samples (Bertazzolo et al. 2012). Neoplasms are also often discovered as incidental findings during abdominal surgery for unrelated disease processes (Bertazzolo et al. 2012).

Other than ovariohysterectomy and surgical debulking, there are no well-established treatment protocols for ovarian carcinoma in dogs. Description of chemotherapeutic strategies are limited to few case reports. Intravenous and intracavitary platinum chemotherapeutics (carboplatin, cisplatin) with and without combined paclitaxel, a chemotherapeutic targeting microtubules, have been reported in individual case reports of dogs with metastatic ovarian carcinoma with variable (8 months – 3 years) survival times (Olsen et al. 1994; Hidaka et al. 2014; Best and Frimberger 2017).

The role of BRCA1 and BRCA2 in canine and feline cancer

The genetic mechanisms of ovarian carcinoma in other mammals, including dogs, has not been reported, to our knowledge. Research on BRCA1 and BRCA2 germline mutations in domestic dogs and cats are limited to mammary cancer (Flisikowski et al. 2015). In dogs, exon 11 of BRCA2 was sequenced in mammary tumor tissue and normal mammary gland, and several single nucleotide polymorphisms (SNPs) resulting in missense amino acid changes in mammary tumors were found (Hsu et al. 2010). In another study, multiple SNPS in exon 11 of BRCA2 in regions encoding BRC repeats were found in canine mammary carcinomas, and the c.2383A>C (T1425P) SNP in BRC repeat 3 was predicted to be the most damaging based on protein and mutation prediction software programs (Ozmen et al. 2017). A mammalian two-hybrid assay was also used to show that the T1425P missense variant in canine BRCA2 BRC repeat 3 had reduced binding affinity to RAD51 (Ochiai et al. 2015). Loss of heterozygosity in BRCA2 was also found in mammary carcinoma of one dog using microsatellite markers (Yoshikawa et al. 2008). In English Springer Spaniels in Sweden, a breed that has high prevalence of mammary carcinoma, several tumor suppressor genes were evaluated by association analysis for SNPs, and those in BRCA1 and BRCA2 were significantly associated with mammary carcinoma in this breed (Rivera et al. 2009). In cats, feline mammary adenocarcinomas that exhibit a basal-like morphology similar to human breast cancer associated with BRCA mutations were evaluated for loss of heterozygosity in BRCA1 and BRCA2 genes, and no evidence of LOH was found (Wiese et al. 2013). Overall, the extent to which BRCA1 and BRCA2 germline mutations are involved in canine or feline mammary cancer remains speculative.

Ovarian Carcinoma in Jaguars: Current Knowledge

In the early 1990's, Dr. Linda Munson discovered that jaguars from North American zoological institutions had a high prevalence of ovarian papillary cystadenocarcinoma (ovarian carcinoma). In a survey of twelve jaguars, nine had ovarian carcinoma, all of which demonstrated stromal invasion and implantation to adjacent serosal surfaces (Munson 1994). Prior to this study, reports of this neoplasm in jaguars were limited to rare individual case reports (Maryamma et al. 1974; Bossart and Hubbell 1983). This was a surprising finding, as ovarian carcinoma is very rare in domestic cats, and had not been reported in any other zoo or wild felid species (Gelberg and McEntee 1985). Felids in zoological institutions are commonly contracepted with long-acting exogenous progestins, such as melengestrol acetate (MGA). Munson and others found an association with MGA exposure and endometrial hyperplasia, endometrial carcinoma, and mammary carcinoma in many zoo felid species, including jaguars (Harrenstein et al. 1996; Kazensky et al. 1998; Munson et al. 2002; Hope and Deem 2006; McAloose et al. 2007).

Because jaguars were the only felid species that also developed ovarian carcinoma, Munson was the first to propose that these neoplasms develop via an alternative pathogenesis independent of progestin exposure, and that zoo jaguars may have an underlying genetic predisposition associated with the high prevalence of ovarian carcinoma. This hypothesis is the basis for the work presented in this dissertation. The opportunity to study the genetic pathogenesis of ovarian carcinoma in jaguars, which may represent a natural model of inherited ovarian cancer similar to hereditary breast and ovarian cancer in humans, could also contribute to the understanding of ovarian cancer in general.

Central Hypothesis and Specific Aims

The central hypothesis of this dissertation is that the high prevalence of ovarian carcinomas in jaguars is associated with a germline mutation in BRCA1, BRCA2, or other tumor suppressor gene known to be involved in human hereditary breast and ovarian cancer.

The specific aims of this dissertation are as follows:

(1) Characterize reproductive tract and mammary gland lesions in female jaguars with emphasis on ovarian carcinoma phenotype, and identify clinical, historical and hormonal/reproductive factors associated with ovarian carcinoma in the affected population (Chapter 2).

(2) Develop resources to study ovarian carcinoma in jaguars, including establishing a DNA archive from the North American zoo population, and developing in-vitro and in-vivo resources (cell lines and cell line derived mouse xenograft models) to study the pathogenesis of ovarian carcinoma (Chapter 3).

(3) Assess the genetic diversity of the North American zoo jaguar population compared to wild populations by development of a multiplex microsatellite genotyping panel using a universal primer method (Chapter 4).

(4) Identify candidate germline variants associated with ovarian carcinoma in paired jaguar samples (ovarian carcinoma DNA and constitutive DNA - normal tissue or blood) using a candidate gene and targeted next-generation sequencing approach (Chapter 5).

REFERENCES

REFERENCES

Agnew DA, MacLachlan NJ (2017) Tumors of the Genital System In: Tumors of Domestic Animals 5th edition. Ed: Meuten DJ. John Wiley & Sons, Inc. Ames IA, pp. 689-722

Akihara Y, Shimoyama Y, Kawasako K (2007) Immunohistochemical evaluation of canine ovarian tumors. J Vet Med Sci 69(7):703-708

Aprelikova ON, Fang BS, Meissner EG, et al. (1999) BRCA1-associated growth arrest is RB-dependent. PNAS 96(21):11866-11871

Banco B, Antuofermo E, Borzacchiello G, et al. (2011) Canine ovarian tumors: an immunohistochemical study with HBME-1 antibody. Journal of Veterinary Diagnostic Investigation 23(5):977-981

Barua A, Bitterman P, Abramowicz JS, et al. (2009) Histopathology of ovarian tumors in laying hens, a preclinical model of human ovarian cancer. Int J Gynecol Cancer 19(4):531-539

Bell DA (2005) Origins and molecular pathology of ovarian cancer. Mod Pathol18:S19-S32

Bell D, The Cancer Genome Atlas Research Network et al. (2011) Integrated genomic analyses of ovarian carcinoma. Nature 474:609 doi:10.1038/nature10166

Bertazzolo W, Bonfanti U, Mazzotti S, et al. (2012) Cytologic features and diagnostic accuracy of analysis of effusions for detection of ovarian carcinoma in dogs. Veterinary Clinical Pathology 41(1):127-132

Bonadona V, et al. (2011) Cancer risk associated with germline mutations in MLH1, MSH2 and MSH6 Genes in Lynch Syndrome. JAMA. 305(22): 2304-2310

Bork P, Blomberg N, Nilges M (1996) Internal repeats in the BRCA2 protein sequence. Nature Genetics 13:22-23

Borzacchiello G, Russo V, Russo M (2007) Immunohistochemical expression of cyclooxygenase-2 in canine ovarian carcinomas. J Vet Med A 54:247-249

Bossart GD, Hubbell G (1986) Ovarian papillary cystadenocarcinoma in a jaguar. J Zoo Anim Med. 14(1):73-76

Bowtell DD, et al. (2015) Rethinking ovarian cancer II: reducing mortality from high-grade serous ovarian cancer. Nature Reviews Cancer 15:668-679

Brown S, Hankinson S (2015) Endogenous estrogens and the risk of breast, endometrial, and ovarian cancers. Steroids: http://dx.doi.org/10.1016/j.steroids.2014.12.013

Buller RE, Lallas TA, Shahin MS, et al. (2001) The p53 mutational spectrum associated with BRCA1 mutant ovarian cancer. Clinical Cancer Research 7:831-838

Carreira A, Kowalczykowski SC (2011) Two classes of BRC repeats in BRCA2 promote RAD51 nucleoprotein filament function by distinct mechanisms. PNAS 108(26): 10448-10453

Clark SL, Rodriguez AM, Snyder RR, Hankins GDV, Boehning G. (2012) Structure-function of the tumor suppressor BRCA1. Computational and structural biotechnology journal 1:1 e201204005

Coleman RL, Monk BJ, Sood AK et al. (2013) Latest research and clinical treatment of advanced-stage epithelial ovarian cancer. Nat Rev Clin Oncol 10(4):211-224

Corner S, Parys M, Moresco A, Yuzbasiyan-Gurkan V, Agnew D (2017) Ovarian adenocarcinoma in jaguars (*Panthera onca*): a naturally-occurring model of human inherited ovarian cancer? [abstract]. In: Proceedings of the 11th Biennial Ovarian Cancer Research Symposium; Sep 12-13, 2016; Seattle, WA. Philadelphia (PA): AACR; Clin Cancer Res 23:11 Suppl Abstract

D'Andrea AD, Grompe M (2003) The Fanconi anaemia/BRCA pathway. Nature Reviews Cancer 3:23-34

Daly MB, Dresher CW, Yates MS, et al. (2015) Salpingectomy as a means to reduce ovarian cancer risk. Cancer Prev Res (Phila) 8(5):342-348

Dasari S, Tchounwou PB (2014) Cisplatin in cancer therapy: molecular mechanisms of action. Eur J Pharmacol 0:364-378

Dobson JM (2013) Breed-predispositions to cancer in pedigree dogs. ISRN Veterinary Science. 2013: 1-23

Dziadkowiec KN, et al. (2016) PARP inhibitors: review of mechanisms of action and BRCA1/2 mutation targeting. Menopause Rev 15(4):215-219

Felder M, Kapur A, Gonzalez-Bosquet J, et al. (2014) MUC16 (CA125):tumor biomarker to cancer therapy, a work in progress. Molecular Cancer 13:129

Flisikowski K, Flisikowska T, Sikorska A, et al. (2015) Germline gene polymorphisms predisposing domestic mammals to carcinogenesis. Veterinary and Comparative Oncology 15(2):289-298

Futreal PA, Liu Q, Shattuck-Eidens D, et al. (1994) BRCA1 mutations in primary breast and ovarian cancer. Science 266:120-122

Gayther SA, Russell P, Harrington P, et al. (1999) The contribution of germline BRCA1 and BRCA2 mutations to familial ovarian cancer: no evidence for other ovarian cancer-susceptibility genes. Am J Hum Genet 65:1021-1029

Gelberg HB, McEntee K (1985) Feline ovarian neoplasms. *Vet Pathol*. 22:572-576

George SHL, Shaw P (2014) BRCA and early events in the development of serous ovarian cancer. Frontiers in Oncology 4(5): doi: 10.3389/fonc.2014.00005

Gilks CB (2004) Subclassification of ovarian surface epithelial tumors based on correlation of histologic and molecular pathology data. International Journal of Gynecological Pathology 23:200-205

Gregson RL, Lewis DJ, Abbott DP (1984) Spontaneous ovarian neoplasms of the laboratory rat. Vet Pathol 21:292-299

Hakem R, Luis de la Pompa J, Mak TW (1998) Developmental studies of BRCA1 and BRCA2 knock-out mice. Journal of Mammary Gland Biology and Neoplasia 3(4):431-432

Hall JM, Lee MK, Newman B et al. (1990) Linkage of early-onset familial breast cancer to chromosome 17q21. Science 250(4988):1684-9

Harrenstein LA, Munson L, Seal US (1996) Mammary cancer in captive wild felids and risk factors for its development: a retrospective study of the clinical behavior of 31 cases. J Zoo Wildl Med 27(4): 468-476

Hasan N, Ohman AW, Diulescu DM (2015) The promise and challenge of ovarian cancer models. Transl Cancer Res 4(1):14-28

Havrilesky LJ et al. (2013) Oral contraceptive pills as primary prevention for ovarian cancer. Obstetrics & Gynecology 122(1): 139-147

Hawkridge AM (2014) The chicken model of spontaneous ovarian cancer. Proteomics Clin Appl 8:689-699

Hearle N, et al. (2006) Frequency and spectrum of cancers in the Peutz-Jeghers syndrome. Clin Canc Res 12(10):3209-3215

Hee Han K, Kim MK, Kim HS, Chung HH, Song YS (2013) Protective effects of progesterone during pregnancy against ovarian cancer. Journal of Cancer Prevention 18(2): 113-122

Henderson JT, Webber EM, Sawaya GF (2018) Screening for ovarian cancer: updated evidence report and systematic review for the US preventive service task force. JAMA 319(6):595-606

Hikada Y, Kodama A, Tamura R, et al. (2014) A canine case of ovarian carcinoma with pulmonary metastasis treated with surgery and paclitaxel/platinum combination chemotherapy. Journal of Japan Veterinary Cancer Society 5(1):6-11

Ho CL, Kurman RJ, Delhari R, et al. (2004) Mutations of BRAF and KRAS precede the development of ovarian serous borderline tumors. Cancer Research 64:6915-6918

Ho SM (2003) Estrogen, progesterone and epithelial ovarian cancer. Reproductive Biology and Endocrinology 1:73

Hope K, Deem SL (2006) Retrospective study of morbidity and mortality of captive jaguars (*Panthera onca*) in North America, 1982-2002. Zoo Biol 25:501-512

Hoppenot C, Eckert MA, Tienda SM, Lengyel E (2018) Who are the long-term survivors of high grade serous ovarian cancer? Gynecologic Oncology 148:204-212

Howlett NG, Taniguchi T, Olson S, et al. (2002) Biallellic inactivation of BRCA2 in Fanconi anemia. Science 297:606-609

Hsu WL, Huang YH, Chang TJ, et al. (2010) Single nucleotide variation in exon 11 of canine BRCA2 in healthy and cancerous mammary tissue. The Veterinary Journal 184:351-356

Jelovac D, Armstrong DK (2011) Recent progress in the diagnosis and treatment of ovarian cancer. Ca Cancer J Clin 61(3):183-203

Kampan NC, Madondo MT, McNally OM, et al. (2015) Paclitaxel and its evolving role in the management of ovarian cancer. Biomed Research International 2015: http://dx.doi.org/10.1155/2015/413076

Kanchi KL, Johnson KJ, Lu C et al. (2013) Integrated analysis of germline and somatic variants in ovarian cancer. Nature Communcations 5:3156 DOI: 10.1038/ncomms4156

Karst AM, Drapkin R (2010) Ovarian cancer pathogenesis: a model in evolution. Journal of Oncology 2010: doi:10.1155/2010/932371

Kazensky CA, Munson L, Seal US (1998) The effects of melengestrol acetate on the ovaries of captive wild felids. J Zoo Wildl Med 29(1): 1-5

Kennedy PC, Cullen JM, Edwards JF, Goldschmidt MH, Larsen S, Munson L, Nielsen S (2007) WHO Tumor Fascicles: Histological Classification of Tumors of the Genital System of Domestic Animals. Armed Forces Institute of Pathology, Washington, DC

Kennedy RD, D'Andrea AD (2018) The Fanconi anemia/BRCA pathway: new faces in the crowd. Genes & Development 19:2925-2940

Kurman RJ, Carcangiu ML, Herrington CS, Young RH (2014) WHO Classification of Tumours of Female Reproductive Organs. International Agency for Research on Cancer (IARC), Lyon, France

Labidi-Galy SI, Papp E, Hallberg D et al. (2017) High grade serous ovarian carcinomas arise in the fallopian tube. Nature Communications 8: 1093 DOI: 10.1038/s41467-017-00962-1

Lakhani S, Ellis IO, Schnitt SJ, Hoon Tan P, van de Vijver MJ (2012) WHO Classification of Tumours of the Breast. International Agency for Research on Cancer (IARC), Lyon, France. Pp 171-194

Lawrenson K, et al. (2015) Common variants at the CHEK2 gene locus and risk of epithelial ovarian cancer. Cacinogenesis 36(11):1341-1353

Lee Y, Miron A, Drapkin R, et al. (2007) A candidate precursor to serous carcinoma that originates in the distal fallopian tube. Journal of Pathology 211:26-35

Lenhard M, Tereza L, Heublein S, et al. (2012) Steroid hormone receptor expression in ovarian cancer: progesterone receptor B as prognostic marker for patient survival. BMC Cancer 12:553

Li ML, Greenberg RA (2012) Links between genome integrity and BRCA1 tumor suppression. Trends in Biomedical Sciences 37(10):418-424

Liaw D, et al. (1997) Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nature Genet 16:64-67

Lim D, Oliva E (2013) Precursors and pathogenesis of ovarian carcinoma. Pathology 45(3): 229-242

Lukanova A, Kaaks R (2005) Endogenous hormones and ovarian cancer: epidemiology and current hypotheses. Cancer Epidemiol Biomarkers Prev 14(1): 98-107

Malkin D (2011) Li-Fraumeni syndrome. Genes Cancer 2(4):475-484

Marr-Belvin AK, Bailey CC, Knight HL et al. (2010) Ovarian pathology in rhesus macaques: a 12-year retrospective. J Med Primatol 39(3):170-176

Martins FC, Santiago I, Trinh A, et al. (2014) Combined image and genomic analysis of high-grade serous ovarian cancer reveals PTEN loss as a common driver event and prognostic classifier. Genome Biology 15:526

Maryamma KI, Sivadas CG, Krishnan Nair M, Rajan A (1974) Cystadenocarcinoma of ovary with leiomyoma of uterus in a jaguar (*Panthera onca*). Indian Vet J 51:269-270

Maxwell KN, Wubbenhorst B, Wenz BM et al. (2017) BRCA locus-specific loss of heterozygosity in germline BRCA1 and BRCA2 carriers. Nature Communications 319: doi:10.1038/s41467-017-00388-9

McAloose D, Munson L, Naydan DK (2007) Histologic features of mammary carcinomas in zoo felids treated with melengestrol acetate (MGA) contraceptives. *Vet Pathol.* 44:320-326

Meindl A, Ditsch N, Kast K, Rhiem K, Schmutzler R (2011) Hereditary breast and ovarian cancer. Med Dtsch Arztebl Int 108(19): 323-330

Miki Y, Swensen J, Shattuck-Eidens D, et al. (1994) A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science 266(5182):66-71

Mittal K, Soslow R, McCluggage WG (2008) Application of immunohistochemistry to gynecologic pathology. Arch Pathol Lab Med 132:402-423

Monk BJ, Minion LE, Coleman RL (2016) Anti-angiogenic agents in ovarian cancer: past, present, and future. Annals of Oncology 27(Supplement 1):I33-I39

Moore CM, Hubbard GB, Leland MM, et al. (2002) Spontaneous ovarian tumors in twelve baboons: a review of ovarian neoplasms in non-human primates. J Med Primatol 32:48-56

Munson L (1994) A high prevalence of ovarian papillary cystadenocarcinomas in jaguars (*Panthera onca*). Vet Pathol (Abstract) 31:5

Munson L, Gardner IA, Mason RJ, Chassy LM, Seal US (2002) Endometrial hyperplasia and mineralization in zoo felids treated with melengestrol acetate contraceptives. *Vet Pathol.* 39:419-427

Nalepa G, Clapp DW (2018) Fanconi anaemia and cancer: an intricate relationship. Nature Reviews Cancer 18:168-185

Narod SA, Feunteun J, Lynch HT et al. (1991) Familial breast-ovarian cancer locus on chromosome 17q12-q23. Lancet 338(8759):82-3

Nevanlinna H, Bartek J. (2006) The CHEK2 gene and inherited breast cancer susceptibility. Oncogene. 25:5912-5919

Nguyen L, Cardenas-Goicoechea J, Gordon P et al. (2013) Biomarkers for early detection of ovarian cancer. Women's Health 9(2):171-187

Norris HJ, Garner FM, Taylor HB (1969) Pathology of feline ovarian neoplasms. The Journal of Pathology 138-143

Ochiai K, Oonuma TI, Yoshikawa Y, et al. (2015) Polymorphisms of canine BRCA2 BRC repeats affecting interaction with RAD51. Biomedical Research (Tokyo) 36(2):155-158

Oikonomou E, Koustas E, Goulielmaki M, et al. (2014) BRAF vs RAS oncogenes: are mutations of the same pathway equal? Differential signaling and therapeutic implications. Oncotarget 5(23): 11752-11777

Olsen J, Komtebedde J, Lackner A, et al. (1994) Cytoreductive treatment of ovarian carcinoma in a dog. Journal of Veterinary Internal Medicine 8(2):133-135

Ozmen O, Kul S, Risvanli A, et al. (2017) Somatic SNPs of the BRCA2 gene at the fragments encoding RAD51 binding sites of canine mammary tumors. Veterinary and Comparative Oncology 15(4):1479-1486

Patnaik AK, Greenlee PG (1987) Canine ovarian neoplasms: a clinicopathologic study of 71 cases, including histology of 12 granulosa cell tumors. Vet Pathol 24:509-514

Pelttari LM, et al. (2011) RAD51C is a susceptibility gene for ovarian cancer. *Hum Mol Gen*. 20(16): 3278-3288

Permuth-Wey J, Lawrenson K, Shen HC, et al. (2013) Identification and molecular characterization of a new ovarian cancer susceptibility locus at 17q21.31. Nat Commun 4:1627 doi:10.1038/ncomms2613

Petrucelli N, Daly MB, Feldman GL (2010) Hereditary breast and ovarian cancer due to mutations in BRCA1 and BRCA2. Genetics in Medicine 12(5): 245-259

Phelan CM, Kuchenbaecker KB, Tyrer JP, et al. (2017) Identification of 12 new susceptibility loci for different histotypes of epithelial ovarian cancer. Nature Genetics 49:680-691

Prat J, Jarboe E (2014) Ovarian Epithelial-Stromal Tumors. Serous Tumors. In: Mutter GL, Prat J (Eds) Pathology of the female reproductive tract. Third Ed, New York, NY, Elsevier pp. 564-590.

Rafnar T, et al. (2011) Mutations in BRIP1 confer high risk of ovarian cancer. Nat Genet 43(11):1104-1109

Reid BM, Permuth JB, Sellers TA (2017) Epidemiology of ovarian cancer: a review. Cancer Biol Med 9-32 doi: 10.20892/j.issn.2095-3941.2016.0084

Riccardi E, Greco V, Verganti S, et al. (2007) Immunohistochemical diagnosis of canine ovarian epithelial and granulosa cell tumors. J Vet Diagn Invest 19:431-435

Rebbeck TR, Mitra N, Wan F, et al. (2015) Association of type and location of BRCA1 and BRCA2 mutations with risk of breast and ovarian cancer. JAMA 313(13): 1347-1361

Rivera P, Melin M, Biagi T, et al. (2009) Mammary tumor development in dogs is associated with BRCA1 and BRCA2. Cancer Research 69:8770-8774

Robles-Díaz L, Goldfrank DJ, Kauff ND et al. (2004) Hereditary ovarian cancer in Ashkenazi Jews. Familial Cancer 3:259-264

Roy R, Chun J, Powell SN (2012) BRCA1 and BRCA2: different roles in a common pathway of genome protection. Nat Rev Cancer 12(1):68-78

Sawyer SL, Tian L, et al. (2015) Biallelic mutations in BRCA1 cause a new Fanconi anemia subtype. Cancer Discov 5(2):135-142

Shahid T, Soroka J, Kong EH et al. (2014) Structure and mechanism of action of the BRCA2 breast cancer tumor suppressor. Nature Structural & Molecular Biology 21(11):962-970

Shattuck-Eidens D, Oliphant A, McClure M, et al. (1997) BRCA1 sequence analysis in women at high risk for susceptibility mutations. JAMA 278(15):1242-1250

Shih IM, Kurman RJ (2004) Ovarian tumorigenesis: a proposed model based on morphological and molecular genetic analysis. American Journal of Pathology 164(5):1511-1518

Sieh W, Salvador S, McGuire V, et al. (2013) Tubal ligation and risk of ovarian cancer subtypes: a pooled analysis of case-control studies. International Journal of Epidemiology 42:579-589

Skandalis SS, Afratis N, Smirlaki G, et al. (2014) Cross-talk between estradiol receptor and EGFR/IGF-IR signaling pathways in estrogen-responsive breast cancers: Focus on the role and impact of proteoglycans. Matrix Biology 35:182-193

Sun H, Zhang X, Sun D, et al. (2017) COX-2 expression in ovarian cancer: an updated meta-analysis. Oncotarget 8(50):88152-88162

Takahashi H, Behbakht K, McGovern PE et al. (1995) Mutation analysis of the BRCA1 gene in ovarian cancers. Cancer Research 55:2998-3002

Tal A, Arbel-Goren R, Stavans J (2009) Cancer-associated mutations in BRC domains of BRCA2 affect homologous recombination induced by RAD51. J Mol Biol 393:1007-1012

Thorstenson YR, et al. (2003) Contributions of ATM mutations to familial breast and ovarian cancer. Cancer Res. 63:3325-3333

Tone AA, Begley H, Sharma M, et al. (2008) Gene expression profiles of luteal phase fallopian tube epithelium from BRCA mutation carriers resemble high-grade serous carcinoma. Clin Cancer Res 14(13):4067-4078
Vachon CM, Mink PJ, Janney CA et al. (2001) Association of parity and ovarian cancer risk by family history of breast or ovarian cancer in a population-based study. Epidemiology 13(1): 66-71

Vaclová T et al. (2015) DNA repair capacity is impaired in healthy BRCA1 heterozygous mutation carriers. Breast Cancer Res Treat. 152:271-282

van Asperen CJ, Brohet RM, Meijers-Heijboer EJ, Hoogerbrugge N, Verhoef S, Vasen HF, Ausems MG, Menko FH, Gomez Garcia EB, Klijn JG, Hogervorst FB, van Houwelingen JC, et al. (2005) Cancer risks in BRCA2 families: estimates for sites other than breast and ovary. J Med Genet 42(9):711–719

Vanderhyden BC, Shaw TJ, Ethier JF (2003) Animal models of ovarian cancer. Reprod Biol Endocrinol 1:67

Voutsadakis IA (2016) Hormone receptors in serous ovarian carcinoma: prognosis, pathogenesis, and treatment considerations. Libertas Academia 10:17-25

Walsh T, et al. (2011) Mutations in 12 genes for inherited ovarian, fallopian tube and peritoneal carcinoma identified by massively parallel sequencing. *PNAS*.108(44):10832-10837

Warren M, Lord CJ, Masabanda J, et al. (2003) Phenotypic effects of heterozygosity for a BRCA2 mutation. Human Molecular Genetics 12(20):2645-2656

Wong AKC, Pero R, Ormonde PA, et al. (1997) RAD51 interacts with the evolutionarily conserved BRC motifs in the human breast cancer susceptibility gene brca2* The Journal of Biological Chemistry 272(51):31941-31944

Wooster R, Neuhausen SL, Mangion J, et al. (1994) Localization of a breast cancer susceptibility gene, BRCA2, to chromosome 13q12-13. Science 265(5181):2088

Yaziji H, Battifora H, Barry TS, et al. (2006) Evaluation of 12 antibodies for distinguishing epithelioid mesothelioma from adenocarcinoma: identification of a three-antibody immunohistochemical panel with maximal sensitivity and specificity. Mod Pathol 19:514-523

Yoshida K, Miki Y (2004) Role of BRCA1 and BRCA2 as regulators of DNA repair, transcription, and cell cycle in response to DNA damage. Cancer Sci 95(11):866-871

Yoshikawa Y, Morimatsu M, Ochiai K, et al. (2008) Novel variations and loss of heterozygosity of BRCA2 identified in a dog with mammary tumors. AJVR 69(10):1323-1328

Zhang F, et al. (2009) PALB2 links BRCA1 and BRCA2 in the DNA-damage response. Curr Biol. 19:524-529

Zhao W, Steinfeld JB, Liang F, et al. (2017) BRCA1-BARD1 promotes RAD51-mediated homologous DNA pairing. Nature 550:360

CHAPTER 2

Characterization of reproductive tract and mammary gland lesions in 56 female jaguars (*Panthera onca*) from North American zoological institutions confirms high prevalence of ovarian carcinoma

Abstract

Jaguars and other zoo felids have a high prevalence of reproductive tract and mammary gland lesions, including endometrial hyperplasia, endometrial carcinoma, and mammary carcinoma that are associated with the use of exogenous progestin contraceptives such as melengestrol acetate (MGA). Additionally, ovarian papillary cystadenocarcinoma (ovarian carcinoma) has been reported with high frequency in the jaguar and has never been reported in other zoo felids. A retrospective analysis of reproductive tracts and mammary glands from 56 female jaguars between 2-27 years of age (mean=16.2 +/- 4.9; median=16.5 years) submitted by 38 zoological institutions to the Association of Zoos & Aquariums (AZA) Reproductive Management Center - Reproductive Health Surveillance Program (RHSP) was undertaken to determine the prevalence of ovarian carcinoma and other reproductive lesions in this species. Reproductive and mammary neoplasia (mammary, endometrial, or ovarian carcinoma) were the most common causes of death in jaguars (n=25; 44%). Endometrial hyperplasia, endometrial carcinoma, and mammary carcinoma were common in jaguars and were associated with MGA contraceptive exposure, as for other felids. Uterine leiomyoma was the most common benign neoplasm in jaguars (n=21; 38.8%). Ovarian carcinoma was identified in 26 of 49 jaguars with ovaries submitted (53%), and occurred in aged animals (mean = 17 years; range 8-27 years), but some jaguars were still of reproductive age. Neoplasms were bilateral in all cases, had stromal invasion, were composed of cystic, papillary, and solid regions, and commonly extended into the adjacent mesentery and uterine serosa. Ovarian carcinoma was the cause of death in 6 jaguars (10.7% of study population). No metastases to distant organs were present, however 8 (31%) had metastasis by peritoneal implantation.

Ovarian carcinomas (n=3) were positive for estrogen receptor alpha (ERα), progesterone receptor (PR), and cytokeratin 7 (CK7), and were negative for calretinin, similar to serous ovarian carcinomas in humans. Ovarian carcinoma was not associated with MGA contraception (*P*=0.1). Pedigree analysis of a subset of jaguars with ovarian carcinoma was suggestive of an autosomal dominant pattern of inheritance, similar to inherited germline mutations in tumor suppressor genes such as BRCA1 and BRCA2 in hereditary breast and ovarian cancer in women. The high prevalence of ovarian carcinoma in jaguars that is not predicated by exogenous progestin exposure suggests an inherited genetic predisposition is a potential mechanism of carcinogenesis.

Introduction

Jaguars (*Panthera onca*) are classified as near threatened by the International Union for the Conservation of Species (IUCN) and are endangered in the United States (https://www.fws.gov/endangered/). Due to continued habitat loss and other threats to survival, maintaining a healthy breeding population in zoological institutions may become increasingly more important for species survival (Quigley et al. 2017). High prevalence of reproductive tract and mammary gland lesions such as endometrial hyperplasia, endometrial carcinomas, and mammary carcinomas have been reported in zoo felids, including jaguars, and are associated with the use of long-acting exogenous progestin contraceptives (Munson et al. 1995; Harrenstein et al. 1996; Chassy et al. 2002; Munson et al. 2002; Munson 2006; McAloose et al. 2007). Exogenous progestins are also associated with endometrial hyperplasia and carcinoma, pyometra, and mammary carcinoma in domestic dogs and cats (Hayden et al. 1989; Schlafer and Gifford, 2008; Keskin et al. 2009).

Historically, one of the most common progestin contraceptives used in zoo felids was melengestrol acetate (MGA), delivered in the form of a long-acting subcutaneous implant, the mechanism of action primarily centered on the endometrium and alteration of uterine motility (Munson 2006). The carcinogenic effects of MGA and other progestins are not well-characterized, but are thought to arise from the combined exposure to a potent exogenous progestin, as well as continued endogenous estrogen and progesterone exposure due to the inability of MGA to suppress folliculogenesis and ovulation (Kazensky et al. 1998; Munson 2006).

Ovarian papillary cystadenocarcinoma (ovarian carcinoma) has been reported only in the jaguar, and seems to occur independent of exogenous progestin exposure (Maryamma KI et al. 1974; Bossart and Hubbell 1983; Munson 1994; Kazensky 1998; Hope and Deem 2006; Bryan et al. 2015). Among domestic animals, ovarian epithelial neoplasms are common only in the dog, and extremely rare in domestic cats (Gelberg and McEntee 1985; Patnaik and Greenlee 1987). Due to the apparent high prevalence of ovarian carcinoma that is unique to jaguars, and lack of a clear association with progestin contraceptives, a genetic predisposition has been suggested as a potential mechanism of carcinogenesis (Munson and Moresco, 2007). In women, germline mutations in tumor suppressor genes such as BRCA1 and BRCA2 are associated with hereditary ovarian and breast cancer (Welcsh and King, 2001). The overall high prevalence of reproductive neoplasia in jaguars has been reported in studies including all zoo felids and one analysis of morbidity and mortality in zoo jaguars (Harrenstein 1996; Kazensky 1998; Hope and Deem 2006; McAloose 2007).

The apparent high prevalence of ovarian carcinoma in jaguars, in addition to other reproductive neoplasia warrants an in-depth analysis of overall reproductive tract and mammary gland lesions in this species. The overall goals of this retrospective study were to (1) characterize and evaluate the prevalence of reproductive tract lesions in female jaguars from North American zoological institutions with emphasis on ovarian carcinoma, (2) identify significant associations of reproductive lesions in jaguars with known risk-factors in zoo felids such as progestin contraceptives, and (3) perform pedigree analysis to evaluate the prevalence of ovarian carcinoma and other neoplasia within jaguar family groups.

Materials and Methods

Study population and case selection

The Reproductive Health Surveillance Program (RHSP), in partnership with the Association of Zoos & Aquariums Reproductive Management Center (AZA-RMC), evaluates and archives reproductive tracts from zoo animals for disease surveillance. The RHSP has collected samples from 90 jaguars, including tissues and blood. Jaguar samples have been collected from 50 different zoological institutions, and include 71 females and 19 males. All tissues in this study are approved for use under IACUC exemption at Michigan State University. Reproductive tracts (uterus and/or both ovaries) and occasionally mammary glands were submitted by zoological institutions after ovariohysterectomy or necropsy from 59 female jaguars. 56 were chosen for inclusion in this study based on availability of hematoxylin & eosin slides and/or formalin-fixed tissues.

Three jaguars, one with mammary carcinoma, one with bilateral ovarian carcinoma, and one apparently healthy animal were excluded from the study because identifications could not be verified in the AZA North American Regional Jaguar Studbook. Jaguar reproductive tracts were collected between the years 1988-2018, from 38 different zoological institutions.

In some jaguars, the entire reproductive tract (uterus and both ovaries) was not available for examination. Jaguars were included in the study if the case had one or more of the following for histologic evaluation: Both ovaries, uterus, or mammary gland. In total, 49/56 (87.5%) jaguars had both ovaries available for histologic evaluation, and 54/56 (96%) had uterus available for histologic evaluation. Slides or tissues were available for only a subset of jaguars diagnosed with mammary carcinoma based on necropsy and histopathology records (11/18, 61%). Historical data were collected from all jaguars, including birth and death dates, cause of death, progestin contraceptive history (categorized as exposed or never exposed to MGA or other contraceptive), parity or nulliparity, number of lifetime litters, and interval between the last litter and death. In this study, 25 (44.6%) jaguars had previous exposure to MGA contraceptives, 24 (42.8%) were never exposed, and 7 (12.5 %) had unknown contraceptive history.

Histologic evaluation

Tissues were fixed in 10% neutral-buffered formalin by the submitting institution and representative sections of uterus (uterine body, both horns), ovary (one longitudinal section of both ovaries +/- oviduct), mammary gland (at least one section), and any gross lesions were paraffin-embedded, cut into 5 μm sections and stained with hematoxylin & eosin (HE) for histologic evaluation.

Uterine, mammary, and ovarian neoplasms were characterized according to the World Health Organization (WHO) histological classification of tumors of the genital system or mammary gland of domestic animals (Misdorp et al. 1999; Kennedy et al. 2007). Endometrial hyperplasia was categorized as described previously for zoo canids and felids (Munson et al. 2002; Moresco et al. 2009). Histologic criteria for endometrial hyperplasia grading are summarized in Appendix Table 2.5. Sections of uterus were also examined for adenomyosis, hydrometra, atrophy, inflammation, and mineralization. For ovarian carcinomas, several histologic features were assessed (Appendix Table 2.7). Mitotic count was calculated as the total number of mitoses within 10 high-powered (400x), random fields. Poorly cellular, cystic or hemorrhagic regions were avoided. Ovarian carcinomas were also assessed for the presence or absence of necrosis, hemorrhage, mineralization, lymphocytic inflammation, neutrophilic inflammation, and stromal invasion. Cytoplasmic and nuclear pleomorphism was assessed as mild, moderate, or marked based on degree of anisokaryosis and anisocytosis, presence of multinucleated cells, densely clumped or vesicular chromatin, and presence of one or multiple nucleoli. Criteria for malignancy included presence or absence of metastatic implantation, extension beyond the ovarian bursa, necrosis, hemorrhage, stromal invasion, mitotic count, and cytoplasmic or nuclear pleomorphism based on domestic animal and human literature (Kennedy et al. 2007; Kurman et al. 2014; Meinhold-Heerlein et al. 2016; Agnew and MachLachlan 2017). Other ovarian lesions assessed included ovarian cysts, interstitial gland hyperplasia, and ovarian surface epithelium hyperplasia.

Mammary carcinoma grading

Mammary carcinomas were classified according to WHO guidelines in domestic animals and categorized based on the predominant histologic patterns of tubular, papillary, solid, cribriform or cystic, and all predominant patterns were listed (Misdorp et al. 1999). Mammary carcinomas were assigned a grade of I, II, or III by the sum of the scores (1, 2 or 3) for percent tubule formation, mitotic count, and nuclear morphology as previously described (Elston and Ellis, 1991). This grading system is also used in zoo felids and has been reported to be of prognostic significance in domestic cats, but with a caution that more standardization is needed (Misdorp et al. 1999; McAloose et al. 2007; Zappulli et al. 2015). Mitotic counts were performed using one H&E section, and ten high-powered, random fields were assessed. Slides or tissues were available for only a subset of jaguars diagnosed with mammary carcinoma based on necropsy and histopathology records (11/18, 61%). A tumor grade was reported only for those cases in which slides were available from the primary tumor site (n=11).

Immunohistochemistry

Immunohistochemistry was performed at MSU Veterinary Diagnostic Laboratory according to established protocols. Due to variable time (years) in formalin of the archival samples and concurrent use of FFPE blocks for next-generation sequencing experiments, an initial pilot study was performed on ovarian carcinomas from three jaguars based on block availability and minimal time in formalin. Additional immunohistochemical studies are currently underway. Briefly, 5 μm sections from each block were deparaffinized in xylene and rehydrated in sequential concentrations of alcohol followed by rinsing in distilled water. General characteristics of each antibody are listed in Table 2.1.

Antigen retrieval was performed using the Bond Epitope Retrieval Solution 1 or 2 (ER1, ER2; Leica Biosystems, Buffalo Grove, IL) for calretinin and cytokeratin 7, respectively. IHC was performed on a BOND-MAX Automated Staining System (Leica Biosystems), and labeled with 3,3'-diaminobenzidine chromagen and hematoxylin counterstain. For ERα and PR, IHC was performed on a Ventana BenchMark XT automated immunostainer and Ventana iView detection kit (Ventana Medical Systems, Tucson, AZ). Domestic cat and jaguar tissues were used as positive controls for each marker to ensure antibody cross-reactivity in the jaguar and site-specificity in normal tissues.

Positive controls were as follows: iris, testis, brain (calretinin), lung (cytokeratin 7), domestic cat and jaguar uterus and ovary (cytokeratin 7, ERα, PR). Negative controls replaced the primary antibody with buffer. Immunoreactivity was characterized by staining intensity (weak, moderate, strong), percentage of positive cells, and location (cytoplasmic and/or nuclear). Immunoreactivity for all antibodies was assigned a score of 0, 1, 2, or 3 based on the percentage of positive neoplastic cells (0 = negative, 1 = <10% positive, 2 = 10–50% positive, 3 = >50% positive).

Table 2.1: General characteristics of antibodies used for immunohistochemistry pilot study of jaguar ovarian carcinomas

Antibody	Clone	Source	Dilution
Estrogen receptor α	Rabbit monoclonal SP1	Ventana Medical Systems, Tucson AZ	1:50
Progesterone receptor	Rabbit monoclonal 1E2	Ventana Medical Systems, Tucson AZ	1:50
Cytokeratin 7	Mouse monoclonal OV-TL 12/30	Agilent Technologies (Dako), Santa Clara CA	1:75
Calretinin	Mouse monoclonal	Agilent Technologies (Dako), Santa Clara, CA	1:100

Statistical analysis

Data for continuous variables were analyzed using Student's unpaired two-tailed *t*-test or nonparametric equivalent (Mann-Whitney test) when data were not normally distributed. Normality was assessed by the D'Agostino-Pearson test. Continuous numerical data were reported as means +/- standard deviation and median. Fisher's exact tests were used for categorical variables. Significance level was set at $P<0.05$, and all statistical analyses were performed using GraphPad Prism 7 (v7.0d).

For statistical analysis, only jaguars with complete MGA contraceptive history and parity information were included (n=49). For comparison of histologic findings with parity, MGA contraception, or concurrent histologic lesions, only those jaguars with both ovaries (n=40) or uterus (n=54) were included to confirm absence of lesions. Mammary glands were only submitted if disease was present, and were excluded from statistical analysis due to lack of jaguars with normal mammary glands for comparison. Endometrial hyperplasia was categorized as mild (Grade 1 or absent) or advanced (Grade 2 and 3). All other lesions were categorized as present or absent.

Results

Reproductive tracts from 56 female jaguars were collected between years 1988-2018 from 38 North American zoological institutions after ovariohysterectomy or necropsy. Jaguars were between 2-27 years of age (mean=16.2 +/- 4.9; median=16.5 years). The age, cause of death, contraceptive exposure history, tissues examined, and main reproductive lesions for all study jaguars are listed in Appendix Table 2.6.

Reproductive (ovarian, endometrial) or mammary carcinoma was the most common cause of death in this study population (n=25, 44%). Mammary carcinoma was the most common (n=15; 26%), followed by ovarian carcinoma (n=6, 10.7%) and endometrial carcinoma (n=4, 7.1%). Specific cause of death was not reported for 19 jaguars, but of these cases, 7 had ovarian carcinoma, 1 had concurrent ovarian and endometrial carcinoma, and 1 had mammary carcinoma. Other causes of death in this population, each occurring in a single jaguar, included septic peritonitis, trauma, hepatocellular carcinoma, cholangiocarcinoma, soft tissue sarcoma, mesothelioma, unspecified metastatic carcinoma, congestive heart failure, and chronic renal failure.

Figures 2.1 and 2.2 represent the two most complete pedigrees from this study population, summarizing the distribution of malignant reproductive or mammary neoplasia within family groups. Figure 2.1 includes 9 female jaguars with multiple reproductive carcinomas in 2 generations. Seven jaguars had ovarian carcinoma, including two pairs of siblings, and one dam and two offspring. In Figure 2.2, one dam and two offspring (half-siblings) had ovarian carcinoma.

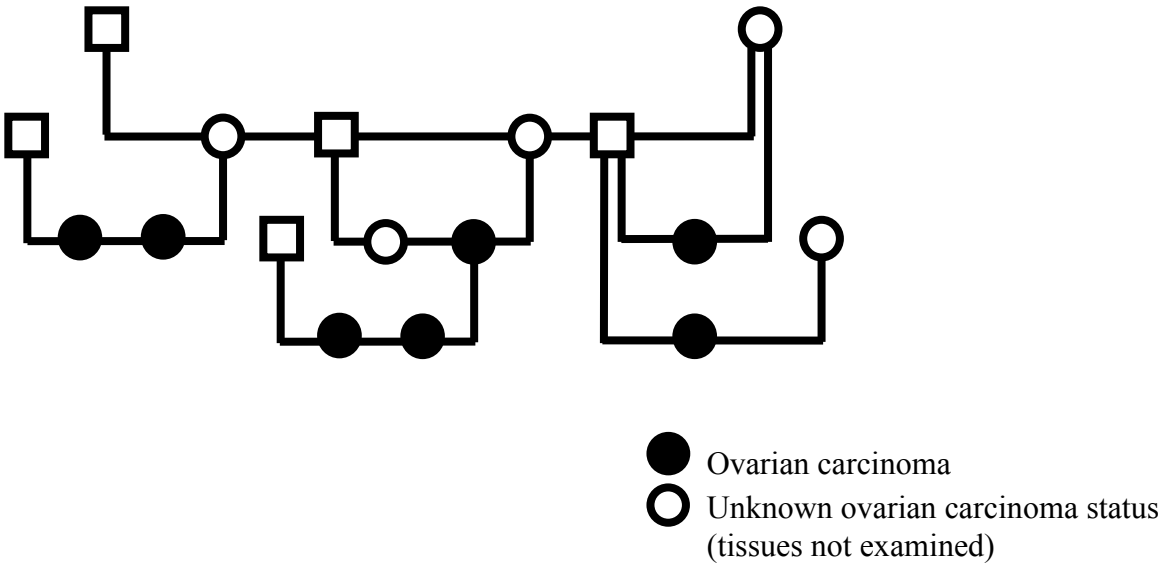

Figure 2.1: Pedigree of a subset of jaguars with ovarian carcinoma. This pedigree represents four generations. Jaguars labeled white have unknown ovarian lesion status (ovaries were not available for examination).

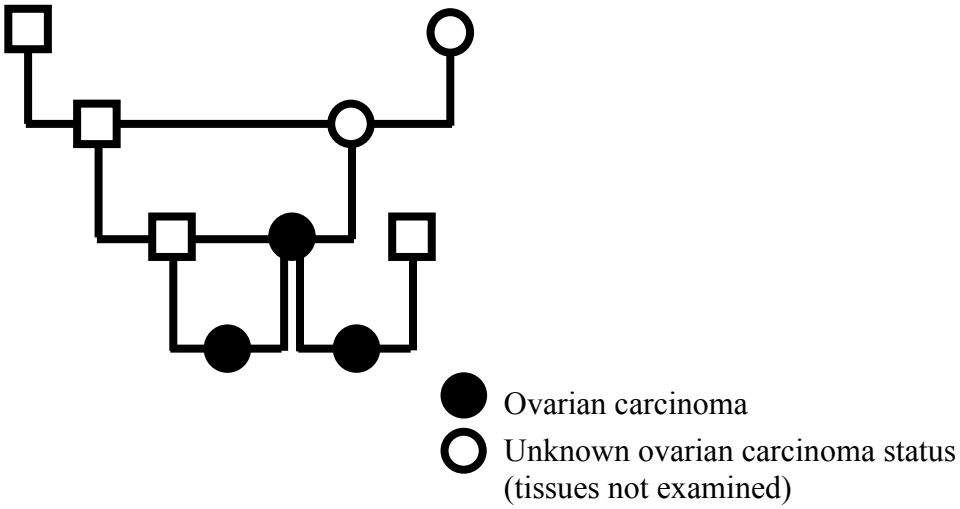

Figure 2.2: Pedigree of a subset of jaguars with ovarian carcinoma. This pedigree represents four generations. Jaguars labeled white have unknown ovarian lesion status (ovaries were not available for examination).

Ovary

Main histologic findings and prevalence of ovarian lesions in 49 jaguars are summarized in Table 2.2.

Table 2.2: Histologic lesions of the ovary in North American zoo jaguars (n=49)

Lesion	Number affected (%)
Carcinoma (papillary cystadenocarcinoma)	26 (53%)
Cystadenoma	1 (2%)
Sex cord-stromal tumor	2 (4%)
Leiomyoma	4 (8.2%)
Metastatic Neoplasia	5 (10%)
Endometrial carcinoma	2 (4%)
Mammary carcinoma	3 (6.1%)
Interstitial gland hyperplasia with luteinization	7 (14.2%)
Ovarian surface epithelium hyperplasia	9 (18.3%)
Cystic rete ovarii	14 (28.5%)

The most prevalent lesion was ovarian carcinoma. Of the 49 jaguars with both ovaries available for examination, 26 had ovarian carcinoma (53%). Age distribution of affected jaguars is depicted in Figure 2.4. The mean age at death, which is equivalent to the age at diagnosis, of jaguars with ovarian carcinoma (n=26) was 17 years (+/-4.73; median = 19 years), and age at death of those without ovarian carcinoma (n=17) was 15.5 years (+/- 4.7; median = 17 years). Mean age at death was not significantly different between jaguars with and without ovarian carcinoma (P=0.25). In jaguars without ovarian carcinoma 8 jaguars had either mammary or endometrial carcinoma. Of those animals with ovarian carcinoma, 8 also had mammary carcinoma. All jaguars with endometrial or mammary carcinoma died at a significantly (P=0.01) younger age (n=24; mean 15.21 years +/- 4.5; median=15) than those with ovarian carcinoma alone (n=15; mean 18.8 years +/- 3.5; median=20).

Ovarian carcinomas were bilateral in all cases, and 8 (31%) also had metastasis by peritoneal implantation. No metastases to distant organs were reported. Grossly, neoplasms were composed of multiple, variably-sized cystic, papillary, and fewer solid masses that replaced and obscured both ovaries and extended to the surrounding mesovarium, mesosalpinx, mesometrium, and uterine serosa (Figures 2.3 and 2.7-5). Histologically, neoplastic cells were arranged in papillary projections or cords among variable amounts of fibrous stroma (Figure 2.3). Stromal invasion was present in all cases. Cells were cuboidal to columnar, and in single or multiple layers in some areas. Nuclei were oval to round with dense to vesicular chromatin. Other major histologic findings of all 26 ovarian carcinomas are listed in Table 2.8. Mitotic counts (total number of mitoses within 10 HPF) were low overall and ranged from 2-15 (mean 5.0 +/- 3.31). Seven neoplasms had areas of necrosis (27%) with neutrophilic inflammation, and 18 (69%) had mild stromal lymphocytic inflammation. Multifocal hemorrhage was present in 14 (54%) neoplasms. Mineralization was also common, and was observed in 18 (69%) neoplasms. Cytoplasmic and nuclear pleomorphism was mild in the majority of cases (n=19; 73%). Few neoplasms had moderate (n=3; 11.5%) or marked (n=1; 3.8%) pleomorphism (Appendix Figure 2.10-2).

A collision tumor was present in one case, with ovarian carcinoma and metastatic carcinoma present in the same section (Appendix Figure 2.10-1). In total, 5 jaguars (10%) had metastasis of endometrial or mammary carcinoma to the ovary.

Of jaguars with ovarian carcinoma, 11 (35%) had MGA contraceptive exposure, and 13 jaguars had MGA exposure without ovarian carcinoma (76%). Three jaguars with ovarian carcinoma were removed from statistical analysis due to unknown contraceptive status. MGA contraceptive exposure was not significantly different between jaguars with and without ovarian carcinoma (P=0.1; Figure 2.5). There were also no statistically significant differences in parity (P=0.31), lifetime number of litters (P=0.055), or time interval between last litter and death (P=0.18).

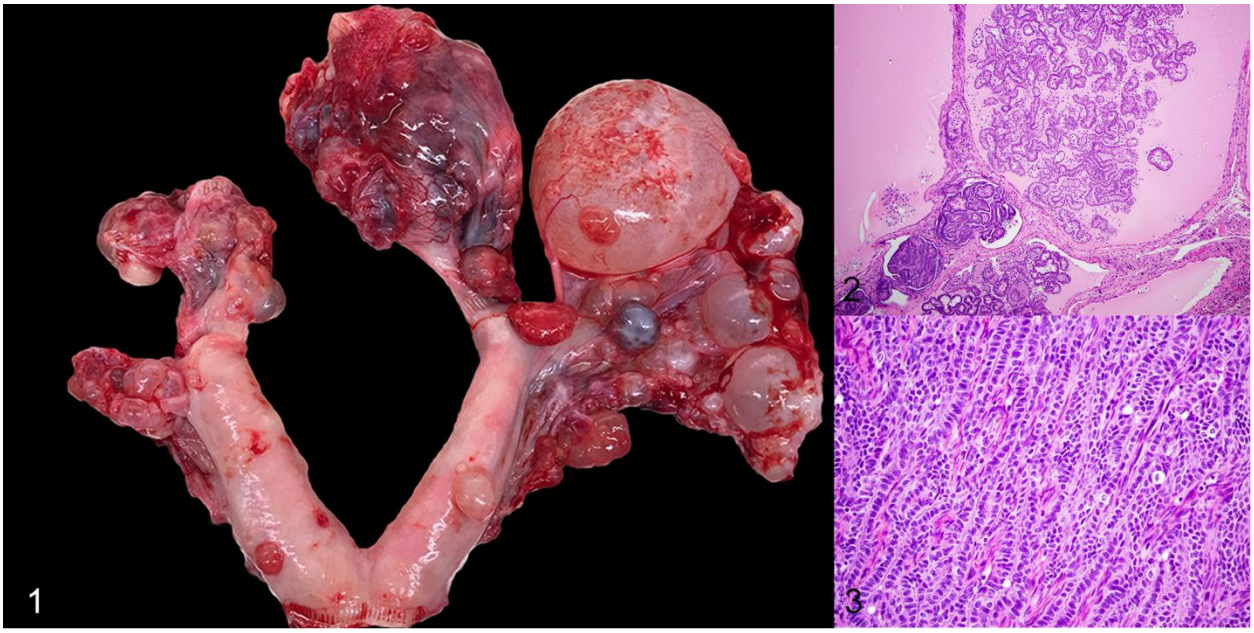

Figure 2.3: Ovarian carcinoma, jaguar (*Panthera onca*). (1) A multinodular neoplasm with cystic and solid regions bilaterally effaces both ovaries and extends into the mesovarium, mesosalpinx, mesometrium, and uterine perimetrium. (2) Neoplastic cells are arranged in papillary projections that extend into variably-sized cystic spaces filled with eosinophilic fluid. (3) Neoplastic cells in solid regions are arranged in tightly-packed cords with columnar epithelium and oval nuclei with minimal anisocytosis and anisokaryosis. Hematoxylin & Eosin.

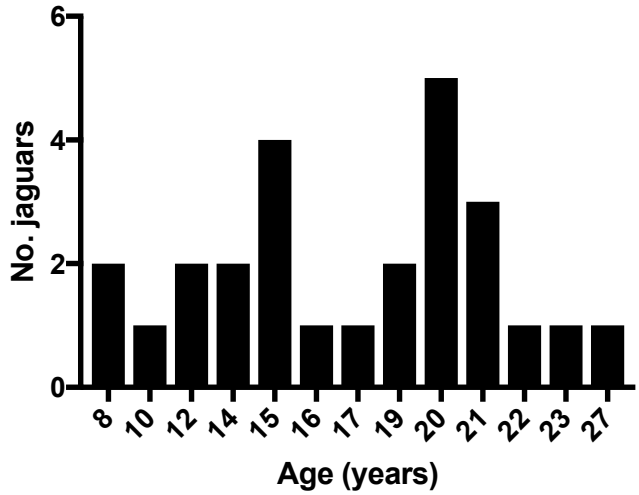

Figure 2.4: Age distribution of jaguars diagnosed with ovarian carcinoma (Range = 8-27 years). All jaguars were diagnosed at or near the time of death (Mean age =17.08 +/- 4.7 years; Median = 19 years).

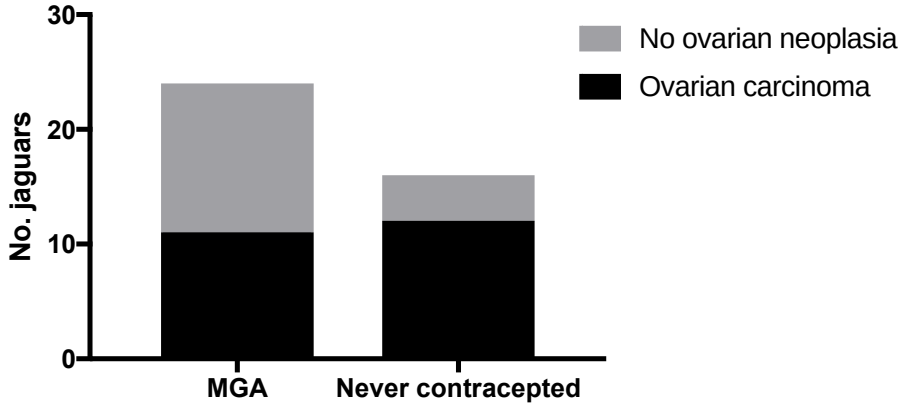

Figure 2.5: Number of jaguars ever exposed to progestin contraceptives (MGA) or never contracepted in jaguars without ovarian carcinoma (n=17) compared to those with ovarian carcinoma (n=23). Prevalence of ovarian carcinoma was similar between jaguars with previous MGA exposure and those without (*P*=0.1).

Immunohistochemistry was performed on three ovarian carcinomas as a pilot study (Case Nos. 7, 21, 40) from three recently acquired cases (Figure 2.4). Formalin-fixation in general can mask antigen detection due to cross-linking of amino acids, and prolonged formalin fixation additionally can result in excessive background staining and poor antigen detection (Ramos-Vara 2005). Since many of these tissues were fixed in formalin for prolonged periods (up to 30 years), a subset of neoplasms from more recently acquired cases were chosen to first ensure site specificity and species cross-reactivity. All three jaguar ovarian carcinomas in this pilot study had positive nuclear immunoreactivity for estrogen receptor α (Immunoreactivity score = 3, weak to strong staining intensity) and progesterone receptor (Immunoreactivity score = 2, weak to strong staining intensity), but overall had a lower percentage of progesterone-positive neoplastic cells (30-40%) compared to ERα (>50%). All ovarian carcinomas also had positive cytoplasmic immunoreactivity for cytokeratin 7 (Immunoreactivity score = 3, strong staining intensity). Ovarian carcinomas were negative for calretinin.

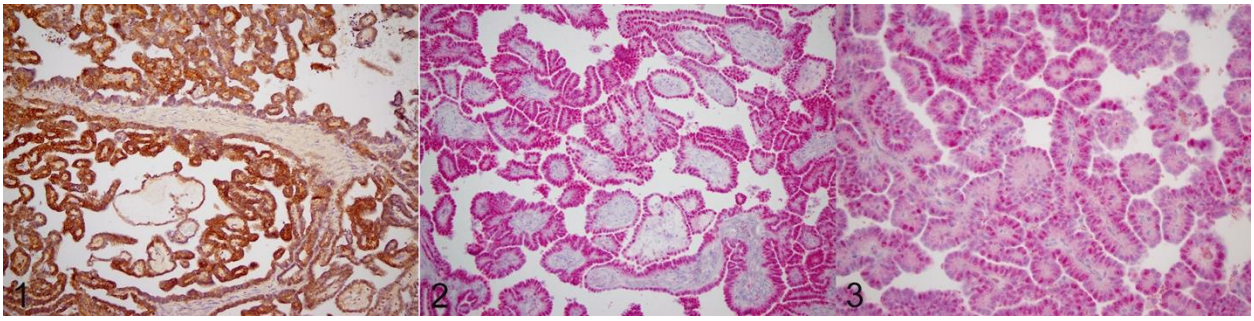

Figure 2.6: Ovarian carcinoma, jaguar (*Panthera onca*). Neoplastic cells have positive cytoplasmic immunoreactivity for cytokeratin 7 (1), and nuclear immunoreactivity for estrogen receptor α (2) and progesterone receptor (3).

One jaguar had a benign ovarian epithelial neoplasm extending from the ovarian surface epithelium, and was classified as cystadenoma (Appendix Figure 2.10-4). This neoplasm was unilateral, approximately 1 cm diameter, and cystic with papillary projections of neoplastic cells extending into the cystic space. No mitoses were observed, and cells had minimal anisocytosis and anisokaryosis.

Two jaguars (4%) had neoplasms categorized as sex cord-stromal tumors. One neoplasm was a unilateral, well-demarcated, round, focal, approximately 2 cm diameter mass that compressed the adjacent ovarian cortex. This neoplasm was composed of cells with an abundant, pale eosinophilic, finely granular cytoplasm and round, vesicular nuclei, and were of luteinized morphology. Some cells also had intracytoplasmic vacuoles. Contraceptive history of this jaguar was unknown. The second jaguar had diffuse infiltration of the ovarian stroma with cells ranging from luteinized, spindloid, to granulosa cell morphology with numerous Call-Exner bodies. This neoplasm had marked anisocytosis and anisokaryosis, and numerous mitotic figures (Appendix Figure 2.11-8,9). This jaguar also had ovarian carcinoma, and was never treated with progestin contraceptives.

Four jaguars (8.2%) had ovarian medullary leiomyomas. These neoplasms were well-demarcated and composed of interlacing fascicles and streams of smooth myocytes (Appendix Figure 2.10-5). Cystic rete ovarii were common, occurring in 14 (28.5%) jaguars (Appendix Figure 2.10-6), and were associated with mild compression of the ovarian cortex in some cases. Cysts were located within the ovarian hilus and variably sized. Cysts were lined by a single layer of cuboidal to attenuated epithelium. Cystic rete ovarii occurred commonly regardless of MGA exposure status ($P>0.05$). Segmental ovarian surface epithelium (OSE) hyperplasia was present in 9 (18.3%) jaguars (Appendix Figure 2.10-3).

Cells had cytoplasmic hypertrophy, were cuboidal to columnar and piled in some areas. Ovarian cortical and medullary interstitial gland hyperplasia with luteinization was present in 7 jaguars (14.2%, Appendix Figure 2.11). Nests of interstitial gland cells were increased in number and had marked cytoplasmic and nuclear pleomorphism, and abundant, eosinophilic, finely granular to vacuolated cytoplasm. Cells had positive cytoplasmic and nuclear immunoreactivity for calretinin and PR (Appendix Figure 2.11-5,6). OSE hyperplasia and interstitial gland hyperplasia with luteinization were present in jaguars regardless of ovarian carcinoma or MGA contraceptive exposure status (P=>0.05).

Uterus

Main histologic findings and prevalence of uterine lesions in 54 jaguars are summarized in Table 2.3.

Table 2.3: Histologic lesions of the uterus in North American zoo jaguars (n=54)

Lesion	**Number affected (%)**
Endometrial carcinoma	6 (11%)
Endometrial hyperplasia	33 (61%)
Mild (Grade 1)	12 (22%)
Moderate (Grade 2)	8 (14.8%)
Severe (Grade 3)	13 (24%)
Adenomyosis	15 (27.7%)
Leiomyoma	21 (38.8%)
Hydrometra	7 (12.9%)
Endometrial atrophy	5 (9.2%)
Endometrial mineralization	16 (29.6%)
Neutrophilic endometritis/Pyometra	8 (14.8%)

The most prevalent uterine lesion was endometrial hyperplasia (n=33; 61%). Lesions consisted of a combination of cystic and adenomatous hyperplasia, along with surface epithelium hyperplasia (Figure 2.7, 1-3). Mild endometrial hyperplasia (n=12; 22%) consisted of few, randomly scattered cystic glands and adenomatous hyperplasia with multifocal piling of cells 2-3 layers thick. Most jaguars (n=21; 63%) with endometrial hyperplasia were categorized as advanced (Grade 2 or 3). Advanced hyperplasia consisted of a markedly thickened endometrium with moderate to marked cystic and adenomatous hyperplasia, irregular, papillary projections of branching glands, and multifocal stromal fibrosis. Cells were cuboidal to columnar with vesicular nuclei, and moderately pleomorphic. MGA exposure was not significantly different between jaguars with grade 1 endometrial hyperplasia and without (P=0.37). In contrast, all jaguars with advanced endometrial hyperplasia had previous MGA contraceptive exposure. Endometrial mineralization occurred in 16 (29.6%) jaguars, and was always associated with advanced endometrial hyperplasia lesions.

Six jaguars (11%) had endometrial carcinoma, and all six also had concurrent advanced (grade 2 or 3) endometrial hyperplasia (Figure 2.7-6). Neoplasms were composed of epithelial cells arranged in irregular, branching glands, tubules, or nests within a dense fibrous stroma that obscured and invaded the myometrium and perimetrium. Neoplasms had numerous mitoses and moderate anisocytosis and anisokaryosis, and large regions of necrosis. Mean age at death for jaguars with endometrial carcinoma was 15.5 years (+/- 5.54; median 14 years). One additional jaguar had carcinoma invading the uterus, but was classified as metastatic mammary carcinoma based on histologic appearance. Two jaguars had metastatic endometrial carcinoma, including to the ovary. Of those animals with endometrial carcinoma, all but one had previous MGA exposure (n=5; 83%). The remaining animal had unknown contraceptive status.

Leiomyomas were the second most prevalent uterine lesion in this population (n=21; 38.8%). Leiomyomas were variably-sized with multiple neoplasms present in most cases. Uterine leiomyomas were common in jaguars with and without progestin contraceptive exposure (*P*=0.13), and in both parous and nulliparous animals (*P*=>0.99).

Adenomyosis was a common finding in jaguars with and without endometrial hyperplasia (*P*=>0.05), and was not associated with MGA exposure (*P*=0.74). Adenomyosis was characterized by few, randomly scattered, individual or small clusters of endometrial glands and scant endometrial stroma within the myometrium. Endometrial atrophy in all cases occurred concurrently with hydrometra (Figure 2.7-4). Endometrial stroma and glands were circumferentially compressed and flattened with mild stromal fibrosis. Hydrometra was characterized by distension of the uterine lumen by eosinophilic, homogenous fluid without concurrent inflammation. Hydrometra was not significantly associated with MGA exposure (*P*=0.38).

Neutrophilic endometritis and pyometra was present in 8 jaguars (14.8%), and were not associated with MGA exposure (*P*=0.11) or advanced hyperplasia (*P*=0.23). These lesions were categorized as any neutrophilic inflammation within the uterine lumen and/or endometrium.

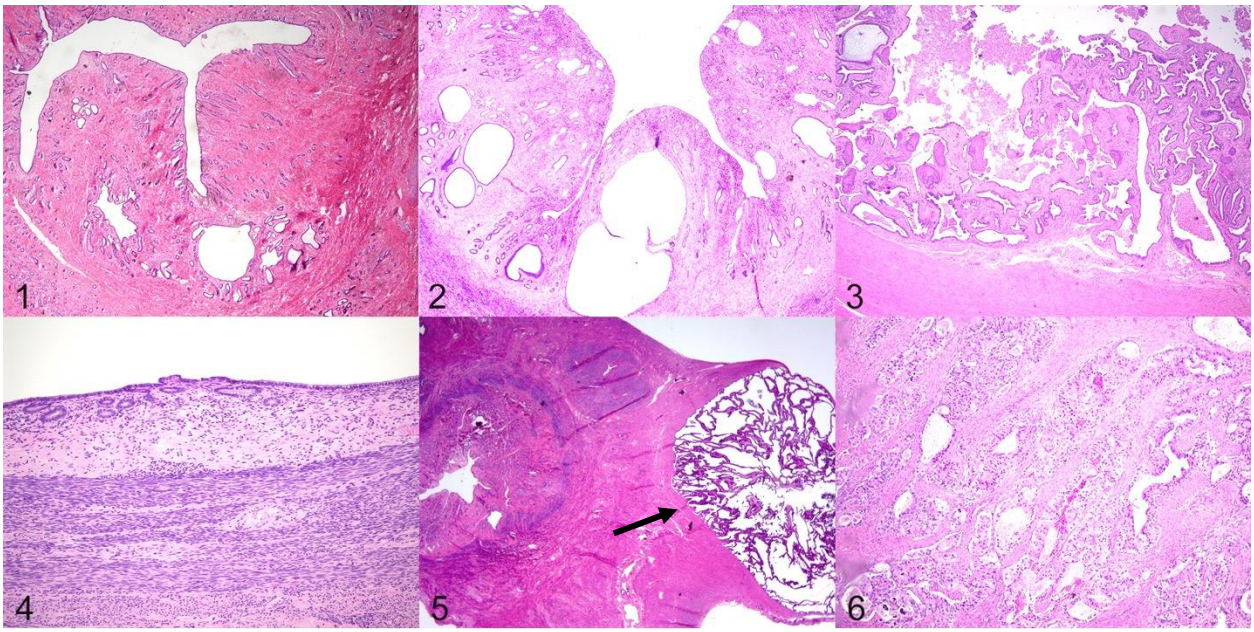

Figure 2.7: Uterus, jaguar (*Panthera onca*), Hematoxylin & Eosin. (1) Endometrial hyperplasia, grade I. Few randomly scattered, cystic glands. (2) Endometrial hyperplasia, grade II. Thickened endometrium with numerous cystic glands throughout all layers. (3) Endometrial hyperplasia, grade III. Severe adenomatous and cystic hyperplasia with papillary projections and mineralization. (4) Endometrial atrophy and mild fibrosis secondary to hydrometra. (5) Metastatic implantation of ovarian carcinoma to the uterine perimetrium (arrow). Neoplastic cells are arranged in cystic spaces with numerous papillary projections. (6) Endometrial carcinoma. Neoplastic cells are arranged in irregular, branching glands that invade and obscure the myometrium.

Mammary Gland

Mammary glands were primarily only submitted to the RHSP if mammary carcinoma was present. Therefore, the prevalence of other mammary gland non-neoplastic lesions, such as hyperplastic or inflammatory lesions, could not be assessed in this jaguar population. In this study, 18 (32%) jaguars had mammary carcinoma. Mean age at death for jaguars with mammary carcinoma was 15.11 years (+/- 4.4; median 15.5 years). Eleven cases were confirmed by histopathology, and the remaining were diagnosed based on necropsy and histopathology reports. Histologic characteristics and grade of the 11 cases for which tissues were available are summarized in Table 2.4. Most neoplasms had a combination of multiple histologic subtypes,

most commonly tubular, papillary, and cystic patterns (Figure 2.8). Two neoplasms also had a solid pattern, and 3 had a cribriform pattern. The cribriform pattern consisted of closely apposed neoplastic cells arranged in cords lining small, linear (duct-like) spaces rather than discrete tubules. Most mammary carcinomas were Grade II (n=7; 63%) or III (n=2; 18%). Two (18%) were classified as Grade I, and these animals did not have metastatic disease. Of the 18 mammary carcinomas, 11 were metastatic, 3 did not have evidence of metastasis, and the remaining cases were unknown. The most common sites of metastasis were the liver, lung, and lymph nodes. Other sites of metastasis included the kidney, uterus, ovary, brain, and omentum.

Statistical analyses were not performed on mammary carcinomas due to lack of unaffected jaguars for comparison, however 13 of 18 jaguars (72%) had previous MGA contraceptive exposure. Three additional cases had unknown MGA status (16.6%), and two (11%) had never been exposed to exogenous progestins.

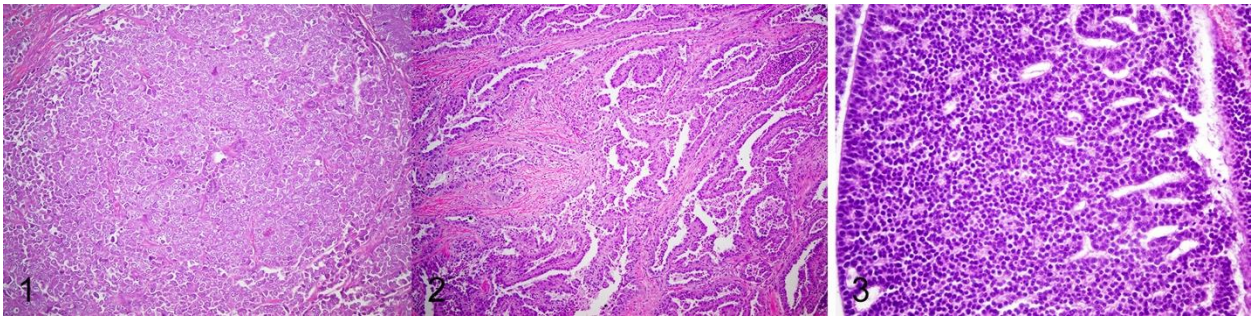

Figure 2.8: Mammary carcinoma, jaguar (*Panthera onca*), Hematoxylin & Eosin. (1) Solid carcinoma, grade II. (2) Papillary carcinoma, grade II. (3) Cribriform carcinoma, grade III.

Table 2.4: Jaguar mammary carcinoma (n=11). Morphologic characteristics, scores of major histologic features, overall grade, progestin contraceptive history and presence of metastases.

Case No.	Progestin (MGA) contraceptive exposure	Predominant histologic patterns	% Tubule formation score	Mitotic count score	Nuclear morphology score	Grade	Metastasis
2	Y	Tubular, papillary, cystic	2	2	2	II	Y; Liver, ovary
6	Unk	Tubular, papillary, cystic	2	1	2	I	N
12	Y	Tubular, papillary, cystic	2	2	2	II	N
15	Y	Papillary, cystic	3	3	3	III	Y; Lymph node, brain, lung, uterus
20	Y	Solid	3	2	2	II	Y; Liver, kidney, lung, lymph node
24	Y	Tubular, cystic, cribriform	2	2	2	II	Unk
26	Y	Solid	3	2	2	II	Unk
31	N	Tubular, papillary	2	3	3	III	Y; Liver, kidney, lung, uterus, omentum
33	Y	Tubular	1	2	2	I	N
38	Unk	Cribriform, cystic, tubular	2	3	2	II	Unk
53	Unk	Cribriform, cystic, papillary	3	2	1	II	Unk

Summary of reproductive tract and mammary gland neoplasia prevalence

Overall, 42 (75%) jaguars had at least one type of benign or malignant neoplasia in this study population (Figure 2.9). Ovarian carcinoma was the most common (n=26), followed by uterine leiomyoma (n=21), mammary carcinoma (n=18), endometrial carcinoma (n=6), ovarian sex cord-stromal tumor (n=2), and ovarian cystadenoma (n=1). Most jaguars (n=24) had more than one type of neoplasia. Of the malignant neoplasia, concurrent ovarian carcinoma and

mammary carcinoma was diagnosed in 10 jaguars. One jaguar had both ovarian carcinoma and endometrial carcinoma, and one additional jaguar had ovarian carcinoma and malignant ovarian sex cord-stromal tumor.

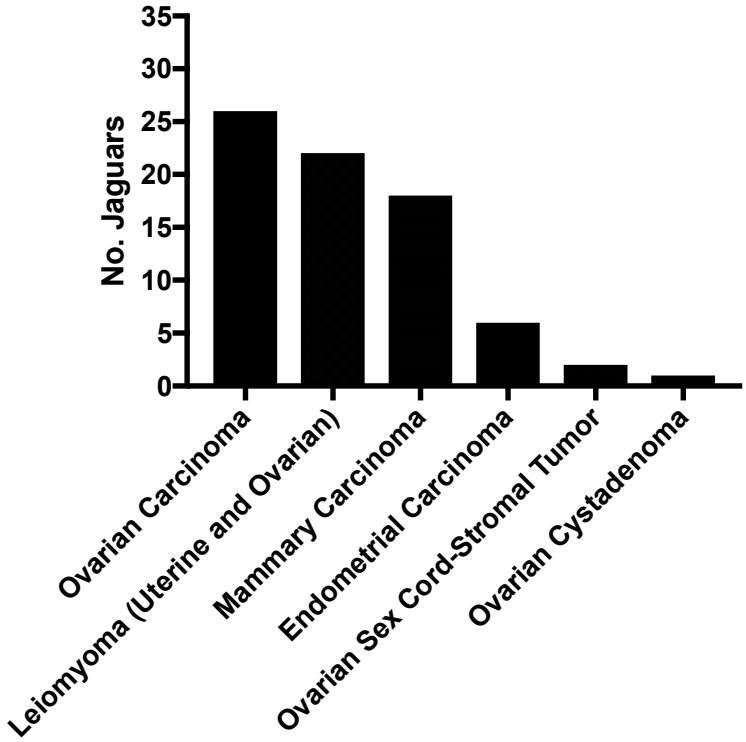

Figure 2.9: Prevalence of reproductive tract or mammary gland neoplasia in the jaguar study population (n=56). Ovarian carcinoma was the most common (n=26), followed by uterine leiomyoma (n=21), mammary carcinoma (n=18), endometrial carcinoma (n=6), ovarian sex cord-stromal tumor (n=2), and ovarian cystadenoma (n=1).

Discussion

In this retrospective analysis of reproductive tracts and mammary glands from 56 jaguars, malignant neoplasia (including endometrial carcinoma, mammary carcinoma, and ovarian carcinoma) was highly prevalent, and was also the most common cause of death in this study population. In a retrospective study of morbidity and mortality in zoo jaguars, reproductive diseases were the most commonly reported cause of death in adult female jaguars (Hope and Deem 2006). Notably, ovarian carcinoma was also highly prevalent in jaguars, and was the cause of death in 6 of 26 (23%) animals due to peritoneal implantation. Among the 14 additional felid species with archived reproductive tissue at the RHSP, ovarian carcinoma was found only in jaguars, and has never been reported in other zoo or wild felids, to our knowledge (Agnew and Moresco, unpublished data). Histologically, these neoplasms were composed of cystic, papillary, and solid regions, similar to ovarian carcinoma reported in dogs and serous ovarian carcinomas in women (Kennedy et al. 2007; Kurman et al. 2014).

Criteria for malignancy in dogs and humans include extension beyond the ovarian bursa, high mitotic count, stromal invasion, necrosis, hemorrhage, and peritoneal implantation (Kennedy et al. 2007; Agnew and MacLachlan 2014; Kurman et al. 2014). In humans, malignant ovarian epithelial neoplasms are divided into low and high-grade serous carcinomas based on morphologic characteristics and somatic mutation profiles (Kurman et al. 2014). Low-grade serous carcinomas are less common and have a more uniform cellular morphology and fewer mitoses than high-grade carcinomas, and are associated with somatic mutations in KRAS and BRAF oncogenes (Kurman et al. 2014). Benign cystadenomas arise from the ovarian surface epithelium or inclusion cysts in humans, and can progress to low-grade carcinomas (Lim and Oliva 2013). Only one jaguar in this study had a benign epithelial ovarian neoplasm, classified as

a cystadenoma. OSE hyperplasia was present in 9 jaguars, but was not associated with ovarian carcinoma cases, and thus was not interpreted as a possible precursor lesion in jaguars. The OSE can also proliferate in response to stimuli such as ovulation (Kennedy et al. 2007).

High-grade serous carcinomas have a high mitotic count, marked pleomorphism, and are associated with somatic p53 mutations, and are the most common type associated with hereditary ovarian cancer (Kurman et al. 2014). All jaguar ovarian carcinomas had malignant characteristics of stromal invasion, with a low to moderate mitotic count and mild to moderate pleomorphism, with few neoplasms exhibiting a higher mitotic count and more pleomorphic neoplastic cells (Appendix Table 2.7).

In dogs, ovarian carcinomas arise from the ovarian surface epithelium (OSE) or subepithelial structures (Agnew and MacLachlan 2014). In humans, ovarian carcinoma was previously thought to arise from the OSE, but more recently, a precursor lesion has been identified in the fallopian tube, with a similar mutation profile as high-grade serous ovarian carcinoma, and is now considered the site of origin. (Labidi-Galy et al. 2017). In jaguar cases where oviduct was available for examination (n=20), no lesions were observed, other than ovarian carcinoma implanted on the serosal surface and surrounding mesosalpinx.

Jaguar ovarian carcinomas all had positive immunoreactivity for ERα, PR, and cytokeratin 7, similar to humans (Kurman et a. 2014). Ovarian carcinomas in humans are usually ERα positive, and fewer are PR positive. Prognostic significance of hormone receptor expression in ovarian carcinoma is not as well-characterized as in breast cancer, but few studies have shown that PR expression in human serous ovarian carcinoma is associated with better prognosis, as endogenous progesterone is associated with decreased risk of ovarian cancer (Sieh et al. 2013).

Cytokeratin 7 is a low molecular weight intermediate filament that is commonly found in epithelium of the breast, ovary, lung, and urothelium but not stratified squamous or gastrointestinal epithelium, and ovarian carcinomas are usually CK7 positive in humans and dogs (Chu et al. 2000; Cathro and Stoler 2002, Riccardi E et al. 2007). Jaguar ovarian carcinomas were also calretinin negative. Calretinin is a calcium binding protein that is found in locations such as neurons and stromal cells of the ovary (Deavers et al. 2003; Yaziji H et al. 2006). Ovarian sex cord-stromal tumors and mesotheliomas in general are usually immunoreactive for calretinin, and ovarian epithelial neoplasms are usually negative (Deavers et al. 2003; Mittal and Soslow 2008).

Jaguars had high prevalence of ovarian carcinoma regardless of MGA contraceptive exposure, unlike the significant association of mammary and endometrial carcinoma with MGA contraceptives in zoo felids, including jaguars in this study (Munson et al. 1995; Harrenstein et al. 1996; McAloose et al. 2007). The occurrence of ovarian carcinoma independent of MGA exposure is consistent with findings in other studies with fewer jaguars (Munson 1994; Kazensky et al. 1998). In humans, the role of exogenous and endogenous estrogen and progesterone in the pathogenesis of ovarian cancer is not as well-characterized as for breast cancer. For example, combined estrogen and progesterone hormone replacement therapy in postmenopausal women has been shown to both increase and decrease the risk of ovarian cancer in different studies, and combined estrogen-progesterone oral contraceptive use in premenopausal women is protective (Ho 2003; Havrilesky et al. 2013). Endogenous estrogen is a risk factor for ovarian carcinoma, supported by evidence that any event that decreases the lifetime number of ovulations, such as parity, is protective, and increased number of lifetime ovulations, such as early menarche or late menopause increase the risk of ovarian cancer (Lukanova and Kaaks 2005; Brown and

Hankinson 2015). Estrogen is a mitogen and stimulates cell proliferation by activation of growth factors, such as insulin-like growth factor and epidermal growth factor, which can act as oncogenes (Skandalis et al. 2014). In contrast, endogenous progesterone decreases the risk of ovarian cancer in women, inducing apoptosis in ovarian cancer cells (Ho 2003; Hee Han et al. 2013).

Family history of ovarian cancer is a well-known risk factor in humans (Lynch et al. 2009). In dogs, there is no known breed predisposition to ovarian cancer (Dobson 2013). The jaguar pedigrees in this study demonstrate multiple occurrences of ovarian cancer within two generations, with a dam and both female offspring affected in some cases. This is similar to the autosomal dominant pattern of inheritance of germline mutations in hereditary ovarian and breast cancer in women (Petrucelli et al. 2010). In humans, hereditary ovarian cancer accounts for a small percentage of cases, and are often associated with mutations in tumor suppressor genes associated with DNA repair, most commonly BRCA1 and BRCA2 (Lynch et al. 2009). Since ovarian carcinoma does not fit the typical association with MGA exposure as other neoplasms in zoo felids, Munson et al. hypothesized that the pathogenesis of ovarian carcinoma in jaguars may also involve a genetic predisposition, such as a germline mutation in a tumor suppressor gene (Munson and Moresco 2007). Studies are currently underway in our laboratory to elucidate the genetic pathogenesis of ovarian carcinoma in jaguars (Corner et al. 2017).

In women, nulliparity is a risk factor for ovarian cancer, and parity is considered protective, increasing with each live birth (Lukanova et al. 2005, Vachon et al. 2001). In jaguars, there was no statistically significant difference in parity, lifetime number of litters, or time interval between last litter and death between jaguars with and without ovarian carcinoma.

However, management decisions and other factors also play a role in breeding decisions overall, including the age at which a jaguar stops breeding.

Ovarian carcinoma is most often diagnosed in postmenopausal women (Duska et al. 1999, Vachon et al. 2001, Wright et al. 2009). In dogs, median age of ovarian carcinoma diagnosis was 10 years (Patnaik and Greenlee, 1987). The average age of jaguars with ovarian carcinoma was also in the adult to geriatric range (mean = 17 years +/-4.73; median = 19 years), but nearly half of jaguars (n=11) with ovarian carcinoma were still considered of breeding age (≤ 15 years). Jaguars in captivity historically have produced litters between the ages of 2-15 years, with a mean and median age of dam at first litter of 3.9 years and 4.7 years, respectively (S. Johnson, AZA North American Regional Jaguar Studbook), The youngest jaguar with ovarian carcinoma was 8 years old, though this jaguar was euthanized due to concurrent mammary carcinoma. Since most ovarian carcinoma cases were diagnosed at or near the time of death, the initial onset of disease and potential effects on reproduction remain unknown. Three jaguars with ovarian carcinoma had a prior history of prolonged reproductive cycling abnormalities such as lack of return to estrus, complete lack of behavioral estrus, and prolonged nulliparity when housed with a breeding male. There was also no statistically significant difference between the litter size in jaguars with and without ovarian carcinoma.

Jaguars have litter sizes between 1 and 4 offspring, and jaguars from the North American zoo population most commonly have small litter sizes of 1-2 offspring (S. Johnson, AZA North American Regional Jaguar Studbook). In women, any history of infertility is also a risk factor for ovarian carcinoma (Reid et al. 2017). Evaluating the relationship between ovarian carcinoma and clinical effects on reproduction in the jaguar population is also difficult to assess due to the high prevalence of concurrent reproductive diseases, including neoplasia and endometrial hyperplasia.

Although not significantly different, the younger age at death in those animals without ovarian carcinoma compared to jaguars with ovarian carcinoma may be due to the presence of endometrial and mammary carcinoma in some animals without concurrent ovarian carcinoma. Jaguars died at a significantly younger age (P=0.01) with endometrial and mammary carcinoma than to those with ovarian carcinoma and no other cancers. In large felids, including jaguars in this study, mammary carcinoma is aggressive and often associated with widespread metastasis (Harrenstein 1996; McAloose 2007; Munson and Moresco 2007).

Ovarian interstitial gland hyperplasia was present in 7 jaguars (14.2%). Cells had marked cytoplasmic and nuclear pleomorphism and had a luteinized morphology, which has not been described in other zoo felids, to our knowledge. Cells were positive for calretinin and PR in one case, indicative of sex cord-stromal origin and potential responsiveness to progesterone. Examples of histologically normal interstitial gland cells in other felids are depicted in Appendix Figure 2.11, 1-3. Many species have variable amounts of cortical interstitial glands that change in morphologic appearance under hormonal influence and at various stages of ovulation and throughout gestation, although their function remains largely unknown (Mossman and Duke, 1973; Perez et al. 1999). In humans, ovarian stromal hyperplasia and hyperthecosis is a condition characterized by nodular or diffuse proliferation of luteinized stromal cells in the ovary that is associated with clinical signs related to elevated androgen exposure (Kurman et al. 2014, Veras et al. 2014). The clinical significance of interstitial gland hyperplasia in jaguars is unknown. In one jaguar, there was diffuse infiltration throughout the ovary, and this case was classified as a sex cord-stroma tumor due to the high number of mitoses, cellular pleomorphism, and replacement of normal follicular structures. One additional sex cord-stromal tumor was present in one jaguar, which was a discrete nodule composed of luteinized sex cord-stromal cells.

We chose to leave these diagnoses using the broad sex cord-stromal tumor terminology, as these neoplasms can be derived from many cell types and can be difficult to differentiate (Agnew and MacLachlan 2017).

Cystic rete ovarii was the most common non-neoplastic ovarian cystic lesion found in jaguars. Cystic rete ovarii is also common in both zoo felids and domestic cats (Gelberg et al. 1984; Kazensky et al. 1998; Kennedy et al. 2007). This lesion was not associated with MGA contraceptives in this study, consistent with findings in other zoo felids (Kazensky et al. 1998). The only lesion found concurrently with rete cysts was mild compression of the ovarian cortex in some cases. Otherwise, this lesion was presumed to be incidental in jaguars.

Advanced endometrial hyperplasia, endometrial carcinoma, and mammary carcinoma were all highly prevalent in this jaguar population, and associated with MGA contraceptive exposure similar to previous reports in zoo felids and domestic dogs and cats (Hayden et al. 1989; Munson et al. 1995; Selman et al. 1995; Harrenstein et al. 1998; Munson et al. 2002; McAloose et al. 2007; Keskin et al. 2009). The molecular mechanisms of exogenous progestin exposure and effects on target tissues in zoo felids and domestic animals are poorly understood. In zoo felids, MGA does not suppress folliculogenesis or ovulation (Kazensky et al. 1998; Munson et al. 2006). Progesterone-only contraceptives in women act primarily at the level of the uterus and cervix to prevent implantation, with inconsistent suppression of ovulation, but do not have an association with increased risk of breast cancer like the combined estrogen-progesterone contraceptives (Samson et al. 2016).

In zoo felids, the combined exposure to a potent exogenous progestin (MGA) that stimulates endometrial and mammary gland proliferation along with simultaneous influence by endogenous

estrogen and progesterone may play a causal role in lesion development (Munson 2006). In humans, estrogen plays a role in carcinogenesis of breast and endometrial cancers as a known mitogen and ability to upregulate growth factors to stimulate cellular proliferation (Kim et al. 2013). The role of progesterone in breast cancer development is more complicated, as progesterone can both inhibit and stimulate proliferation in normal and diseased breast and reproductive organs (Kim et al. 2013).

Endometrial hyperplasia was the most prevalent uterine lesion in jaguars, with most cases classified as Grade 2 or 3 (advanced). Endometrial hyperplasia and carcinoma were associated with MGA contraceptive exposure, as reported other zoo felid studies (Munson et al. 1995; Munson et al. 2002). Endometrial mineralization is a common sequela to advanced endometrial hyperplasia in zoo felids, and in this study was found only in jaguars with advanced hyperplastic lesions (Munson et al. 2002). Six jaguars had endometrial carcinoma, and all had concurrent advanced (grade 2 or 3) endometrial hyperplasia. This is similar to women, where endometrial hyperplasia increases the risk of endometrial carcinoma (Lacey Jr et al. 2007). Other endometrial lesions in jaguars, including adenomyosis, hydrometra with endometrial atrophy, and neutrophilic endometritis/pyometra occurred in a smaller percentage of animals and were not associated with MGA exposure in these jaguars, as for other zoo felids (Munson et al. 2002).

Mammary carcinoma was the most common cause of death (n=15; 26%) in this study population. These neoplasms were commonly high grade (grade II or III), and most (61%) had metastasized. Mammary carcinomas in zoo felids metastasize rapidly and to multiple distant sites (Harrenstein et al. 1996; McAloose et al. 2007; Munson and Moresco 2007).

Although statistical analysis was not performed in these cases, the majority jaguars with mammary carcinoma had previous progestin contraceptive exposure, consistent with findings in other zoo felids (Harrenstein et al. 1996; McAloose et al. 2007).

The high prevalence of malignant neoplasia associated with MGA contraceptive exposure in jaguars, and advanced endometrial hyperplasia that could play a role in infertility, is consistent with that found in other zoo felids, and warrants the current recommendations to avoid exogenous progestin use in felids.

Leiomyomas were the second most common uterine lesion in jaguars (n=21; 38.8%) after endometrial hyperplasia. Leiomyomas are very common in humans and animals, including domestic animals and other zoo felids (Chassy et al. 2002; Kennedy et al. 2007; Rice et al. 2012). In this study, the uterine myometrium was the most common location, and four leiomyomas were located within the ovarian smooth muscle. Neoplasms were often multiple, but with no evidence of malignant morphology. Leiomyomas occurred regardless of MGA exposure or parity, which is consistent with a previous study involving all zoo felids (Chassy et al. 2002). These neoplasms were considered incidental findings in jaguars, and were associated with any specific clinical signs in this study, similar to the findings of leiomyoma in zoo felids in general (Chassy et al. 2002). Leiomyomas are extremely common in women and proliferation is influenced by both estrogen and progesterone (Flake et al. 2003).

In conclusion, jaguars have a high prevalence of reproductive tract and mammary gland carcinomas, which were also the most common cause of mortality in this study population. Like other zoo felids, most of these lesions, including endometrial hyperplasia and carcinoma, and mammary carcinoma, are associated with previous MGA contraceptive exposure.

Notably, jaguars have an extremely high prevalence of ovarian carcinoma (53%) that has been reported only in this species among the wild felids. Ovarian carcinomas occur in aged jaguars (mean 17 years), but also in animals of breeding age, and are not associated with MGA exposure. These neoplasms occur within the jaguar pedigree in an autosomal dominant pattern, similar to hereditary breast and ovarian cancer in humans associated with germline mutations in tumor suppressor genes such as BRCA1 and BRCA2. Because of the high prevalence of proliferative lesions and malignant reproductive and mammary cancer in zoo felids, including jaguars, MGA contraceptive use is now recommended only on rare occasions in zoo felids, and Deslorelin, a gonadotropin releasing hormone (GnRH) agonist, is now the preferred exogenous hormonal contraceptive due to minimal side effects. Limitations of this study include the retrospective nature, with incomplete tissue sets and historical data in some cases, and the inability to identify true risk factors for disease. Statistical analyses should also be interpreted with caution due to the small numbers in group comparisons. Additional studies are warranted to understand the molecular pathogenesis of progestin contraceptives and effects on target tissues in zoo felids. Studies are currently underway in our laboratory to elucidate the genetic mechanisms of carcinogenesis of ovarian carcinoma in jaguars.

APPENDIX

Table 2.5: Reproductive Health Surveillance Program: Felid Reproductive Pathology Criteria

General information

1. Pathology accession #: Number given by RHSP when tissues were received
2. ISIS #: From zoo record
3. Zoo ID: From zoo record
4. Studbook: From zoo record, not all species have a studbook
5. Name: Name of animal
6. Species: As written by the submitting institution
7. Location: Institution
8. Age (years):
9. Date of Birth:
10. Date of Death:
11. Parity: 0, 1, 2, 3, etc., number of pregnancies
12. OVH or NX?: How was the tract obtained
13. History and implant information: Text of all reproductive history
14. Contraceptive: Was this animal contracepted? Y/N
15. MGA: Was MGA the contraceptive used? Y/N
16. Dose: If available
17. Duration: in months: How long was the animal contracepted
18. Number of implants: Total number of implant given to this animal
19. Exposure: Temporary, intermittent, continuous
20. Other contraceptive type: list any other contraceptives used for this animal
21. Dose: of other contraceptives used
22. Duration: of other contraceptives used

HISTOPATHOLOGY

Ovary

23. Ovary: Were ovaries submitted?
24. POC Parovarian cyst (Wolffian duct remnants): Cystic structures located along the outside of the oviduct or ovary. May have prominent musculature with inner longitudinal and outer circular muscle bundles
25. Follicle 3 (tertiary follicle): Are follicles that have a single large cavity or antrum lined by several layers of granulosa cells.
26. Corpora lutea: Composed of large, polygonal cells with abundant, pale eosinophilic cytoplasm (luteinized granulosa cells)
27. Rete cyst: Cystic structure(s) with flat to cuboidal single layer of epithelium. Should be in the medulla

Table 2.5 (Cont'd)

28. Rete hyperplasia:

29. Subepithelial structure cyst: Small cystic structures found directly under the surface of the ovary. Form from invaginations of the ovarian serosal surface.

30. Subepithelial structure hyperplasia:

31. Ovary mineral: Presence of basophilic granular material

32. Ovary primary tumor: Name type

33. Ovary metastatic tumor: Name type

34. Oviduct: Is the oviduct present in the section?

Endometrium

35. Uterus: Was the uterus submitted?

36. Endometrial atrophy: Endometrial tissue is thinner than normal, usually with decreased amount of glandular tissue.

37. Endometrial hyperplasia: Thickening of the endometrial tissue, for classification of type, see below

38. Grade endometrial hyperplasia: 0= no hyperplastic changes; 1= minimal to mild proliferative and/or cystic changes in glands or surface epithelium without an increase in overall endometrial height; 2= moderate hyperplastic and /or cystic changes with an increased endometrial thickness of < 2 times normal; 3= severe hyperplastic and /or cystic changes with an increased endometrial thickness of > 2 times normal; NE= not evaluated.

39. Adenomatous hyperplasia: Proliferative glands with epithelial budding, branching, infolding, tall columnar pseudostratified epithelium. Mitoses present often with back to back glands

40. Cystic hyperplasia: Cystic endometrial hyperplasia characterized by distended glands that compress adjacent stroma with evidence of proliferation in some glands.

41. Superficial hyperplasia: Y/N

42. Endometrial dysplasia: Y/N

43. Endometrial polyp: Focal luminal projection with a varying mix of benign glands and stroma.

44. Adenomyosis: Nests of glands AND stroma within the myometrium that are distinctly separate from the endometrium. Be careful of plane of section and the glands near endocervix extend deeper into the myometrium so be conservative in this region.

45. Uterine mineralization: Basophilic granular material in the glandular or stromal part of the uterus.

46. Vascular mineralization: Presence of basophilic granular material limited to the vascular wall.

47. Fibrosis: Y/N

48. Hydrometra: Presence of serous or mucoid material within the lumen of the uterus.

49. Pyometra: Presence of large numbers of neutrophils within the lumen of the uterus.

50. Neutrophilic metritis: Presence of neutrophils below the luminal surface of the endometrium.

51. Lymphoplasmacytic metritis: Presence of lymphocytes or plasma cells below the luminal surface of the endometrium.

52. Hyaline artery: Arteries and veins especially in myometrium have thick walls with abundant acellular or hypocellular eosinophilic material. Indicative or prior pregnancy. Often looks like a ribbon within the vascular wall.

Table 2.5 (Cont'd)

54. Leiomyosarcoma: Y/N
55. Endometrial carcinoma: Y/N
56. Cervix: Is there cervix present in the section
57. Cervix Dx: Write diagnosis NS= not submitted
Mammary Gland
58. Mammary gland: Was mammary gland submitted?
59. Mammary hyperplasia: Y/N
60. Mammary adenoma: Y/N
61. Mammary adenocarcinoma: Y/N
62. Mammary diagnosis: Write diagnosis NS = not submitted

Table 2.6: Case demographics (age, cause of death, contraceptive exposure, reproductive neoplasia prevalence) of female jaguars (n=56) from North American zoos. N=no, Y=yes, NE=not examined.

Case No.[a]	Age at death or tissue submission (years)	Cause of Death	Progestin (MGA) contraceptive exposure	Ovarian carcinoma	Endometrial carcinoma	Mammary carcinoma	Leiomyoma (any location)
1	2	Euthanized; degenerative joint disease, chronic radial fracture	N	N	N	N	N
2	8	Euthanized; mammary carcinoma	Y	Y	N	Y	Y
3	8	Metastatic mammary carcinoma	Y	Y	N	Y	Y
4	8	Unk	N	N	N	NE	N
5	9	Euthanized; endometrial adenocarcinoma	Y	N	Y	NE	N
6	9	Unk	Unk	N	N	Y	Y
7	10	Euthanized; bilateral ovarian carcinoma	N	Y	N	NE	N
8	11	Unk	N	N	N	N	N
9	12	Unk	N	NE	N	NE	N
10	12	Sudden death	N	N	N	NE	N
11	12	Euthanized; endometrial carcinoma	Y	NE	Y	NE	N
12	12	Diabetic ketoacidosis, mammary adenocarcinoma	Y	N	N	Y	N
13	12	Euthanized; metastatic mammary carcinoma	Y	Y	N	Y	Y
14	12	Unk	Unk	Y	N	NE	N
15	13	Euthanized; metastatic mammary carcinoma	Y	N	N	Y	N
16	13	Unk	Y	N	N	NE	N
17	13	Metastatic endometrial carcinoma	Y	N	Y	NE	Y
18	13	Euthanized; metastatic mammary carcinoma	Y	N	N	Y	N
19	13	Unk	N	NE	N	NE	N
20	14	Euthanized; metastatic mammary carcinoma	Y	Y	N	Y	N
21	14	Euthanized; bilateral ovarian carcinoma	N	Y	N	NE	N

Table 2.6 (Cont'd)

22	15	Euthanized; hepatic adenocarcinoma	Y	Y	N	NE	Y
23	15	Euthanized; mandibular soft tissue sarcoma	Y	Y	N	NE	N
24	15	Mammary carcinoma	Y	Y	N	Y	Y
25	15	Unk	Unk	Y	Y	NE	Y
26	16	Euthanized; mammary carcinoma	Y	Y	N	Y	Y
27	16	Euthanized; hepatic cholangiocarcinoma	Y	N	N	N	N
28	16	Metastatic mammary carcinoma	Y	N	N	Y	N
29	17	NA	N	N	N	NE	N
30	17	Euthanized; metastatic mammary carcinoma	Y	Y	N	Y	N
31	17	Metastatic mammary carcinoma	N	NE	NE	Y	NE
32	18	Unk	Y	N	N	NE	Y
33	19	Euthanized	Y	N	N	Y	Y
34	19	Chronic renal failure	N	N	N	N	N
35	19	Unk	Unk	NE	N	NE	N
36	19	Euthanized; mesothelioma	N	N	N	NE	N
37	19	Metastatic carcinoma	N	Y	N	Y	N
38	19	Mammary carcinoma	Unk	Y	N	Y	Y
39	20	Pheochromocytoma, bilateral ovarian carcinoma	N	Y	N	NE	Y
40	20	Unk	N	Y	N	NE	N
41	20	Euthanized; bilateral ovarian carcinoma	N	Y	N	NE	N
42	20	Unk	N	Y	N	NE	Y
43	20	Euthanized; ovarian carcinoma with peritoneal implantation	N	Y	N	NE	N
44	20	Euthanasia; endometrial carcinoma	Y	N	Y	NE	Y
45	20	NA	N	N	N	NE	N
46	21	Unk	N	N	N	NE	N
47	21	Unk	Y	N	N	NE	N
48	21	Euthanized; congestive heart failure	N	N	N	NE	N
49	21	Unk	N	Y	N	NE	Y
50	21	Euthanized; ovarian carcinoma with peritoneal implantation	N	Y	N	N	Y

Table 2.6 (Cont'd)

51	21	Euthanized; septic peritonitis, migrating foreign body with intestinal perforation	Y	Y	N	NE	N
52	22	Euthanized; metastatic mammary carcinoma and chronic renal failure, pheochromocytoma with vascular invasion	Y	Y	N	Y	Y
53	23	Mammary carcinoma	Unk	NE	NE	Y	NE
54	23	Unk	Unk	Y	N	NE	Y
55	24	Euthanized	Y	NE	Y	NE	Y
56	27	Unk	N	Y	N	NE	Y

[a] Submitting zoological institutions: Akron Zoological Park, OH; Albuquerque Biological Park, NM; Alexandria Zoological Park, LA; BREC's Baton Rouge Zoo, LA; Caldwell Zoo, TX; Chattanooga Zoo at Warner Park, TN; Cheyenne Mountain Zoological Park, CO; Cincinnati Zoo and Botanical Garden, OH; El Paso Zoo, TX; Gladys Porter Zoo, TX; Great Plains Zoo and Delbridge Museum of Natural History, SD; Happy Hollow Zoo, CA; Houston Zoo, TX; Jacksonville Zoo and Gardens, FL; Lee Richardson Zoo, KS; Little Rock Zoological Gardens, AR; Los Angeles Zoo, CA; Louisville Zoological Garden, KY; Maryland Zoo in Baltimore, MD; Memphis Zoological Garden and Aquarium, TN; Micke Grove Zoo, Lodi, CA; Montgomery Zoo, AL; Omaha's Henry Doorly Zoo & Aquarium, NE; Oregon Zoo, OR; Palm Beach Zoo at Dreher Park, FL; Philadelphia Zoo, PA; Phoenix Zoo, AZ; Sacramento Zoo, CA; Saint Louis Zoo, MO; San Antonio Zoological Society, TX; Sedgwick County Zoo, KS; Toledo Zoological Gardens, OH; Tulsa Zoo, OK; Utah's Hogle Zoo, UT; Wildlife Waystation, CA; Woodland Park Zoo, WA; Zoo Atlanta, GA; Zoo World, FL.

Table 2.7: Histologic characteristics of ovarian carcinoma (n=26) in North American zoo jaguars (*Panthera onca*)

Case No.	Unilateral (U) or bilateral (B)	Peritoneal implantation	Mitotic count[a]	Necrosis	Hemorrhage	Mineralization	Lymphocytic inflammation	Neutrophilic inflammation	Cytoplasmic or nuclear pleomorphism[b]	Stromal invasion
2	B	Y	2	N	N	N	Y	N	mild	Y
3	B	N	6	Y	Y	Y	Y	Y	mild	Y
7	B	Y	3	N	Y	Y	Y	N	mild	Y
13	B	N	2	N	Y	Y	N	N	mild	Y
14	B	N	4	N	N	N	Y	N	mild	Y
20	B	N	15	Y	Y	Y	Y	Y	moderate	Y
21	B	Y	6	N	N	Y	Y	N	mild	Y
22	B	N	5	N	Y	Y	Y	N	mild	Y
23	B	N	3	N	N	N	N	N	mild	Y
24	B	N	4	N	N	N	Y	N	mild	Y
25	B	N	10	Y	Y	Y	Y	Y	moderate	Y
26	B	N	13	N	Y	Y	N	N	marked	Y
30	B	Y	6	N	N	Y	Y	N	mild	Y
37	B	N	3	N	Y	Y	Y	N	mild	Y
38	B	N	4	N	N	Y	N	N	mild	Y
39	B	N	2	N	N	Y	Y	N	mild	Y
40	B	N	3	N	Y	N	Y	N	mild	Y
41	B	Y	7	N	Y	Y	Y	N	mild	Y
42	B	N	3	N	N	N	N	N	mild	Y
43	B	Y	7	Y	Y	Y	Y	Y	mild	Y
49	B	N	4	N	N	Y	N	N	mild	Y
50	B	Y	5	Y	Y	Y	Y	Y	mild	Y
51	B	Y	7	Y	Y	Y	Y	Y	moderate	Y
52	B	N	4	Y	Y	Y	Y	Y	mild	Y
54	B	N	2	N	N	N	N	N	mild	Y
56	B	N	2	N	N	N	N	N	mild	Y

[a] Mitotic count is reported as the total number of mitoses in ten high powered fields (400x). Poorly cellular, cystic regions were avoided. Mitotic counts ranged from 2-15 (mean 5.0 +/- 3.31).

[b] Cytoplasmic and nuclear pleomorphism were assessed as mild, moderate, or marked based on degree of anisokaryosis and anisocytosis, presence of multinucleated cells, densely clumped or vesicular chromatin, and presence of nucleoli.

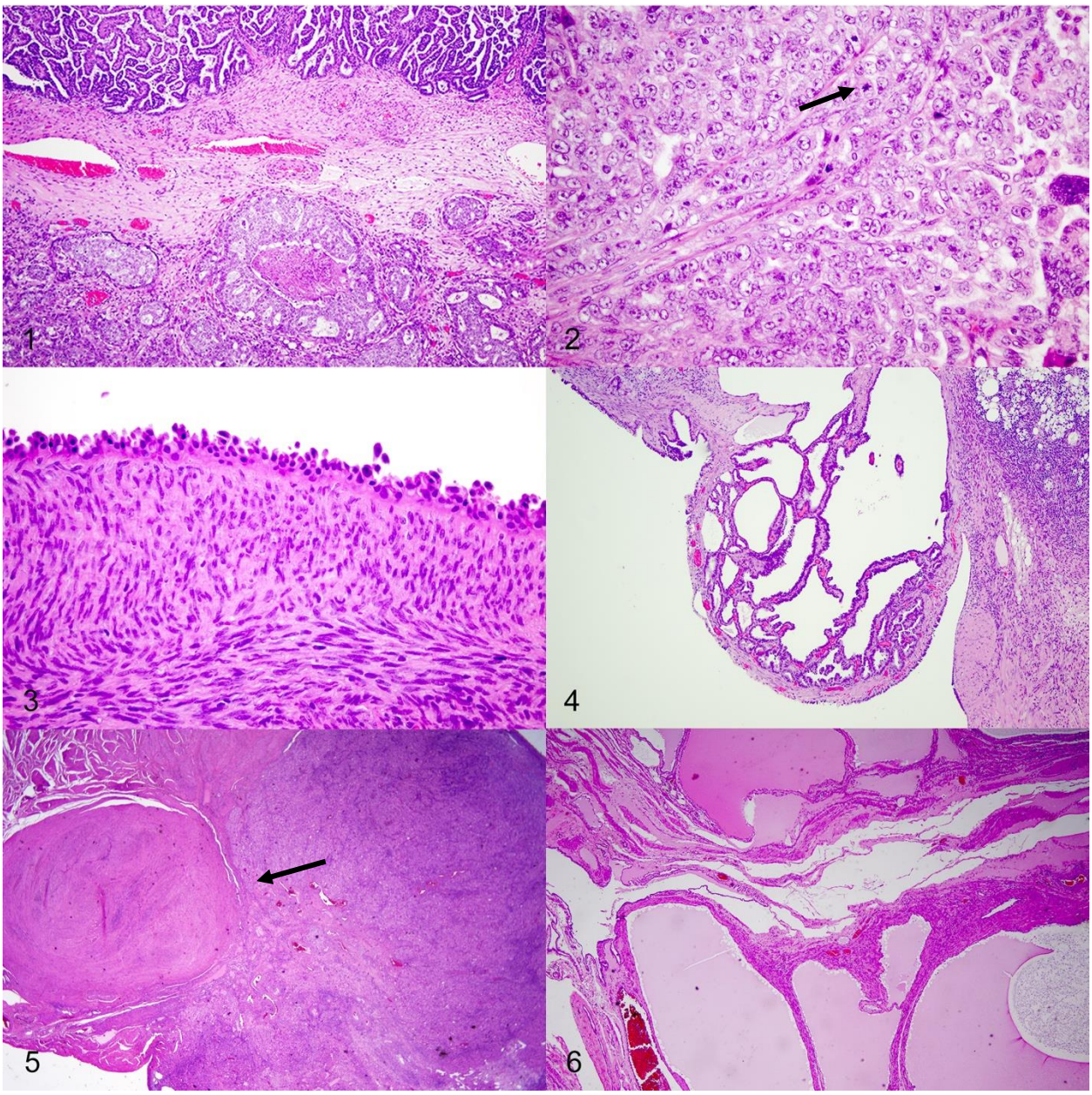

Figure 2.10: Ovary, jaguar (*Panthera onca*), Hematoxylin & Eosin. (1) Ovarian carcinoma, papillary (top) and metastatic mammary carcinoma (bottom). (2) Ovarian carcinoma. Some neoplasms had focal areas of moderate anisocytosis and anisokaryosis with numerous mitoses (arrow). (3) Ovarian surface epithelium hypertrophy and hyperplasia. Cells are piled and disorganized with abundant cytoplasm. (4) Ovarian cystadenoma. An approximately 1cm diameter, unilateral, focal, cystic mass with neoplastic cells arranged in papillary projections. (5) Ovarian leiomyoma (arrow). (6) Ovarian hilus, cystic rete ovarii. Numerous variably-sizes cysts contain eosinophilic fluid and are lined by a single layer of attenuated epithelium.

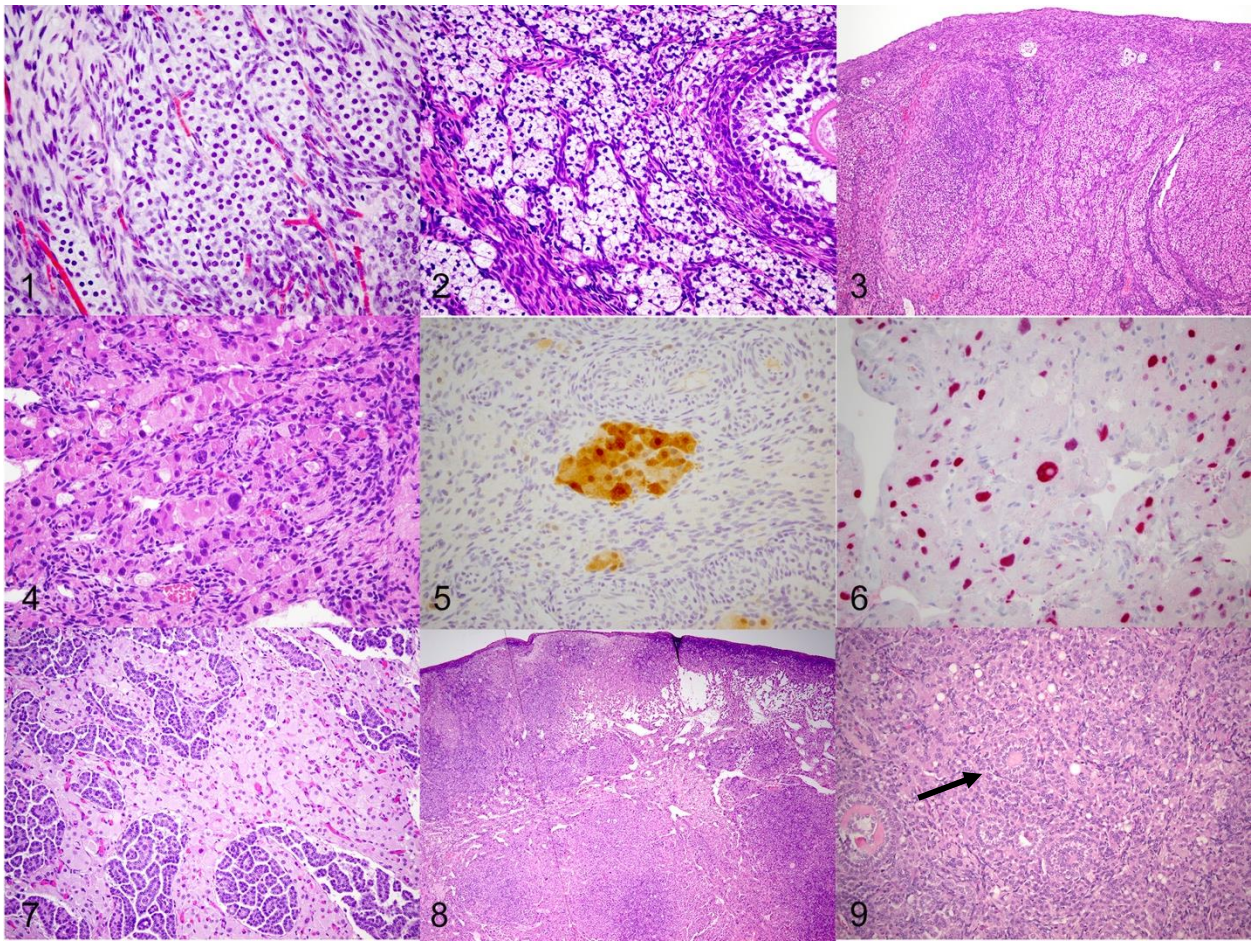

Figure 2.11: Ovary, Hematoxylin & Eosin. Interstitial gland cells, ovarian cortex. Domestic cat (1), mountain lion (*Puma concolor*) (2), and Amur leopard (*Panthera pardus orientalis*) (3). Cells are uniform in size and arranged in cords with a polygonal, foamy to finely granular cytoplasm and round nucleus with dense chromatin. (4) Interstitial gland hyperplasia, ovarian cortex, jaguar (*Panthera onca*). Cells are increased in number and pleomorphic with abundant, eosinophilic, finely granular cytoplasm (luteinized) and large nuclei with moderate anisokaryosis. Cells have strong cytoplasmic and nuclear immunoreactivity for calretinin (5) and nuclear immunoreactivity for progesterone receptor (6). (7) Interstitial gland hyperplasia and papillary ovarian carcinoma, jaguar (*Panthera onca*). Ovarian papillary carcinoma (basophilic cells) separated by abundant interstitial cells with a foamy, pale eosinophilic cytoplasm. (8) Ovarian cortex, jaguar (*Panthera onca*). The ovarian cortex is diffusely expanded by a poorly demarcated, densely cellular neoplasm. (9) Cells are of sex cord-stromal origin with numerous Call-Exner bodies (arrow).

REFERENCES

REFERENCES

Agnew DA, MacLachlan NJ (2017) Tumors of the Genital System In: Tumors of Domestic

Animals 5th edition. Ed: Meuten DJ. John Wiley & Sons, Inc. Ames IA, pp. 689-722

Bossart GD, Hubbell G (1986) Ovarian papillary cystadenocarcinoma in a jaguar. J Zoo Anim Med. 14(1):73-76

Brown S, Hankinson S (2015) Endogenous estrogens and the risk of breast, endometrial, and ovarian cancers. Steroids: http://dx.doi.org/10.1016/j.steroids.2014.12.013

Bryan LK, Edwards JF, Hoppes SM (2015) Pathology in Practice. JAVMA 247(10):1117-1119

Cathro HP, Stoler MH (2002) Expression of cytokeratins 7 and 20 in ovarian neoplasia. Am J Clin Pathol 117:944-951

Chassy LM, Gardner IA, Plotka ED, Munson L (2002) Genital tract smooth muscle tumors are common in zoo felids but are not associated with melengestrol acetate contraceptive treatment. Vet Pathol 39: 379-385

Chu P, Wu E, Weiss LM (2000) Cytokeratin 7 and cytokeratin 20 expression in epithelial neoplasms: a survey of 435 cases. Mod Pathol 13(9) 962-972

Corner S, Parys M, Moresco A, Yuzbasiyan-Gurkan V, Agnew D (2017) Ovarian adenocarcinoma in jaguars (*Panthera onca*): a naturally-occurring model of human inherited ovarian cancer? [abstract]. In: Proceedings of the 11th Biennial Ovarian Cancer Research Symposium; Sep 12-13, 2016; Seattle, WA. Philadelphia (PA): AACR; Clin Cancer Res 23:11 Suppl Abstract

Deavers M, Malpica A, Liu J, Broaddus R, Silva EG (2003) Ovarian sex cord-stromal tumors: an immunohistochemical study including a comparison of calretinin and inhibin. Mod Pathol 16(6): 584-590

Dobson JM (2013) Breed-predispositions to cancer in pedigree dogs. ISRN Veterinary Science. 2013: 1-23

Duska LR, Chang Y, Flynn CE (1999) Epithelial ovarian carcinoma in the reproductive age group. Cancer 85(12): 2623-2629

Elston CW, Ellis IO (1991) Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathol 19:403-410

Flake GP, Anderson J, Dixon D (2002) Etiology and pathogenesis of uterine leiomyomas: a review. Environ Health Perspect 111:1037-1054

Gelberg HB, McEntee K, Heath EH (1984) Feline cystic rete ovarii. Vet Pathol 21: 304-307

Gelberg HB, McEntee K (1985) Feline ovarian neoplasms. *Vet Pathol*. 22:572-576

Harrenstein LA, Munson L, Seal US (1996) Mammary cancer in captive wild felids and risk factors for its development: a retrospective study of the clinical behavior of 31 cases. J Zoo Wildl Med 27(4): 468-476

Havrilesky LJ et al. (2013) Oral contraceptive pills as primary prevention for ovarian cancer. Obstetrics & Gynecology 122(1): 139-147

Hayden DW, Barnes DM, Johnson KH (1989) Morphologic changes in the mammary gland of megestrol acetate-treated and untreated cats: a retrospective study. Vet Pathol 26:104-113

Hee Han K, Kim MK, Kim HS, Chung HH, Song YS (2013) Protective effects of progesterone during pregnancy against ovarian cancer. Journal of Cancer Prevention 18(2): 113-122

Ho SM (2003) Estrogen, progesterone and epithelial ovarian cancer. Reproductive Biology and Endocrinology 1:73

Hope K, Deem SL (2006) Retrospective study of morbidity and mortality of captive jaguars (*Panthera onca*) in North America, 1982-2002. Zoo Biol 25:501-512

Kazensky CA, Munson L, Seal US (1998) The effects of melengestrol acetate on the ovaries of captive wild felids. J Zoo Wildl Med 29(1): 1-5

Kennedy PC, Cullen JM, Edwards JF, Goldschmidt MH, Larsen S, Munson L, Nielsen S (2007) WHO Tumor Fascicles: Histological Classification of Tumors of the Genital System of Domestic Animals. Armed Forces Institute of Pathology, Washington, DC

Keskin A, Yilmazbas G, Yilmaz R et al. (2009) Pathological abnormalities after long-term administration of medroxyprogesterone acetate in a queen. Journal of Feline Medicine and Surgery 11:518-521

Kim JJ, Kurita T, Bulun SE (2013) Progesterone action in endometrial cancer, endometriosis, uterine fibroids, and breast cancer. Endocrine reviews 34(1):130-162

Kurman RJ, Carcangiu ML, Herrington CS and Young RH (2014) WHO Classification of Tumours of Female Reproductive Organs. International Agency for Research on Cancer (IARC), Lyon, France

Labidi-Galy SI, Papp E, Hallberg D et al. (2017) High grade serous ovarian carcinomas arise in the fallopian tube. Nature Communications 8: 1093 DOI: 10.1038/s41467-017-00962-1

Lacey Jr JV, Loffe OB, Ronnett BM, et al. (2008) Endometrial carcinoma risk among women diagnosed with endometrial hyperplasia: the 34-year experience in a large health plan. British Journal of Cancer 98:45-53

Lim D, Oliva E (2013) Precursors and pathogenesis of ovarian carcinoma. Pathology 45(3): 229-242

Lukanova A, Kaaks R (2005) Endogenous hormones and ovarian cancer: epidemiology and current hypotheses. Cancer Epidemiol Biomarkers Prev 14(1): 98-107

Lynch HT, Casey MJ, Snyder CL et al. (2009) Hereditary ovarian cancer: molecular genetics, pathology, management, and heterogeneity. Mol Oncol 3(2): 97-137

Maryamma KI, Sivadas CG, Krishnan Nair M, Rajan A (1974) Cystadenocarcinoma of ovary with leiomyoma of uterus in a jaguar (*Panthera onca*). Indian Vet J 51:269-270

McAloose D, Munson L, Naydan DK (2007) Histologic features of mammary carcinomas in zoo felids treated with melengestrol acetate (MGA) contraceptives. *Vet Pathol.* 44:320-326

Meinhold-Heerlein I, Fotopoulou C, Harter P (2016) The new WHO classification of ovarian, fallopian tube, and primary peritoneal cancer and its clinical implications. Arch Gynecol Obstet 293:695-700

Misdorp W, Else RW, Hellmén E, Lipscomb TP (1999) Histological classification of mammary tumors of the dog and the cat. Armed Forces Institute of Pathology, Washington DC

Mittal K, Soslow R (2008) Application of immunohistochemistry to gynecologic pathology. Arch Pathol Lab Med 132: 402-423

Moresco A, Munson L, Gardner IA (2009) Naturally occurring and melengestrol acetate-associated reproductive tract lesions in zoo canids. Vet Pathol 46:1117-1128

Mossman HW, Duke KL (1973) Ovarian interstitial gland tissues. In: Comparative morphology of the mammalian ovary. The University of Wisconsin Press, Madison, WI pp 166-202

Munson L (1994) A high prevalence of ovarian papillary cystadenocarcinomas in jaguars (*Panthera onca*). Vet Pathol (Abstract) 31:5

Munson L (2006) Contraception in felids. *Theriogenology* 66: 126-134

Munson L, Gardner IA, Mason RJ, Chassy LM, Seal US (2002) Endometrial hyperplasia and mineralization in zoo felids treated with melengestrol acetate contraceptives. *Vet Pathol.* 39:419-427

Munson L, Moresco A (2007) Comparative pathology of mammary gland cancers in domestic and wild animals. *Breast Disease* 28: 7-21

Munson L, Stokes JE, Harrenstein LA (1995) Uterine cancer in zoo felids on progestin contraceptives. Vet Pathol (Abstract) 32: 578

Patnaik AK, Greenlee PG (1987) Canine ovarian neoplasms: a clinicopathologic study of 71 cases, including histology of 12 granulosa cell tumors. Vet Pathol 24:509-514

Perez JF, Conley AJ, Dieter JA, Sanz-Ortega J, Lasley BL (1999) Studies on the origin of interstitial tissue and the incidence of endometrial hyperplasia in domestic and feral cats. General and Comparative Endocrinology 116:10-20

Petrucelli N, Daly MB, Feldman GL (2010) Hereditary breast and ovarian cancer due to mutations in BRCA1 and BRCA2. Genetics in Medicine 12(5): 245-259

Ramos-Vara JA. (2005) Technical aspects of immunohistochemistry. Vet Pathol 42:405-426

Reid BM, Permuth JB, Sellers TA (2017) Epidemiology of ovarian cancer: a review. Cancer Biol Med doi: 10.20892/j.issn.2095-3941.2016.0084

Riccardi E, Greco V, Verganti S, Finazzi M (2007) Immunohistochemical diagnosis of canine ovarian epithelial and granulosa cell tumors. J Vet Diagn Invest 19:431-435

Rice KE, Secrist JR, Woodrow EL (2012) Etiology, diagnosis, and management of uterine leiomyomas. Journal of Midwifery & Women's Health 57(3): 241-247

Quigley, H., Foster, R., Petracca, L., Payan, E., Salom, R. & Harmsen, B. 2017. *Panthera onca*. The IUCN Red List of Threatened Species 2017: e.T15953A50658693

Samson M, Porter N, Orekoya O et al. (2016) Progestin and breast cancer risk: a systematic review. Breast Cancer Res Treat 155(1): 3-12

Schlafer DH, Gifford AT (2008) Cystic endometrial hyperplasia, pseudo-placentational endometrial hyperplasia, and other cystic conditions of the canine and feline uterus. Theriogenology 70:349-358

Selman PJ, van Garderen E, Mol JA et al. (1995) Comparison of the histological changes in the dog after treatment with the progestins medroxyprogesterone acetate and proligestone. Veterinary Quarterly 17:128-133

Skandalis SS, Afratis N, Smirlaki G, et al. (2014) Cross-talk between estradiol receptor and EGFR/IGF-IR signaling pathways in estrogen-responsive breast cancers: Focus on the role and impact of proteoglycans. Matrix Biology 35:182-193

Sieh W et al. (2013) Associations between hormone receptor expression and ovarian cancer survival: an Ovarian Tumor Tissue Analysis consortium study. Lancet Oncol 14(9): 853-862

Vachon CM, Mink PJ, Janney CA et al. (2001) Association of parity and ovarian cancer risk by family history of breast or ovarian cancer in a population-based study. Epidemiology 13(1): 66-71

Veras EF, Crow JH, Robboy SJ (2014) Non-neoplastic and tumor-like lesions of the ovary. In: Mutter GL, Prat J (Eds) Pathology of the female reproductive tract. Third Ed, New York, NY, Elsevier

Welcsh PL and King MC (2001) BRCA1 and BRCA2 and the genetics of breast and ovarian cancer. Human Molecular Genetics 10(7): 705-713

Wright JD, Shah M, Mathew L, et al. (2009) Fertility preservation in young women with epithelial ovarian cancer. Cancer DOI: 10.1002/cncr.24461

Yaziji H et al. (2006) Evaluation of 12 antibodies for distinguishing epithelioid mesothelioma from adenocarcinoma: identification of a three-antibody immunohistochemical panel with maximal sensitivity and specificity. Mod Pathol 19:514-523

Zappulli V, Rasotto R, Caliari D et al. (2015) Prognostic evaluation of feline mammary carcinomas: a review of the literature. Vet Pathol 52(1): 46-60

CHAPTER 3

Resource Development: Establishment of a jaguar DNA archive, ovarian carcinoma cell lines, and a xenograft mouse model pilot study

Abstract

Jaguars are an endangered species and represent the only felid in the Americas from the genus *Panthera*. Wild jaguars are threatened by habitat loss and other human impacts, and maintaining a healthy breeding population in zoological institutions is important for survival. Ovarian carcinoma occurs commonly in adult jaguars housed in United States zoological institutions, and is a significant cause of mortality in this population. There is a paucity of resources, including genomic information, in-vitro, or in-vivo models for the study of wild and zoo animal natural diseases. For example, the first whole genome from a jaguar has been sequenced within the past year. Additionally, to our knowledge, in-vitro or in-vivo methods for the study of cancer or other diseases in jaguars has not been described. The Association of Zoos & Aquariums (AZA) Reproductive Management Center's (RMC) Reproductive Health Surveillance Program (RHSP) at Michigan State University collects and archives reproductive tracts from all zoo animals for ongoing studies of contraceptive use and disease surveillance. Over the past 35 years, the RHSP has received samples (whole blood and/or reproductive tracts) from 90 jaguars housed at zoological institutions throughout North America, including 19 males and 71 females. The specific aims of this study were to (1) develop resources for the characterization of jaguar ovarian carcinoma, including collection of representative DNA from the North American jaguar population, (2) establish a jaguar ovarian carcinoma cell line, and (3) create an in-vivo model of jaguar ovarian carcinoma using an ovarian carcinoma cell line derived mouse xenograft method. We collected reproductive tracts from 59 female jaguars, and have also established a DNA archive from 45 jaguars for use in this study. Jaguar ovarian carcinoma cell lines were initiated from two jaguar neoplasms, and a cell-line derived xenograft mouse model was also attempted using two jaguar ovarian carcinoma cell lines.

Introduction

Jaguars are classified as near threatened throughout their geographic range, are endangered in the United States, and represent the only felid in the Americas from the genus *Panthera* (Quigley et al. 2017). Wild jaguars are threatened by habitat loss and other human impacts, and maintaining a healthy breeding population in zoological institutions is important for the overall population. Although rare in domestic cats, ovarian carcinoma is common in adult jaguars housed in United States zoological institutions, and is a significant cause of mortality in this population (Gelberg and McEntee 1985; Bossart and Hubbell 1986; Munson 1994; Hope and Deep 2006; Bryan et al. 2015). This neoplasm in jaguars is not well-characterized, and the pathogenesis and effects on reproduction are currently unknown. Mammary and endometrial carcinoma in zoo felids are associated with progestin contraceptive use, such as melengestrol acetate (Munson et al. 1995; Harrenstein et al. 1996; Munson et al. 2002; Munson 2006; McAloose et al. 2007; Munson and Moresco 2007). However, the same association has not been found with ovarian neoplasia in zoo felids (Kazensky et al. 1998). Therefore, an inherited predisposition to ovarian carcinoma is proposed as a mechanism of tumorigenesis in jaguars. The overall aim of this study is to develop resources to investigate the genetic mechanisms of carcinogenesis of this common neoplasm in jaguars. The Association of Zoos and Aquariums (AZA) along with the American Association of Zoo Veterinarians (AAZV) supports the Reproductive Management Center (RMC) located at the St. Louis Zoo.

The RMC oversees the use of contraceptives in zoo animals and provides consultation on lifetime reproductive planning and contraceptive products for use in managing and sustaining genetically valuable zoo populations of mammalian wildlife. The RMC partners with the Reproductive Health Surveillance Program (RHSP), located at Michigan State University.

The RHSP collects and archives reproductive tracts from many zoo species, including jaguars, to monitor reproductive pathology that may be associated with contraceptive use. Jaguar samples used to study ovarian carcinoma are received through the RHSP. To study the underlying genetic mechanisms of ovarian carcinoma, an archive of representative DNA from jaguars in the North American zoo population is also needed.

Cancer cell lines are valuable in-vitro resources for studies including understanding the molecular pathogenesis of disease, high-throughput drug screening, and functional testing using technologies such as CRISPR (Wilding and Bodmer 2014). In humans, ovarian cancer cell lines are difficult to establish, requiring specific culture media for different tumor subtypes and other specialized methods (Ince et al. 2015). Successful ovarian cancer cell lines have been established mainly from ascites fluid rather than the solid component of tumors (Shepherd et al. 2006; Ince et al. 2015). However, some ovarian cancer cell lines have been established that recapitulate tumor morphology and molecular signatures of the neoplasm (Ince et al. 2015). Development of a jaguar ovarian carcinoma cell line would be useful for future studies. A jaguar ovarian carcinoma cell line will serve as a source of fresh neoplastic cells for DNA and RNA studies, such as whole exome or genome sequencing, RNA-seq, assessing the effects of exogenous hormones on cell growth and viability, high-throughput drug screening, and functional studies. To our knowledge, cell lines from any jaguar cell type have not been described previously. In wild felids, short-term culture of the ovarian cortex has been performed for oocyte cryopreservation (Wiedemann et al. 2013). The establishment of ovarian cancer cell lines from domestic or other animals has also not been reported. Among domestic animals, ovarian epithelial cancer is common only in the dog (Patnaik and Greenlee 1987).

Although we rarely have the opportunity to obtain fresh tumor tissue from jaguars, creating a jaguar ovarian carcinoma cell line would be useful for downstream studies.

Cancer cell line and patient-derived xenograft (PDX) mouse models of cancer in humans are an essential tool for pre-clinical studies, such as evaluation of drug efficacy within a model that better represents tumor heterogeneity and microenvironment than in-vitro models (Cho et al. 2016). In human ovarian cancer, both PDX and genetically engineered mouse models are used extensively (Shaw et al. 2004; Connolly and Hensley 2009; Archiveert 2011; Alkema et al. 2015; Bobbs et al. 2015; Columbo et al. 2015; Harrington et al. 2016). In addition to in-vitro tools such as cell lines, in-vivo models of jaguar ovarian carcinoma would allow for additional downstream studies to not only understand the pathogenesis of disease in jaguars, but to also evaluate drug efficacy in-vivo. Because ovarian carcinoma cells are difficult to propagate in-vitro, a xenograft mouse model would also be an alternative source of fresh tumor tissue for study. Currently, jaguars are diagnosed very late in the disease process or at necropsy, and treatment is rarely initiated. Additional tools are needed for future studies to better understand the disease process and to explore options for earlier diagnosis and intervention.

The specific aims of this study were to (1) develop resources for the characterization of jaguar ovarian carcinoma, including collection and archiving of representative DNA from the North American jaguar population, (2) establish a jaguar ovarian carcinoma cell line, and (3) create an in-vivo model of jaguar ovarian carcinoma using a xenograft mouse model.

Materials and Methods

Jaguar sample collection and establishment of jaguar DNA archive

All jaguar and other felid samples, including tissue and blood, are approved for use under a Michigan State University Institutional Animal Care and Use Committee (IACUC) Exemption.

An ongoing request to zoological institutions for jaguar reproductive tissues (male and female) and whole blood is in place through the Association of Zoos & Aquariums (AZA) Reproductive Management Center (RMC). The RMC partners with the Reproductive Health Surveillance Program (RHSP) at Michigan State University that collects and archives reproductive tracts from all zoo animals for studies on contraceptive use and general disease surveillance. Jaguar reproductive tracts are voluntarily submitted to the RHSP for use in this ovarian carcinoma study from those animals that have undergone necropsy or ovariohysterectomy. Tissues are submitted in 10% neutral buffered formalin, or fresh when available. Jaguar whole blood is also requested from males and females and is submitted in RNALater, on filter paper as a dried blood spot, or in EDTA.

A DNA archive from male and female jaguars from the North American zoological population has been initiated at Michigan State University for ongoing genetics studies. DNA extraction from whole blood stored in RNALater or EDTA was performed using the DNEasy Blood & Tissue Kit (Qiagen, Hilden Germany). DNA stored on filter paper was extracted using the QIAmp DNA Mini Kit (Qiagen). After extraction, DNA was quantified using a Qubit Fluorometer (ThermoFisher Scientific, Waltham MA). For those animals in which formalin-fixed tissue was the only source of DNA, extraction was performed using the QIAmp DNA FFPE Tissue Kit (Qiagen). All DNA samples were stored long-term at -80°C.

Formalin-fixed reproductive tissues from jaguars were examined for gross lesions and trimmed according to an established protocol that includes representative sections of uterus, both ovaries, oviduct, and mammary gland. Trimmed tissues were submitted to Michigan State University Investigative Histopathology Laboratory. Tissues were embedded in paraffin, and 5μm sections were routinely stained with hematoxylin & eosin.

All histologic lesions were then characterized and recorded as part of the larger jaguar ovarian carcinoma study. All jaguar samples submitted were labeled with individual barcodes unique for each case and logged into FreezerWorks® software.

Establishment of jaguar ovarian carcinoma cell lines

Establishment of ovarian carcinoma cell lines from two jaguars was initiated, based on availability of fresh tissues. For cell line 1, fresh reproductive tract (uterus and both ovaries) was shipped overnight from the submitting zoological institution. This reproductive tract was from a 13-year-old, intact female jaguar that was euthanized after abdominal exploration. Bilateral ovarian carcinoma with peritoneal implantation was detected. Cell line 2 was from a 10-year-old, intact female jaguar that was also diagnosed with bilateral ovarian carcinoma on abdominal exploration. An ovariohysterectomy was performed on this animal, and the fresh uterus and ovaries were sent to Michigan State University.

To generate the cell cultures, multiple 1-2 mm explants were placed into 25 cm^2 culture flasks (Corning, Sigma-Aldrich, St. Louis MO), using a previously described method for culture of domestic animal reproductive tissues (Munson et al. 1988; Moresco 2009). Fluid from cystic regions of the neoplasm was also aspirated and plated into culture flasks. Explants were plated with and without collagenase.

For those explants treated with collagenase, minced explants were added to culture flasks with 1% collagenase (C9891, Sigma-Aldrich) dissolved in 20 ml Hank's balanced salt solution (ThermoFisher Scientific, Waltham MA) and incubated for 1 hour before transfer to complete culture media.

Culture media consisted of Dulbecco modified eagle medium (DMEM – ThermoFisher Scientific), 10% fetal bovine serum (Sigma-Aldrich, St. Louis MO), 5 μg/ml insulin-transferrin-sodium selenite media supplement (Sigma-Aldrich), 100 μg/ml streptomycin, 100 U penicillin and 0.25 μg/ml amphotericin B (ThermoFisher Scientific), 10 ng/ml epidermal growth factor (Life Technologies, Carlsbad CA), and gentamicin (50 μg/ml). Gentamicin was removed from culture media after the first passage. Cell lines were incubated at 37°C with 5% CO_2. After 90% confluence, cells were passaged using 0.25% trypsin (Life Technologies, Carlsbad CA). Cell morphology was visualized using an inverted microscope (Nikon Eclipse TS100).

Cells were maintained in 25 cm^2 culture flasks, media was changed every 3-4 days, and cells were trypsinized after flasks were confluent. After each passage, 1/3 of the cells were saved for cryopreservation. For long-term storage of cell lines, cells were stored in 1 ml cryovials in culture media as described above without gentamicin, and supplemented with 20% fetal bovine serum and 10% di-methyl sulfoxide (DMSO). Cells were transferred to -80°C overnight then placed in liquid nitrogen. Additional freezing media was tested for long-term storage of cells, including Cryostor (StemCell Technologies, Vancouver BC) and replacement of DMSO with 10% glycerol. Cell count and viability were measured using a trypan blue method with a Countess automated cell counter (ThermoFisher Scientific).

DNA from each cell line was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden Germany).

Characterization of jaguar cell line immunophenotype and cell line validation

Cell pellets from jaguar cell lines 1 and 2 were preserved in 10% neutral buffered formalin. Cells were paraffin embedded and 5 μm sections were made in preparation for immunohistochemistry, which was performed at MSU Veterinary Diagnostic Laboratory (estrogen receptor, progesterone receptor, cytokeratin 7) and MSU Investigative Histopathology Laboratory (AE1/AE3, vimentin) according to laboratory protocol for each antibody. Antibodies and general characteristics of each are listed in Table 3.1. Immunohistochemical labeling was characterized by location (nuclear and/or cytoplasmic) and staining intensity (weak, moderate, strong). Negative controls replaced the primary antibody with buffer. Domestic cat and jaguar uterus and ovary were used as positive controls for each marker to ensure antibody cross-reactivity in the jaguar and site-specificity in normal tissues.

Table 3.1: List of antibodies for jaguar cell line characterization

Antibody	**Clone**	**Brand**	**Dilution**	**Incubation Time (minutes)**	**Incubation Temperature (°C)**
Estrogen receptor α	Rabbit monoclonal SP1	Ventana Medical Systems, Tucson AZ	1:50	32	37
Progesterone receptor	Rabbit monoclonal 1E2	Ventana Medical Systems, Tucson AZ	1:50	16	37
AE1/AE3	Mouse monoclonal	Merck Millipore, Burlington MA	1:300	60	37
Cytokeratin 7	Mouse monoclonal OV-TL 12/30	Dako Agilent, Santa Clara CA	1:75	30	37
Vimentin	Mouse monoclonal	ThermoFisher Scientific, Waltham MA	1:150	60	37

Xenograft mouse model of jaguar ovarian carcinoma

This study was performed under the approval of Michigan State University Institutional Animal Care and Use Committee (IACUC), Animal Use Form (AUF) 09/15-133-00.

An in-vivo pilot study was initiated to establish a source of neoplastic cells from jaguar ovarian carcinoma for downstream studies. Two 6-week old, immunodeficient female mice (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ, The Jackson Laboratory, Bar Harbor ME) were used. Mice were housed at the MSU IVIS Spectrum Imaging Core and acclimated for 1 week before injection of tumor cells.

Cryopreserved jaguar ovarian carcinoma cell lines 1 and 2 were removed from liquid nitrogen storage and plated in 25 cm^2 flasks in media described previously. Cells were grown to confluence, and $1x10^6$ cells from each cell line were prepared for subcutaneous injection. Cells were resuspended in culture media without antibiotics, and basement membrane matrix (BD Matrigel, BD Biosciences, Franklin Lakes NJ) in a 1:1 ratio, with a total volume of 200 μl for injection.

Subcutaneous injection of cancer cells from culture was performed according to established protocols (Morton and Houghton 2007). Briefly, mice were anesthetized using 1-5% isoflurane delivered by induction chamber, then mask. $1x10^6$ cells from jaguar cell line 1 and jaguar cell line 2 were prepared for subcutaneous injection into two mice (one for each cell line). The injection site (left hind flank) was disinfected using an ethanol pad. Cells were injected subcutaneously using a 0.5cc tuberculin syringe and 25-gauge needle using aseptic technique. Mice were monitored every 48 hours for clinical signs and tumor presence/growth was assessed (Appendix Table 3.4). Subcutaneous tumor size was measured with a caliper using the following equation: D_1=length, D_2=width, D_3=thickness/height; Volume (mm^3) = $0.523x(D_1xD_2xD_3)$.

Mice were monitored for a variety of clinical signs associated with tumor progression. Humane endpoints were established and adhered to according to the AUF. Mice were also monitored routinely by Campus Animal Resources (CAR) staff veterinarians.

Mice were humanely euthanized via CO_2 exposure according to IACUC protocol after 60 days of no visible tumor progression or adverse clinical signs. Tissues were preserved in 10% neutral buffered formalin and histopathology of all tissues were reviewed, including the injection site. Tissue at the injection site was also frozen and stored at -80°C. DNA was extracted from tissue at the injection site using the DNeasy blood & tissue kit (Qiagen, Hilden Germany), according to manufacturer's protocol. PCR amplification of a multiplex of 12 jaguar microsatellite loci was performed to confirm or rule-out the presence of jaguar tissue at the injection site (Corner et al. under review; also see Chapter 4).

Results

Sample collection and establishment of a jaguar DNA archive

Over the past 35 years, the RHSP has collected samples from 90 jaguars, including tissues and blood. The submitting institution, type of samples collected (whole blood, H&E slides, formalin-fixed paraffin embedded tissue, other formalin-fixed tissue, and fresh or frozen tissue), and samples added to the MSU jaguar DNA archive are summarized in Appendix Table 3.3. Jaguar samples were submitted from 50 different zoological institutions, and included 71 females and 19 males. Reproductive tracts were collected from 59 females and have been used to characterize reproductive lesions in the jaguar, including ovarian carcinoma.

In total, DNA was extracted and archived from 45 jaguars based on availability and quality of blood or tissue for extraction. We received two fresh reproductive tracts from female jaguars with bilateral ovarian carcinoma that were used to develop jaguar cell lines 1 and 2.

Historically, jaguar whole blood was requested to be sent to the RHSP in RNALater and on filter paper. DNA quantity was higher after extraction of RNALater-stored whole blood (mean 18.7 ng/ul; range 5-58 ng/ul) compared to filter paper (mean 2 ng/ul; range 1.5-3 ng/ul). RNALater-stored whole blood had comparable DNA quantity to blood stored at -80°C in EDTA. DNA quantity extracted from formalin-fixed, paraffin embedded tissues was highly variable (0-50 ng/ul). In addition to the samples already collected, reproductive tracts and whole blood from female and male jaguars continue to be collected and archived for future use.

Establishment of jaguar ovarian carcinoma cell lines and characterization of jaguar cell line immunophenotype

Cell line origin, cytologic characteristics, and immunophenotype of the cell line and corresponding tumor are summarized in Table 3.2. Cellular morphology was similar in both cell lines. Cells had an epithelial morphology, and were polygonal or rarely spindloid and organized in cohesive clusters (Figure 3.1). Scattered multinucleated cells and those exhibiting cytomegaly and karyomegaly were present. Jaguar cell lines 1 and 2 survived for 8 or 5 passages, respectively. Cells initially had rapid growth, but by the third passage, cell proliferation slowed and eventually progressed to rapid and widespread cell death.

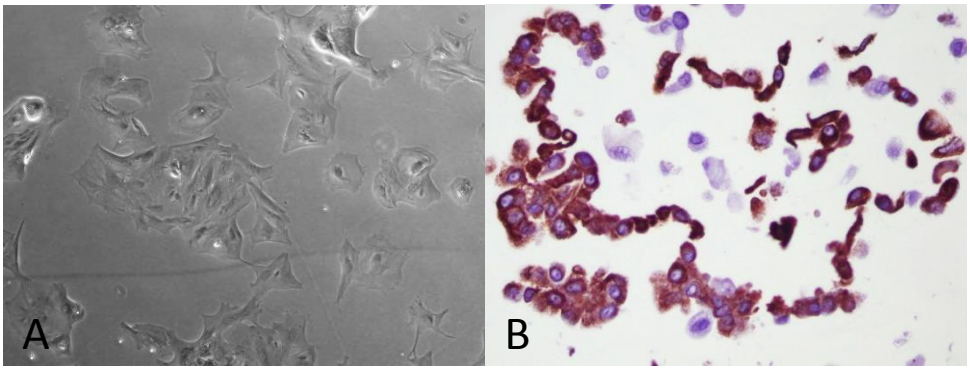

Figure 3.1: Ovarian carcinoma neoplastic cells, Jaguar Cell Line 1. (A) Polygonal epithelial cells in small cohesive clusters with minimal cytoplasmic and nuclear pleomorphism. (B) AE1/AE3 immunohistochemistry. Cultured cells have positive cytoplasmic immunoreactivity for broad cytokeratins.

Cell lines had strong cytoplasmic immunoreactivity for broad cytokeratins (AE1/AE3) and Cytokeratin 7, had nuclear immunoreactivity for estrogen receptor (strong), and progesterone receptor (weak), and were vimentin negative. The immunophenotype corresponded with that of the originating tumors, which were also evaluated by immunohistochemistry using the same antibody panel. These results support that the cells were of ovarian carcinoma origin.

To validate the origin of cell lines and confirm that each originated from a different jaguar, cell lines 1 and 2 were genotyped using a novel set of 12 microsatellite markers validated for use in this species, which were developed in our laboratory. The microsatellite panel confirmed that each cell line was derived from a unique jaguar, and all alleles from each cell line corresponded with the alleles present in the blood from each jaguar from which the cells originated. (Corner et al. under review; also see Chapter 4).

Table 3.2: Characteristics of jaguar ovarian carcinoma cell lines and corresponding tumor immunophenotype

Cell line	Diagnosis	Jaguar age	Total No. Passages	Cytologic characteristics	Cell Line Immunophenotype	Tumor Immunophenotype
1	Bilateral ovarian carcinoma with peritoneal metastasis	13	8	Cohesive clusters of polygonal to spindloid cells	AE1/AE3 + CK7 + ER+ (strong) PR+ (weak) Vimentin -	CK7+ ER+ (strong) PR+ (strong)
2	Bilateral ovarian carcinoma with peritoneal metastasis	15	5	Cohesive clusters of polygonal to spindloid cells	AE1/AE3 + CK7 + ER+ (strong) PR+ (weak) Vimentin -	CK7+ ER+ (strong) PR+ (weak)

Both cell lines had overall poor survival after short-term (days) and long-term (months) cryopreservation. Cells were cryopreserved in antibiotic-free general media supplemented with 20% fetal bovine serum and 10% of either DMSO or glycerol. Cells were also cryopreserved in a commercially-available, DMSO-based freezing media (Cryostor, StemCell Technologies, Vancouver, BC). Cell viability of both cell lines was similar for all three cryopreservation methods, and was measured at approximately 50% using a trypan blue method for detection.

Xenograft mouse model of jaguar ovarian carcinoma

Subcutaneous injection of $1x10^6$ cells from each cell line was performed in immunodeficient female mice (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ, The Jackson Laboratory, Bar Harbor ME). One mouse was injected for each cell line, due to the low number of cells available for injection. There was no visible tumor growth in the subcutaneous tissues and no adverse clinical signs after 60 days. After euthanasia, histopathology on all mouse tissues, including the

injection site region, were evaluated on routine H&E stained slides by a veterinary pathologist (SC). No abnormalities or evidence of neoplasia were detected.

DNA extracted from the injection site region was genotyped using the jaguar-specific microsatellite 12-plex, and no jaguar DNA was detected in either mouse.

Discussion

There is a paucity of resources, including genomic information, in-vitro, or in-vivo models for the study of wild and zoo animal natural diseases. For example, in the jaguar, the first whole genome was sequenced within the past year (Figueiró et al. 2017). Because ovarian carcinoma is common specifically in jaguars, species-specific resources need to be developed to better characterize this disease. The aims of this study were to develop resources to study ovarian carcinoma in jaguars. These included the development of a DNA archive from jaguars in the North American zoo population, a jaguar ovarian carcinoma cell line, and a cell line derived mouse model of jaguar ovarian carcinoma. The DNA archive derived from fresh/frozen tissues, FFPE tissues, and blood samples is now available for future studies (Table 3.3).

Over the past 35 years, samples from 90 jaguars, including reproductive tissues and whole blood, have been collected by the RHSP. Continued sample collection, storage, and entry into the RHSP database will ensure a growing collection of samples to represent DNA from the approximately 124 jaguars currently housed in North American zoological institutions (2016 North American Regional Jaguar Studbook). Reproductive tissues and blood from this collection have been used to characterize the pathology and genetic basis of ovarian carcinoma in jaguars, and have also been used to evaluate the overall genetic diversity of the North American zoo jaguar population.

We have created a DNA archive from 45 jaguars submitted to the RHSP that can continue to be used for future genetics studies.

To establish the DNA archive, DNA was extracted from all samples stored in RNALater or filter paper. We found that DNA quantity of whole blood stored on filter paper was too low for next-generation sequencing studies. Whole blood stored in RNALater had higher DNA yield and was successfully used in a targeted next-generation sequencing assay in our laboratory. For the future, we have asked zoological institutions to send blood either in RNALater or EDTA whole blood shipped on ice for storage rather than on filter paper.

Cell lines from two jaguars with bilateral ovarian carcinoma were initiated. Fresh reproductive tracts were shipped overnight by the submitting institutions. Both cell lines had overall poor growth and longevity, and survived in culture for up to 8 passages. We confirmed that ovarian carcinoma cells grew in culture by the overall cellular morphology and immunohistochemistry. Hormone receptor expression was similar to the originating tumor, and cells were cytokeratin positive, indicating that they were of epithelial cell origin.

We also validated that each cell line was from a different jaguar, and that cells were of jaguar origin, using a novel 12plex of microsatellite markers developed for use in this species. Alleles in each cell line matched those of the blood from each jaguar. Due to poor cell viability and the acquisition of only two fresh tumors, ability to experiment with different types of general culture media and additives were limited. In humans, ovarian cancer cell lines are difficult to establish, requiring specialized culture media for different tumor subtypes (Ince et al. 2015).

Jaguar cell lines also had poor survival using multiple cryopreservation methods. DMSO is commonly used as a cryopreservative for many cell types, but some cells can be sensitive to this cryopreservative (Stacey and Masters 2008; Jang et al. 2017). Glycerol, although used more

commonly for bacteria and animal sperm cryopreservation, can also be used as an alternative to DMSO for mammalian cells (Jang et al. 2017).

In both jaguar cell lines, cell viability after cryopreservation and thawing remained at approximately 50% with DMSO, glycerol, or a commercially-available, DMSO-based complete freezing media (Cryostor, StemCell Technologies, Vancouver BC). One potential problem is that most of the jaguar cells were not frozen during the exponential growth phase, which is recommended for successful cryopreservation (Stacey and Masters 2008). The jaguar cells had rapid growth only initially during early passages. We did not find any difference in cell viability between the early or later passages after cryopreservation of both cell lines. Should additional fresh jaguar tissue become available, future studies should include the testing of a wider array of cell culture media components, including specialized media for human ovarian carcinoma that includes additional growth factors and hormones, depending on the tumor subtype. Also, these tissues were shipped overnight, which may have affected cell viability prior to initiation of culture. Coordination with the submitting institution to start a cell line on-site or providing the institution with aliquots of culture media for storage and shipment of fresh tumor samples may improve culture success.

For development of the cell line derived mouse model of jaguar ovarian carcinoma, subcutaneous injection of $1x10^6$ cells from each cell line was performed in immunodeficient female mice (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ, The Jackson Laboratory, Bar Harbor ME). These mice were chosen because they are broadly immunodeficient, with lack of B, T and natural killer cell function, making them ideal for PDX studies. In domestic cats, subcutaneous xenografts of feline mammary carcinoma have been developed using nude mice (Hassan et al. 2017). The use of cell line derived mouse models of cancer in wild felids has not been described, to our

knowledge. There was no tumor growth in mice after injection of cells from both jaguar cell lines.

A small number of cells were injected ($1x10^6$) due to poor viability of cell lines after cryopreservation. Due to the limited number of cells available, injection was performed in a single mouse for each cell line. In mouse models of human ovarian carcinoma, approximately $5x10^6$ cells are injected per mouse (Connolly and Hensley 2009). The low number of cells injected may have been one reason for failure of tumor growth in this case. Mouse models of human ovarian cancer have been successfully established using subcutaneous, intraperitoneal, and orthotopic (intrabursal) injection of cultured cells (Vanderhyden et al. 2003). However, direct xenotransplantation of patient ovarian carcinoma tissue to create PDX mouse models has been more successful in humans (Alkema et al. 2015; Colombo et al. 2015; Harrington et al. 2016). In our study, the route of injection also may have played a role in failure of tumor growth. We chose a subcutaneous route of injection for ease of tumor growth monitoring. Jaguar cells were not transfected with luciferase for bioluminescence imaging due to the fragility of the cell lines. Ideally, intraperitoneal or orthotopic (intrabursal) injection of cells would have been performed to better recapitulate the tumor microenvironment in-vivo. Direct xenotransplantation of jaguar ovarian carcinoma tissue could alternatively be performed if fresh tissue is available.

In conclusion, we have initiated a DNA archive from jaguars housed in North American zoological institutions for current and future studies of the genetic basis of carcinogenesis of ovarian carcinoma in this population. We have also used this resource to assess the genetic diversity of zoo jaguars. This is an important resource for future studies to maintain the health of North American zoo jaguars. Pilot studies of in-vitro and in-vivo models of jaguar ovarian

carcinoma were also initiated. Jaguar ovarian carcinoma cells grew in culture, but had poor survival and viability after cryopreservation.

Initiation of cell culture on site at the institution where the animal is housed rather than shipping fresh tissues overnight on ice may improve cell viability. Cell line derived xenograft mouse model of ovarian carcinoma were not successful in this initial attempt. Direct xenotransplantation of fresh tumor tissue could be performed as an alternative method. Fresh jaguar tissue is rarely available, but future studies could include more robust experimentation with various culture media, alternative routes of injection, and increased cell quantity. We recommend preserving DNA from jaguar whole blood with storage either in RNALater or EDTA long-term at -80°C for downstream next-generation sequencing and other genomic studies. Filter paper whole blood does not yield DNA quantities needed for next-generation sequencing studies.

APPENDIX

Table 3.3: Jaguar samples submitted to and archived at the Reproductive Health Surveillance Program over an approximately 35-year time period. WB = whole blood; FFPE = Formalin-fixed, paraffin embedded; FF = Formalin-fixed.

Jag ID[a]	Sex	Submitted Sample Type					gDNA Archive MSU
		WB	H&E slides	FFPE blocks	FF tissue	Fresh/ Frozen tissue	
1	M		X				
2	F		X	X			
3	F		X				
4	F		X	X			X
5	F		X	X			X
6	F		X	X			X
7	F		X				
8	M		X	X	X		
9	F		X	X			X
10	F		X	X			X
11	F		X	X	X		
12	F		X	X			
13	F		X				
14	F		X	X			
15	F		X	X			
16	F		X	X			X
17	F		X	X	X		X
18	F		X	X			
19	F		X	X	X		X
20	F		X	X	X		X
21	F		X	X			
22	F		X	X			
23	F		X	X			
24	F		X	X			X
25	F		X		X		
26	F		X	X			X
27	F		X	X			X
28	F		X				
29	F		X	X	X		X
30	F		X	X			
31	F		X	X			X
32	F		X	X			
33	F		X	X			
34	F		X	X	X		
35	M				X		
36	F		X				
37	F		X	X			X
38	F		X	X			X
39	F		X	X			X
40	F		X	X			X
41	F		X	X			
42	F		X	X			
43	F		X	X			X
44	M	X					X
45	F		X	X			
46	F		X	X	X		
47	F		X	X			
48	F	X	X	X			X
49	F	X				X	X
50	M	X					X
51	F		X	X	X		X
52	M		X	X	X		
53	F	X					X
54	F		X	X			

Table 3.3 (Cont'd)

55	F	X	X	X			X
56	F		X		X		
57	M	X					X
58	F		X	X		X	X
59	F	X					X
60	F	X					X
61	M	X					X
62	M	X					X
63	F	X	X	X		X	X
64	F		X	X	X		
65	F		X	X			X
66	F	X	X	X			X
67	F		X	X			
68	F	X					X
69	F	X	X	X	X	X	X
70	M	X					X
71	F	X					X
72	M	X					X
73	F	X					X
74	F	X					X
75	M	X					X
76	F	X					X
77	F	X					X
78	M	X					X
79	F	X					X
80	M	X					X
81	F	X					X
82	F	X	X	X			X
83	M	X					X
84	M	X					X
85	M	X					X
86	F	X	X	X	X	X	X
87	M	X					X
88	M	X					X
89	F		X				
90	F		X	X			X

[a] Submitting zoological institutions: Abilene Zoological Gardens, TX; Akron Zoological Park, OH; Albuquerque Biological Park, NM; Alexandria Zoological Park, LA; Audobon Zoo, LA; BREC's Baton Rouge Zoo, LA; Brevard Zoo, FL; Caldwell Zoo, TX; Chattanooga Zoo at Warner Park, TN; Cheyenne Mountain Zoological Park, CO; Cincinnati Zoo and Botanical Garden, OH; El Paso Zoo, TX; Ellen Trout Zoo, TX; Elmwood Park Zoo, PA; Gladys Porter Zoo, TX; Great Plains Zoo and Delbridge Museum of Natural History, SD; Happy Hollow Zoo, CA; Houston Zoo, TX; Jacksonville Zoo and Gardens, FL; Lee Richardson Zoo, KS; Little Rock Zoological Gardens, AR; The Living Desert Zoo and Gardens, CA; Los Angeles Zoo, CA; Louisville Zoological Garden, KY; Maryland Zoo in Baltimore, MD; Memphis Zoological Garden and Aquarium, TN; Mesker Park Zoo & Botanic Garden, IN; Micke Grove Zoo, Lodi, CA; Montgomery Zoo, AL; Omaha's Henry Doorly Zoo & Aquarium, NE; Oregon Zoo, OR; Palm Beach Zoo at Dreher Park, FL; Philadelphia Zoo, PA; Phoenix Zoo, AZ; Reid Park Zoo, AZ; Sacramento Zoo, CA; Saint Louis Zoo, MO; San Antonio Zoological Society, TX; Sedgwick County Zoo, KS; Smithsonian National Zoological Park, DC; Soco Gardens Zoo, NC; Toledo Zoological Gardens, OH; Tulsa Zoo, OK; Utah's Hogle Zoo, UT; Wild Animal Safari, GA; Wildlife Waystation, CA; Woodland Park Zoo, WA; Zoo Atlanta, GA; Zoo Miami, FL; Zoo World, F

Table 3.4: Mouse Clinical Signs / Tumor Measurement Record

Cage #		**Researcher: Sarah Corner**		**P.I.**: Vilma Yuzbasiyan-Gurkan		**AUF #**: 09/15-133-00	
Rack Position/Ear Mark:		**Strain**: NOD.SCID		**Source:** JAX Lab		**Sex**: Female	**Age (weeks)**: 6wks
Cell line injected/# cells/route: Jaguar ovarian carcinoma, subcutaneous							
Day# post injection							
Date							
Procedure							
Ear clipping							
Cells injection							
Imaging IVIS							
Isoflurane (1-5%)							
Checking							
Weight, g							
Tumor Measurements	L						
	W						
	H						
Tumor Volume [0.523(LxWxH)]							
Ulceration of Tumor (Y / N)							
Drug Treatments							
General Observations							
Body Condition Score							
Activity	Normal						
	Abnormal						
	Not moving						
Appearance (skin)	Normal						
	Jaundice						
	Anemia/Pale						
	Cyanosis (blue)						
Respiration	Normal						
	Increased						
	Labored						
Appetite							
Water intake							
Feces							

REFERENCES

REFERENCES

Alkema NG, Tomar T, Duiker EW, et al. (2015) Biobanking of patient and patient-derived xenograft ovarian tumor tissue: efficient preservation with low and high fetal calf serum based methods. Nature Scientific Reports 5:14495 DOI:10.1038/srep14495

Archiveert RB, Balu-Iyer SV, Odunsi K, Shultz LD, Kelleher RJ Jr, et al. (2011) Humanized mouse model of ovarian cancer recapitulates patient solid tumor progression, ascites formation, and metastasis. PLoS ONE 6(9): e24420

Bobbs AS, Cole JM, Cowden Dahl KD (2015) Emerging and evolving ovarian cancer animal models. Cancer Growth and Metastasis 8(S1):29-36

Bossart GD, Hubbell G (1986) Ovarian papillary cystadenocarcinoma in a jaguar. J Zoo Anim Med. 14(1):73-76

Bryan LK, Edwards JF, Hoppes SM (2015) Pathology in Practice. J Am Vet Med A. 247(10):1117-1119

Cho SY, et al. (2016) An integrative approach to precision cancer medicine using patient-derived xenografts. Mol Cells 39(2):77-86

Colombo PE, du Manoir S, Orsetti B, et al. (2015) Ovarian carcinoma patient derived xenografts reproduce their tumor of origin and preserve an oligoclonal structure. Oncotarget 6(29):28327-28340

Connolly DC, Hensley HH (2009) Xenograft and transgenic mouse models of epithelial ovarian cancer and non-invasive imaging modalities to monitor ovarian tumor growth in situ – applications in evaluating novel therapeutic agents. Curr Protoc Pharmacol. 45(14):14.12.1-14.12.26.

Figueiró HV, Li G, Trindade FJ et al (2017) Genome-wide signatures of complex introgression and adaptive evolution in the big cats. Sci Adv 3(7):e1700299

Gelberg HB, McEntee K (1985) Feline ovarian neoplasms. Vet Pathol. 22:572-576

Hassan et al. (2017) Feline mammary cancer: novel nude mouse model and molecular characterization of invasion and metastasis genes. Vet Pathol 54(1):32-43

Harrenstein LA, Munson L, Seal US (1996) Mammary cancer in captive wild felids and risk factors for its development: a retrospective study of the clinical behavior of 31 cases. J Zoo Wildl Med. 27(4): 468-476

Harrington BS, He Y, Davies CM, et al. (2016) Cell line and patient-derived xenograft models reveal elevated CDCP1 as a target in high-grade serous ovarian cancer. British Journal of Cancer 114:417-426

Hope K, Deem SL (2006) Retrospective study of morbidity and mortality of captive jaguars (Panthera onca) in North America, 1982-2002. Zoo Biol. 25:501-512

Ince TA, et al. (2015) Characterization of twenty-five ovarian tumor cell lines that phenocopy primary tumors. Nat Commun. 6:7419. 2015

Jang TH, Park SC, Yang JH, Kim JY, Seok JH, Park US, Choi CW, Lee SR, Han J (2017) Cryopreservation and its clinical applications. Integr Med Res 6:12-18

Kazensky CA, Munson L, Seal US (1998) The effects of melengestrol acetate on the ovaries of captive wild felids. J Zoo Wildl Med. 29(1): 1-5

McAloose D, Munson L, Naydan DK (2007) Histologic features of mammary carcinomas in zoo felids treated with melengestrol acetate (MGA) contraceptives. Vet Pathol. 44:320-326

Moresco A (2009) The Pro-Carcinogenic Effects of Progestogens on Carnivore Target Tissues. Doctoral Dissertation, University of California, Davis

Morton CL, Houghton PJ (2007) Establishment of human tumor xenografts in immunodeficient mice. Nature Protocols 2(2):247-250

Munson L, Chandler SK, Schlafer DH (1988) Cultivation of bovine fetal and adult endometrial epithelial cells. Journal of Tissue Culture Methods 11:129-133

Munson L (1994) Abstract: A high prevalence of ovarian papillary cystadenocarcinomas in jaguars (Panthera onca). Vet Pathol 31:5

Munson L, Gardner IA, Mason RJ, Chassy LM, Seal US (1995) Endometrial hyperplasia and mineralization in zoo felids treated with melengestrol acetate contraceptives. Vet Pathol. 39:419-427

Munson L, Stokes JE, Harrenstein LA (1995) Abstract: Uterine cancer in zoo felids on progestin contraceptives. Vet Pathol. 32:5

Munson L (2006) Contraception in felids. Theriogenology 66: 126-134

Munson L, Moresco A (2007) Comparative pathology of mammary gland cancers in domestic and wild animals. Breast Disease 28: 7-21

Patnaik AK, Greenlee PG (1987) Canine ovarian neoplasms: a clinicopathologic study of 71 cases, including histology of 12 granulosa cell tumors. Vet Pathol 24:509-514

Quigley H, Foster R, Petracca L, Payan E, Salom R, Harmsen B (2017) Panthera onca. The IUCN Red List of Threatened Species 2017: e.T15953A50658693

Shaw TJ, et al. (2004) Characterization of intraperitoneal, orthotopic and metastatic xenograft models of human ovarian cancer. Mol Ther. 10(6):1032-1042

Shepherd TG, Thériault BL, Campbell EJ, Nachtigal MW (2007) Primary culture of ovarian surface epithelial cells and ascites-derived ovarian cancer cells from patients. Nature Protocols 1(6):2643-2649

Stacey GN, Masters JR (2008) Cryopreservation and archiving of mammalian cell lines. Nature Protocols 3(12):1981-1989

Vanderhyden BC, Shaw TJ, Ethier JF (2003) Animal models of ovarian cancer. Reprod Biol Endocrinol 1:67

Wiedemann C, Zahmel J, Jewgenow K (2013) Short-term culture of ovarian cortex pieces to assess the cryopreservation outcome in wild felids for genome conservation. BMC Vet Res 9:37

Wildling J, Bodmer W (2014) Cancer cell lines for drug discovery and development. Cancer Res 74(9):2377-2384

CHAPTER 4

Development of a single multiplex of twelve new microsatellite markers using a novel universal primer method to evaluate the genetic diversity of jaguars (*Panthera onca*) from North American zoological institutions

Abstract

Maintenance of genetic diversity in both zoo-managed and wild animals is critical to population health. Jaguars are considered near-threatened throughout their geographic range, and are endangered in the United States. Genetic diversity of zoo-managed jaguar populations in North America using microsatellite markers has not been assessed. To evaluate genetic diversity in jaguars housed in North American zoological institutions compared to wild jaguar populations, we created a full multiplex of twelve tetranucleotide (n=10) or pentanucleotide (n=2) autosomal microsatellite loci that have not previously been described for use in jaguars. We used a universal primer approach to develop a complete genotyping panel in a single PCR reaction, which minimizes cost and DNA quantity requirements. A single multiplex of the twelve microsatellite loci was developed using four fluorescent dye-labeled, uniquely designed universal primers, and three amplicon size ranges. A subset of jaguars from North American zoological institutions (n=35, approximately 28% of population) were genotyped using DNA extracted from whole blood. Microsatellite loci were polymorphic with a range of 3-10 alleles (average 6.4 alleles per locus), and expected heterozygosity (H_e) between 0.47 and 0.85. Overall population H_e was 0.73. Probability of identity of individuals (PI) and siblings (PI_{sibs}) were 6.7×10^{-13} and 2.1×10^{-5}, respectively, confirming that this panel is highly powerful and can be used reliably to identify individuals. Using this novel multiplex microsatellite panel, we concluded that jaguars from the North American zoo population have high genetic diversity, comparable to reported wild and other zoo jaguar populations.

Introduction

The jaguar (*Panthera onca*) is the only wild felid from the genus *Panthera* in the Americas, and is considered near threatened by the International Union for the Conservation of Nature (IUCN), with a decreasing population (Quigley et al. 2017). The jaguar is a Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix I species, and is also listed as Endangered by the United States Fish & Wildlife Service. Habitat loss and fragmentation, and human conflict are major threats to species survival in the wild (Haag et al. 2010; Quigley et al. 2017). The overall management of jaguar breeding and genetics in North American zoological institutions is overseen by the Association of Zoos & Aquariums Jaguar Species Survival Plan (https://www.aza.org/species-survival-plan-programs). Minimizing inbreeding depression and the accumulation of deleterious alleles as well as maintaining genetic diversity are important for the long-term genetic management of populations (Frankham 1995).

Breeding recommendations in zoo populations can be determined with techniques such as minimizing kinship using pedigree and studbook information for each species (Ballou et al. 1997; Montgomery et al. 1997). Molecular-based methods, including the use of microsatellite markers to evaluate allele frequencies and heterozygosity have also been used extensively to assess the overall genetic diversity of both wild and zoo-managed animal populations (e.g. Mondol et al. 2009; Rueda-Zozaya et al. 2016; Simons et al. 2012; Beugin et al. 2017). Microsatellites are highly polymorphic, short, tandem nucleotide repeats 1-6 base pairs in length that are present in the noncoding region of the genome. The high mutation rates and polymorphic nature of these repeats are due to strand slippage during DNA replication, resulting in high allelic diversity and heterozygosity, making these ideal markers for population genetics studies (Ellegren 2004; Selkoe and Toonen 2006).

Evaluation of genetic diversity in jaguars from North American zoological institutions using microsatellite markers has not been reported, to our knowledge. Microsatellite markers, mainly dinucleotides and fewer tetranucleotides, have been used in wild felid population studies to assess allelic diversity and heterozygosity (Ruiz-Garcia et al. 2006; Soares et al. 2006; Sharma et al. 2008; Mondol et al. 2009; Palomares et al. 2012; Miller et al. 2014; Roques et al. 2014; Wultsch et al. 2014; Zou et al. 2015; Dou et al. 2016; Zanin et al. 2016; Souza et al. 2017). Many of these studies used microsatellite markers derived from well-characterized *Felis catus* microsatellite loci (Menotti-Raymond and O'Brien 1995; Menotti-Raymond et al. 1999; Driscoll et al. 2002; Menotti-Raymond et al. 2005). Genetic diversity and population structure of wild jaguars have been studied across their geographic range in Central and South America using mitochondrial DNA and microsatellite marker analysis. Jaguars were reported to have moderate to high genetic diversity overall, but with regional loss of genetic variation associated with habitat fragmentation in some cases (Eizirik et al. 2001; Haag et al. 2010; Wultsch et al. 2016). In zoo-managed jaguars, genetic diversity has been assessed using primarily dinucleotide microsatellites in Brazilian, Colombian, and Mexican zoological institutions, where moderate to high levels of allelic diversity and heterozygosity were also reported (Moreno et al. 2006; Rueda-Zozaya et al. 2016; Jimenez Gonzalez et al. 2017).

To assess genetic diversity of jaguars from the North American zoo population compared to wild jaguar populations and other zoo-managed populations, we identified an optimized, highly powerful set of twelve tetranucleotide or pentanucleotide autosomal microsatellite markers for specific use in the jaguar, and generated a full multiplex of the twelve markers using a universal primer method. These microsatellite loci have not previously been used in jaguar population genetics studies, to our knowledge.

The overall aims of this study were (1) to identify and validate twelve highly informative tetra- or pentanucleotide microsatellite markers for use in the jaguar, (2) develop a full multiplex of jaguar microsatellite markers to minimize cost and input DNA requirements using a universal primer method, and (3) use this multiplex to evaluate genetic diversity of jaguars in North American zoological institutions compared to wild and other zoo jaguar populations.

Materials and Methods

Samples

Whole blood from 35 adult jaguars (20 females, 15 males) from 20 North American zoological institutions was submitted to the Association of Zoos & Aquariums Reproductive Management Center's Reproductive Health Surveillance Program at Michigan State University. This represents approximately 28% of the current living population of jaguars housed in North American zoological institutions (n=124), according to the 2016 North American Regional Jaguar Studbook. Whole blood was stored long-term in RNALater or EDTA at -80 °C. Genomic DNA was extracted using the Qiagen DNEasy Blood & Tissue Kit using the manufacturer's protocol (Qiagen, Hilden, Germany) and quantified using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA).

Microsatellite marker identification

Twelve tetra- or pentanucleotide microsatellite repeats were selected, each on a different autosome, using the RepeatMasker track of the UCSC Genome Browser (https://genome.ucsc.edu) domestic cat genome (*Felis catus*_6.2). Microsatellites were first selected based on the *Felis catus* Smith-Waterman (SW) sequence alignment score within RepeatMasker (UCSC Genome Browser, FelCat 5; later genomic builds use a different Smith-Waterman weighting system). Tetranucleotide or pentanucleotide repeats with SW scores between 600-800 were selected. This range was selected to produce near maximal heterozygosity while minimizing mutation rates of tetranucleotide repeats (Venta, unpublished data). Microsatellites were also selected that had at least 8 or more perfect repeats and that did not occur within regions with other repeat elements such as SINEs or LINEs. The twelve selected microsatellite markers were named according to the species *Panthera onca* (PONC) and autosome location (PONCD3, PONCE3, PONCD2, PONCC2, PONCC1, PONCA1, PONCB4, PONCA3, PONCB3, PONCD1, PONCE1, and PONCD4 - Table 1). Autosome locations are based on the domestic cat, however genus *Panthera* species are reported to have the same karyotype as *Felis catus* (Cho YS et al. 2013). *Panthera tigris* has two interchromosomal rearrangements that could potentially affect chromosome location of microsatellite markers; however, our chosen microsatellites fall outside the reported regions (Cho YS et al. 2013).

Locus-specific and universal primer design

Microsatellite marker characteristics and primer sequence information are summarized in Appendix Table 4.2.

Primer design for jaguars was completed using the Amur tiger (*Panthera tigris altaica*) genome, a closely related species within the genus *Panthera* (Johnson et al. 2006, Figueiró et al. 2017). The Amur tiger was chosen based on availability in the NCBI RefSeq Genome Database and sample species availability for confirmation of primer design.

The presence of each microsatellite marker was confirmed in the Amur tiger (*Panthera tigris altaica* PanTig 1.0 assembly) genome using NCBI Blast (Cho et al. 2013). Locus-specific primers were designed using Primer3 v.0.4.0 (Koressaar and Remm 2007; Untergasser et al. 2012). Most forward primers were designed to end with two adenosine nucleotides (AA), which reduces primer-dimer formation (Innis and Gelfand, 1999). A 7-base pair "pigtail" sequence (GTTTCTT) was added to the 5' end of each reverse primer to reduce peak splitting from incomplete adenylation (Brownstein et al. 1996).

To create the single multiplex of 12 microsatellite loci, primers were designed to produce PCR amplicons within three size ranges (120-200, 220-280, or 280-400 base pairs), with four microsatellite loci in each size range. To distinguish between the four microsatellite loci within each amplicon size range, a universal primer labeling method was used (Oetting et al. 1995, Schuelke et al. 2000, Blacket et al. 2012). Rather than direct fluorescent-labeling, each locus-specific forward primer was 5' labeled with an identical sequence tag to one of four fluorescent dye-labeled universal primers (FAM, PET, NED, or VIC). Universal primer sequences were designed to be compatible with the species primer designs and the sequences are as follows:

Uni 1 - FAM: CTCCAACTCACCTCCAACAAA (Integrated DNA Technologies, San Jose, CA)
Uni 2 - PET: AAACCTCTCTCCACACCCAAA (Applied Biosystems, Foster City, CA)
Uni 3 - NED: CTCACCTCCCACTCCACAAA (Applied Biosystems, Foster City, CA)
Uni 4 - VIC: AACTCCACCACTCCCACAAA (Applied Biosystems, Foster City, CA)

PCR amplification

PCR for each microsatellite marker was first run individually to confirm amplification in genomic DNA derived from one Amur tiger tissue sample and run in duplicate in at least 3 jaguars before creating the multiplex. A negative control was run with each PCR reaction.

For the multiplex, a single primer mix was prepared, including locus-specific forward primers and reverse primers for all 12 microsatellite loci, and four universal fluorescent-labeled primers (FAM, PET, NED, VIC) at final concentrations (μM) specified in Table 1. Peak heights were balanced among the four microsatellite markers in each amplicon size range by adjusting primer concentrations before creating the full multiplex of all twelve markers.

PCR reactions were prepared in a final volume of 12.5 μl, and included 10x PCR Buffer (20 mM Tris HCL [pH 8.4] and 50 mM KCl), 0.2 mM dNTPs, 2 mM $MgCl_2$, primer mix (Table 1), 0.04 U/μl AmpliTaq Gold (Applied Biosystems, Foster City, CA), and 2-10 ng DNA. PCR amplification was performed on a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA) under the following cycling conditions: An initial denaturation step of 10 minutes at 94°C, followed by 50 cycles of 1 minute at 94°C, 2 minutes at 57°C, and 3 minutes at 72°C. A final extension step was performed for 60 minutes at 72°C, followed by 20 minutes at 20°C.

Genotyping and statistical analysis

PCR amplicons from the full multiplex of 12 microsatellite markers were visualized on a 1.5% agarose gel and fluorescent labeling of primers was confirmed using a Typhoon FLA 9500 (GE Healthcare Life Sciences, Pittsburgh, PA) before submission for high-resolution genotyping.

PCR amplicons were submitted to Michigan State University Research Technology Support Facility Genomics Core for high-resolution genotyping using a 3730xl DNA Analyzer with a Genescan 500 LIZ dye size standard (Applied Biosystems, Foster City, CA).

Fragment analysis was performed using Peak Scanner Software Version 2.0 (Applied Biosystems, Foster City, CA), and all allele calls were confirmed by direct visualization of all peaks for each jaguar sample.

Statistical analyses, including number of alleles, observed (*Ho*) and expected (H_e) heterozygosity, deviation from Hardy-Weinberg equilibrium (*HWE*), probability of identity (*PI*) and probability of identity of siblings (*PIsibs*) were performed using GenAlEx 6.5 (Peakall and Smouse 2012).

Results

Microsatellite marker selection and multiplex genotyping

Sixteen tetra- or pentanucleotide microsatellites were originally selected for use in the multiplex. Twelve had cross-species amplification in the domestic cat, Amur tiger, and jaguar using the criteria for selection based on *Felis catus* SW score, number of perfect repeats, and lack of additional repeat elements in the flanking sequence, and were chosen for the multiplex. These included 10 tetranucleotides and 2 pentanucleotide repeats from 12 different autosomes. Three other tetranucleotide microsatellites were designed and tested, but were excluded due to

failure of amplification in the jaguar, presumably due to primer mismatches. One additional microsatellite locus was excluded from the multiplex due to the presence of only two alleles in the entire jaguar study population. As little as 2 ng of DNA was used in amplification of the twelve microsatellite multiplex, with a range of 2-10 ng of input DNA.

Genotyping with the full multiplex of twelve microsatellite markers was successful in all 35 jaguars (20 females, 15 males) from 20 North American zoological institutions. Jaguar sample identification number, submitting institution name, and individual allele calls for each microsatellite locus are listed in Appendix Table 4.3. In one jaguar, the PONCE1 microsatellite locus did not amplify with single marker amplification or as part of the multiplex. This was the only indication of a possible null allele in the study population. Off-ladder alleles were detected in two microsatellite markers, PONCC1 and PONCB3. These were confirmed manually by direct peak visualization using the "overlay all" function in Peak Scanner. PCR amplification and genotyping were also repeated for these samples for confirmation. After confirmation, these off-ladder alleles were inferred to be true microvariants in these jaguars.

A chromatogram of the full multiplex from one jaguar is depicted in Figure 4.1. Jaguar amplicon size ranges for the twelve microsatellite markers were between 128-396 base pairs. Microsatellite loci were distinguished by separation of amplicons into three size ranges (128-197, 222-284, and 277-396 base pairs), and the use of a universal primer method, where each forward primer was labeled with one of four fluorescent primer sequence tags (FAM, NET, PET, and VIC) identical to the unique universal primer sequence.

These new sequences were designed to produce minimal primer-dimer formation. Allele calling was easily visualized in all 35 jaguars without stutter band or other genotyping artifact interference. Peak heights were balanced among the four microsatellite markers within each

amplicon size range before integration into the full multiplex. Once combined into the full multiplex, microsatellite markers in the larger amplicon size range had overall lower peak heights compared to markers in the mid- and small amplicon size range. This difference in peak height did not interfere with allele calling in any jaguars genotyped (Figure 4.1).

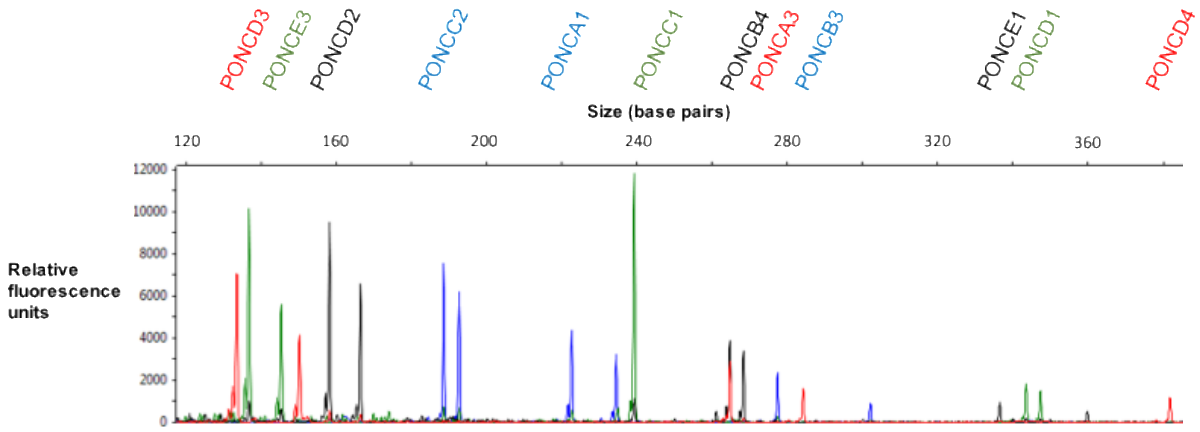

Figure 4.1: Chromatogram representing genotyping results from the full multiplex panel of all 12 microsatellite loci from one jaguar. Capillary electrophoresis was performed using the Applied Biosystems 3730*xl* DNA Analyzer and visualized using Peak Scanner V2.0 (Applied Biosystems). Each peak corresponds to a microsatellite marker amplicon separated by size (base pairs) on the x-axis. Relative fluorescence units are on the y-axis. Peaks are labeled by color according to the corresponding fluorescent primer: Red = PET, Blue = FAM, Black = NED, Green = PET. Peaks are also labeled on the x-axis by the corresponding locus ID. Genescan 500 LIZ dye size standard was used (not pictured).

Genetic diversity and identification of individuals

Number of alleles, *Ho* and H_e for each tetranucleotide (n=10) or pentanucleotide (n=2) microsatellite locus are summarized in Table 4.1. All microsatellites were polymorphic, with a range of 3-10 alleles (average 6.42 alleles per locus). The overall average H_o and H_e were 0.71 (range 0.35-0.91) and 0.73 (range 0.47-0.85), respectively.

Because some of the jaguar samples in this study were from related individuals, statistical analyses were repeated with all first-degree relatives removed, to assess impact on overall heterozygosity. The jaguar study population (n=22) H_o and H_e with first-degree relatives

removed were 0.70 and 0.74, respectively, which was nearly identical to our overall study population (n=35) heterozygosity.

Significant deviations from Hardy-Weinberg equilibrium were detected in six of twelve microsatellite loci (PONCD3, PONCD2, PONCC2, PONCB4, PONCA3, PONCE1). When statistical analysis was performed again with all first-degree relatives removed (n=22), only three microsatellite loci significantly deviated from Hardy-Weinberg (HW) equilibrium (PONCC2, PONCB4, PONCE1). After Bonferroni correction, PONCB4 was no longer significantly deviated from HW equilibrium.

Using the full multiplex, all 35 jaguar samples had a unique genotype. Probability of identity of individuals (*PI*) and siblings (*PIsibs*) were 6.7×10^{-13} and 2.1×10^{-5}, respectively, confirming that this panel can be used reliably to identify individual jaguars.

Table 4.1: Number of alleles, observed (H_o) and expected (H_e) heterozygosity at each microsatellite locus from all 35 jaguars genotyped with the full multiplex of twelve microsatellite markers. The mean jaguar study population H_o and H_e were 0.710 and 0.733, respectively.

Locus	**Number of alleles**	H_o	H_e
PONCD3	5	0.571	0.669
PONCE3	6	0.800	0.759
PONCD2	5	0.686	0.678
PONCC2	3	0.400	0.549
PONCC1	8	0.829	0.832
PONCA1	8	0.686	0.786
PONCB4	6	0.800	0.796
PONCA3	9	0.800	0.803
PONCB3	10	0.914	0.853
PONCD1	5	0.857	0.776
PONCE1	5	0.353	0.468
PONCD4	7	0.829	0.827

Discussion

The present study reports and validates a set of twelve tetra- or pentanucleotide microsatellite markers for use in the jaguar, and the development of a full multiplex panel that can be used to evaluate genetic diversity of jaguars in North American zoological institutions compared to wild and other zoo populations. This multiplex panel significantly reduces costs of genotyping and allows for minimal input DNA requirements of valuable wild animal samples. This is the largest multiplex created using a universal primer method, to our knowledge, and the only full multiplex genotyping panel of tetra- and pentanucleotides reported for use in the jaguar.

The multiplex genotyping panel developed consists of ten tetranucleotide and two pentanucleotide microsatellite repeats. Microsatellites reported in most jaguar studies to date have been derived from those previously characterized in the domestic cat (Menotti-Raymond et al. 2005 and 2009). Many microsatellite loci previously used in jaguar studies to assess genetic diversity are dinucleotide repeats (Moreno et al. 2006; Ruiz-Garcia et al. 2006; Palomares et al. 2012; Roques et al. 2014; Rueda-Zozaya et al. 2016; Zanin et al. 2016). Eizirik et al. proposed the use of tetranucleotides for more accurate allele calling in jaguars, and used ten tetranucleotide markers using microsatellites designed previously for the domestic cat (Eizirik et al. 2008; Menotti-Raymond et al. 1999; Menotti-Raymond et al. 2005). In our study, microsatellites with longer repeat length (tetra- and pentanucleotides) were also selected for multiplexing due to their reproducibility and improved accuracy of allele calls, with less interference from strand slippage during amplification and resultant stutter band formation (Weber et al. 2001; Yuan et al. 1997; Ellegren 2004).

We identified new microsatellites that have not previously been characterized for use in the jaguar, and designed primers to optimize these microsatellite markers for creation of a full multiplex.

We chose microsatellites that were not surrounded by other repeats, to minimize interference with allele calling and amplification. We also used the SW score based on the domestic cat genome to target microsatellite markers predicted to have high heterozygosity. Because of the stringent selection criteria used, we had to remove only 3 of the 15 microsatellite markers originally selected due to failure of amplification in the jaguar. One additional marker was removed due to only two alleles in the entire study population. Additionally, all automated genotyping calls using the Peak Scanner program were accurate after manual verification in all individuals, indicating this panel has reproducible and reliable genotyping results.

We also used a universal primer method, rather than direct fluorescent labeling of locus-specific forward primers, which significantly reduces genotyping costs (Blacket et al. 2012). In our experience, universal primers based on M13 and other sequences are often associated with excessive primer-dimer formation and loss of amplification for many markers. For this jaguar multiplex, we designed new universal primer sequences to minimize primer dimer formation. Although our PCR conditions are somewhat unconventional (e.g. relatively high cycle number), they produce reliable data for this universal primer system. This system uses a standard hot start Taq polymerase rather than other reliable but more expensive systems such as the Qiagen multiplex PCR kit (Qiagen, Hilden, Germany).

Two markers, PONCC2 and PONCE1 were significantly deviated from HW equilibrium after Bonferroni correction and removal of first-degree relatives from analysis due to an excess of homozygosity. These results are expected, as this study population was not from a panmictic population but rather from zoo populations obtained from diverse geographical areas.

The results of this multiplex panel show that jaguars from the North American zoo population have overall high allelic diversity (6.42 average alleles per locus) and heterozygosity (H_e = 0.73). Assessment of genetic diversity was made based on genotyping data from 35 of the 124 jaguars currently housed in North American zoological institutions. Although this represents 28% of the study population, and rare alleles may be missed, this number of individuals is likely an accurate estimate of the predominant microsatellite allele frequencies (Hale et al. 2012). Heterozygosity of North American zoo jaguars is similar to the moderate to high genetic diversity found in jaguars from Mexican (H_e = 0.65; Rueda-Zozaya et al. 2016) and Colombian (H_e= 0.68; Jimenez Gonzalez et al. 2017) zoological institutions. Jaguars from Brazilian zoos were reported to have high genetic diversity based on high number of alleles and polymorphic information content (PIC) using four dinucleotide repeats from the domestic cat (Moreno et al. 2006). Jaguars from our study also had comparable heterozygosity (H_e= 0.73) to those reported in wild populations. In Brazil, wild jaguar populations were reported to have an expected heterozygosity between 0.67 and 0.73 (Eizirik et al. 2008; Haag et al. 2010; Roques et al. 2014). Wild jaguars in Colombia had high genetic diversity, with an expected heterozygosity of 0.84 (Ruiz-Garcia et al. 2006) and 0.87 (Jimenez Gonzalez et al. 2017). In Belize and Mexico, wild jaguars had moderate levels of genetic diversity, with an expected heterozygosity range of 0.57-0.60, and 0.58-0.66, respectively (Wultsch et al. 2014; Wultsch et al. 2016; Zanin et al. 2016).

In two additional studies, genetic diversity of wild jaguars was assessed using samples from a wider geographic range, including Mesoamerican jaguars (H_e = 0.59), and jaguars ranging from Mexico to Brazil (H_e = 0.73) (Eizirik et al. 2001; Wultsch et al. 2016). Comparison of North American zoo jaguar H_e between other reported zoo and wild jaguar populations are depicted in Appendix Figures 4.2 and 4.3.

One potential pitfall of comparing reported heterozygosity from multiple studies is the use of different sets of microsatellite markers in each study. Genetic diversity comparisons between jaguar populations would be more strongly supported if a standard set of molecular markers were used.

In conclusion, jaguars from North American zoological institutions have high allelic diversity and heterozygosity, comparable to those reported in other zoo and wild jaguar populations. From these findings, we can conclude that any inbreeding in the North American zoo population has, to this time, had minimal effect on genetic diversity. We created a novel multiplex microsatellite genotyping panel for use in jaguars that is composed of a highly polymorphic set of markers. This single panel was designed to minimize costs and input DNA requirements, and optimize reproducibility and accuracy of allele calls. This multiplex can also be used to reliably distinguish between individual jaguars and examine relatedness of individuals. This panel has high potential for cross-species utility in other felids in addition to the jaguar and tiger. Initial pilot studies in our laboratory have confirmed that most microsatellites in this multiplex also amplified in other *Panthera* species, such as the snow leopard (*Panthera uncia*), and a more distantly related felid, the fishing cat (*Prionailurus viverrinus*), without any adjustment to primer design. Additional studies should also be performed to assess the usefulness of this panel on non-invasive DNA samples for use in field studies.

APPENDIX

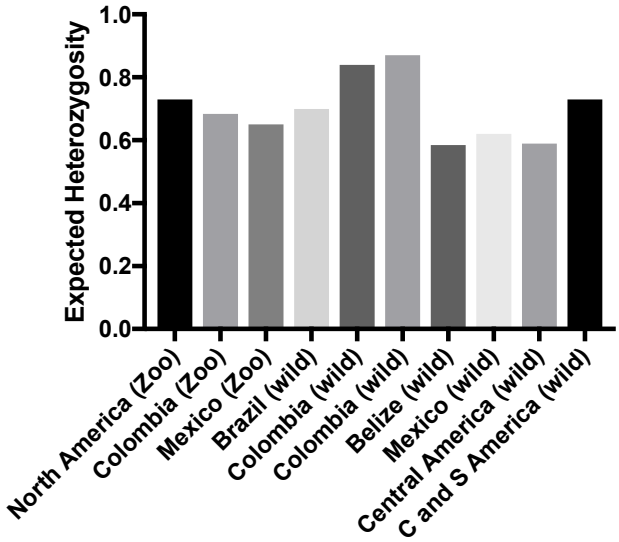

Figure 4.2: Comparison of North American zoo population expected heterozygosity (H_e) from this study with other reported zoo and wild jaguar populations. North American zoo jaguar H_e is similar to Colombian and Mexican zoo jaguar populations (Rueda-Zozaya et al. 2016; Jimenez Gonzalez et al. 2017). Among wild jaguars, populations in South America have higher reported H_e than those from Central American countries (Eizirik et al. 2001 and 2008; Haag et al 2010; Roques et al 2014; Ruiz-garcia et al. 2006; Wultsch et al. 2014 and 2016; Zanin et al. 2016).

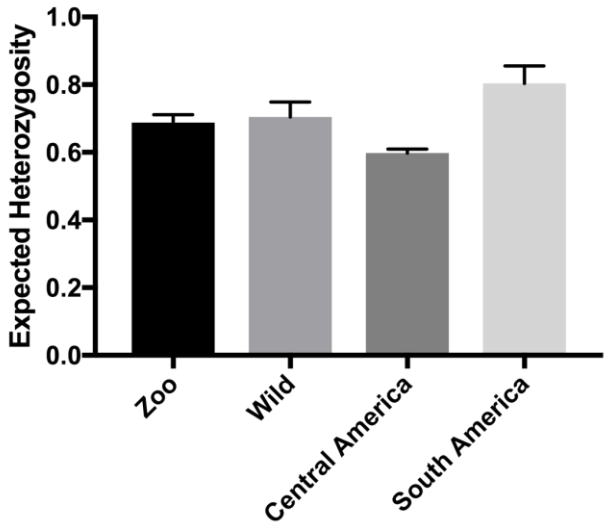

Figure 4.3: Comparison of mean zoo jaguar expected heterozygosity (H_e) with mean H_e of reported wild jaguar populations overall. Wild jaguar populations were then split by general geographic regions (Central or South America). Zoo (H_e = 0.69) and wild (H_e = 0.70) jaguars had nearly identical H_e. Mean H_e of Central American jaguars (H_e = 0.60) was significantly lower than that of South American jaguars (H_e = 0.80) using Student's unpaired, two-tailed *t*-test (*P*=0.01). Bars = Standard error. Eizirik et al. 2001 and 2008; Haag et al 2010; Roques et al 2014; Ruiz-garcia et al. 2006; Wultsch et al. 2014 and 2016; Zanin et al. 2016; Rueda-Zozaya 2016; Jimenez Gonzalez et al. 2017).

Table 4.2: Locus characteristics and multiplex design information for all 12 microsatellite loci identified in the jaguar.

Locus	Fluorescent Label[a]	Primer Sequence[b,c]	Motif	Smith-Waterman Score *Felis catus*[d]	Reference Sequence Number of Repeats *Felis catus*	Reference Sequence Number of Repeats *Panthera tigris altaica*[e]	Reference Sequence amplicon size (base pairs) *Panthera tigris altaica*	Observed Amplicon Size Range (base pairs) *Panthera onca*	Primer concentration (µM)
PONCD3	PET	F: CTTAACCATTGGCTGGATGG	TTTTC	695	13	7	131	128-150	0.024
		R: ACTCCAGCTTTGGAGGACAA							0.24
PONCE3	VIC	F: CTGTTATAGAGGCCAAGAGGAA	TTTA	612	17	12	152	129-154	0.024
		R: TGTCTCATTGGACGGGTCA							0.24
PONCD2	NED	F: CACAACAGGGCTAGGATGAA	GAAA	613	13	16	180	158-175	0.004
		R: GACACAATCTGGCACTGGAA							0.04
PONCC2	FAM	F: TATGGGAGCACCAGAAGGAA	TTTC	688	15	10	197	189-197	0.012
		R: CAGCATGGAGACTGCTTGCAA							0.12
PONCC1	VIC	F: ATCCAGGCTTCCAGTTCAAA	GAAA	772	21	13	236	222-249	0.016
		R: CCACCCTGTGCCACTACAA							0.16
PONCA1	FAM	F: GAGAGGAGCAGCCAGGAAA	GAAA	795	21	25	286	222-250	0.016
		R: ACGGAGAATGCTCCTATGAA							0.16
PONCB4	NED	F: TGGGTGATAAAGCCAAGAAA	TTTC	701	17	17	271	250-268	0.016
		R: GCGAAGGATGTCACCATGAA							0.16
PONCA3	PET	F: AGTGTCTAGAAGGTGAGTGACAGAA	GAAA	664	16	16	258	251-284	0.048
		R: CCCATTATTCTATATGAGGA							0.48
PONCB3	FAM	F: CCAGGAACCCATGATTCAAA	TTTC	711	18	9	385	277-306	0.112
		R: TCCCCTTCCACTTTCAAAAA							1.12
PONCD1	VIC	F: ACCAGGTAGGGGCTTGGTAA	TTCC	752	17	12	351	328-347	0.112
		R: ACCTGTCCCCCATCCTAGAC							1.12
PONCE1	NED	F: GCTCCTCAGGAAGTTGGAAA	TTTTC	711	15	10	390	327-360	0.128
		R: TTGGATTTCCCAGGATGAA							1.28
PONCD4	PET	F: AACCCATGGCTCTACAATCAA	TTTC	783	21	11	370	367-396	0.128
		R: CACAGCATGTGTTTCATTTCAA							1.28

Table 4.2 (Cont'd)

[a]Fluorescent labeled universal primer concentrations used are as follows:
Uni 1 - FAM: 0.32 μM
Uni 2 - PET: 0.64 μM
Uni 3 - NED: 0.32 μM
Uni 4 - VIC: 0.32 μM
[b] A 7-nucleotide (pigtail) sequence (GTTTCTT) was added to the 5' end of each reverse primer.
[c]Primers for *Panthera onca* were designed using Primer3 v.0.4.0 (Koressaar and Remm 2007; Untergasser 2007) using the *Panthera tigris altaica* PanTig 1.0 Assembly (Cho et al. 2013).
[d] *Felis catus* Smith-Waterman score was derived from RepeatMasker within the University of California Santa Cruz Genome Browser (https://genome.ucsc.edu)
[e] Predicted number of repeats for *Panthera tigris altaica* was determined using BLAST (https://blast.ncbi.nlm.nih.gov).

Table 4.3: Allele calls for all 12 microsatellite loci in 35 jaguars from North American zoological institutions.

Jaguar ID[a]	PONCD3		PONCE3		PONCD2		PONCC2		PONCC1		PONCA1		PONCB4		PONCA3		PONCB3		PONCE1		PONCD1		PONCD4	
1	150	150	137	137	158	158	189	189	235	235	222	230	265	268	273	284	277	298	327	337	340	343	374	382
2	150	150	137	137	158	158	193	197	239	243	222	234	265	268	273	276	282	**301**	337	337	343	347	374	382
3	133	133	137	154	167	171	193	193	239	243	234	250	250	254	269	276	287	**296**	337	337	336	336	367	385
4	133	133	141	145	167	171	189	189	235	243	230	242	257	261	251	255	277	306	337	337	328	343	367	374
5	133	139	141	150	162	171	189	193	222	235	234	242	261	261	273	280	282	287	337	337	336	340	367	385
6	133	145	141	141	171	171	193	193	235	249	242	242	257	261	276	276	282	**295**	327	337	340	347	374	378
7	133	145	141	145	167	175	193	193	226	231	234	242	250	261	265	276	**296**	306	327	332	340	343	374	396
8	128	139	141	141	167	171	189	193	226	235	230	238	250	257	269	273	277	302	327	337	328	336	378	382
9	128	139	145	154	171	171	189	193	226	235	230	242	257	261	259	276	287	302	327	337	328	336	382	385
10	133	133	150	154	162	162	189	193	222	239	242	242	254	261	276	280	282	298	337	337	328	336	371	385
11	133	133	141	154	162	171	189	193	222	**233**	234	242	254	261	276	280	282	298	337	337	328	336	371	385
12	133	133	129	141	158	167	189	193	222	239	238	238	250	261	273	280	277	282	327	360	336	340	374	374
13	133	145	141	141	167	171	189	189	**233**	243	242	242	254	261	251	269	287	302	337	337	328	347	367	374
14	133	133	141	154	167	171	189	189	231	243	234	242	254	261	251	269	287	306	337	337	328	347	367	374
15	133	145	141	154	167	171	189	189	231	**233**	234	242	254	254	251	269	287	306	337	337	340	347	367	378
16	133	145	141	154	167	171	189	189	233	235	242	242	254	254	269	273	287	306	337	337	328	328	371	378
17	139	145	141	154	171	171	189	189	226	235	230	234	257	257	273	276	282	302	337	337	328	336	378	385
18	133	139	141	154	171	171	189	193	226	239	234	234	250	254	273	276	282	302	337	337	328	343	371	378
19	133	150	137	145	158	167	189	193	239	239	222	234	265	268	265	284	277	302	337	360	343	347	382	382
20	139	139	145	154	167	171	189	193	**233**	235	230	230	250	257	276	276	277	302	327	337	336	343	374	378
21	133	139	145	154	171	171	189	193	**233**	235	230	242	250	257	255	276	287	302	327	337	336	343	367	378
22	133	139	145	154	167	171	189	189	226	235	230	230	250	257	269	276	287	302	327	337	328	343	367	385
23	133	133	141	145	158	171	189	193	235	239	230	234	254	265	255	276	282	298	327	337	340	343	378	396
24	139	139	137	145	162	171	197	197	231	231	226	226	265	265	276	276	277	287	351	351	340	347	374	374
25	133	139	141	141	162	167	189	189	222	222	226	234	250	261	280	280	282	282	360	360	336	343	378	385

Table 4.3 (Cont'd)

26	133	139	129	141	167	171	193	193	226	235	230	242	257	261	273	276	**301**	306	337	337	328	343	374	382
27	128	133	141	145	167	171	193	193	235	235	238	242	250	261	259	269	277	287	327	337	328	347	367	382
28	133	133	129	141	167	171	193	193	226	231	226	230	250	261	276	276	306	306	337	337	328	343	378	382
29	139	139	141	150	167	171	189	193	222	235	226	234	261	261	280	280	282	291	**NA**	**NA**	340	343	385	385
30	128	133	145	154	162	162	189	189	**233**	239	242	242	254	257	276	276	298	298	337	337	328	328	374	374
31	139	145	141	150	162	167	193	197	231	239	226	230	250	261	273	276	282	291	351	351	340	347	371	382
32	133	139	145	145	167	167	193	193	231	239	230	230	257	257	259	269	277	282	337	337	328	343	374	378
33	133	145	145	154	171	171	189	189	226	**233**	238	246	254	261	269	273	302	306	337	337	328	328	371	382
34	133	133	145	154	167	171	189	189	226	235	234	242	254	257	269	273	302	306	337	337	328	328	378	378
35	139	139	141	145	167	171	193	193	235	235	238	242	254	257	276	280	282	306	337	337	328	340	378	382

NA: Jaguar 29 had no amplification detected in single or multiplex amplification protocol at PONCE1 microsatellite locus (possible null allele).

Alleles in Bold Font: Off ladder alleles in jaguars at PONCC1 and PONCB3 microsatellite loci. These were confirmed manually by peak visualization and in duplicate multiplex amplifications.

[a]Jaguars in this study are from the following zoological institutions: Abilene Zoological Gardens, TX; Akron Zoological Park, OH; Albuquerque Biological Park, NM; Audobon Zoo, LA; Elmwood Park Zoo, PA; Happy Hollow Zoo, CA; Jacksonville Zoo and Gardens, FL; Lee Richardson Zoo, KS; Little Rock Zoological Gardens, AR; The Living Desert Zoo and Gardens, CA; Louisville Zoological Garden, KY; Mesker Park Zoo & Botanic Garden, IN; Philadelphia Zoo, PA; Reid Park Zoo, AZ; Sacramento Zoo, CA; Saint Louis Zoo, MO; Sedgwick County Zoo, KS; Tulsa Zoo, OK; Woodland Park Zoo, WA; Zoo Miami, FL.

REFERENCES

REFERENCES

Ballou JD, Lees C, Faust LJ, Long S, Lynch C, Bingaman Lackey L, Foose TJ (1996) Demographic and genetic management of captive populations. In: Kleiman DG, Thompson K, Kirk-Baer C (ed) Wild Mammals in Captivity. Univ. of Chicago Press, pp 1-73

Beguin MP, Letty J, Kaerle C, Guitton JS, Muselet L, Queney G, Pontier D (2017) A single multiplex of twelve microsatellite markers for the simultaneous study of the brown hare (*Lepus europaeus*) and the mountain hare (*Lepus timidus*). Ecol Evol 7(11):3931-3939

Blacket MJ, Robin C, Good RT, Lee SF, Miller AD (2012) Universal primers for fluorescent labelling of PCR fragments-an efficient and cost-effective approach to genotyping by fluorescence. Mol Ecol Resour 12(3):456-63

Brownstein MJ, Carpten JD, Smith JR (1996) Modulation of non-templated nucleotide addition by Taq DNA polymerase: Primer modifications that facilitate genotyping. BioTechniques 20(6): 1004–1010

Cho YS, Hu L, Hou H et al (2013) The tiger genome and comparative analysis with lion and snow leopard genomes. Nat Commun 4:2433. doi:10.1038/ncomms3433

Dou H, Yang H, Feng L, Mou P, Wang T, Ge J (2016) Estimating the population size and genetic diversity of Amur tigers in Northeast China. PLoS ONE 11(4): e0154254. https://doi.org/10.1371/journal.pone.0154254

Driscoll CA, Menotti-Raymond M, Nelson G, Goldstein D, O'Brien SJ (2002) Genomic microsatellites as evolutionary chronometers: a test in wild cats. Genome Res 12(3):414-423

Eizirik E, Haag T, Santos AS, Salzano FM, Silveira L, Azevedo FCC, Furtado MM (2008) Jaguar conservation genetics. CAT News 4:31-34

Eizirik E, Kim JH, Menotti-Raymond M, Crawshaw P, O'Brien SJ, Johnson W (2001) Phylogeography, population history and conservation genetics of jaguars (*Panthera onca*, Mammalia, Felidae). Mol Ecol 10(1):65-79

Ellegren H (2004) Microsatellites: simple sequences with complex evolution. Nat Rev Genet 5(6):435-45

Figueiró HV, Li G, Trindade FJ et al (2017) Genome-wide signatures of complex introgression and adaptive evolution in the big cats. Sci Adv 3(7):e1700299. DOI: 10.1126/sciadv.1700299

Frankham R (1995) Conservation Genetics. Annu Rev Genetics 29:305-327

Haag T, Santos AS, Sana DA, Morato RG, Cullen Jr. L, Crawshaw Jr. PG, De Angelo C, Di Bitetti MS, Salzano FM, Eizirik E (2010) The effect of habitat fragmentation on the genetic structure of a top predator: loss of diversity and high differentiation among remnant populations of Atlantic Forest jaguars (*Panthera onca*). Mol Ecol 19:4906-4921

Hale ML, Burg TM, Steeves TE (2012) Sampling for microsatellite-based population genetic studies: 25 to 30 individuals per population is enough to accurately estimate allele frequencies. PLoS ONE 7(9): e45170. doi:10.1371/journal.pone.0045170

Innis M, Gelfand DH (1999) Optimization of PCR: conversations between Michael and David. In: Innis M, Gelfand DH, Sninsky JJ (ed) PCR Applications: Protocols for functional genomics. Academic Press, New York. pp 3-22.

Jimenez Gonzalez S, Ruiz Garcia M, Maldonado J, et al. (2017) Genetic characterization of jaguars (*Panthera onca*) in captivity in zoological parks of Colombia. In: Singh KP (ed) Big Cats, 1st edn. Intech, pp 73-91

Johnson WE, Eizirik E, Pecon-Slattery J, Murphy WJ, Antunes A, Teeling E, O'Brien SJ (2006) The late Miocene radiation of modern felidae: a genetic assessment. Science 311 (5757): 73-77

Koressaar T, Remm M (2007) Enhancements and modifications of primer design program Primer3. Bioinformatics 23(10):1289-91

Menotti-Raymond M, David VA, Lyons LA, Schaffer AA, Tomlin JF, Hutton MK, O'Brien SJ (1999) A genetic linkage map of microsatellites in the domestic cat (*Felis catus*). Genomics 57(1):9-23

Menotti-Raymond M, David VA, Wachter LL, Butler JM, O'Brien SJ (2005) An STR forensic typing system for genetic individualization of domestic cat (*Felis catus*) samples. J Forensic Sci 50(5): 1-10

Menotti-Raymond M, O'Brien SJ (1995) Evolutionary conservation of ten microsatellite loci in four species of Felidae. J Hered 86(4): 319-322

Miller Sm, Harper CK, Bloomer P, Hofmeyr J, Funston PJ (2014) Evaluation of microsatellite markers for populations studies and forensic identification of African lions (*Panthera leo*). J Hered 105(6):762-72

Mondol S, Navya R, Athreya V, Sunagar K, Selvaraj VM, Ramakrishnan U (2009) A panel of microsatellites to individually identify leopards and its application to leopard monitoring in human dominated landscapes. BMC Genet 10(79):1-7

Montgomery ME, Ballou JD, Nurthen RK, England PR, Briscoe DA, Frankham R (1997) Minimizing kinship in captive breeding programs. Zoo Biol 16 (5): 377-389

Moreno VR, Griolia AB, Campagnari F, Milazzotto M, Adania CH, Garcia JF, Souza, Barreiros de Souza E (2006) Genetic variability of *Herpailurus yagouaroundi*, *Puma concolor* and *Panthera onca* (Mammalia, Felidae) studied using *Felis catus* microsatellites. Genet Mol Biol *29*(2):290-293

Oetting WS, Lee HK, Flanders DJ, Wiesner GL, Sellers TA, King RA (1995) Linkage analysis with multiplexed short tandem repeat polymorphisms using infrared fluorescence and M13 tailed primers. Genomics 30(3):450-458

Palomares F, Roques S, Chávez C, Silveira L, Keller C, Sollmann R, et al. (2012) High proportion of male faeces in jaguar populations. PLoS ONE 7(12): e52923. https://doi.org/10.1371/journal.pone.0052923

Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. Bioinformatics 28:2537–2539

Quigley, H., Foster, R., Petracca, L., Payan, E., Salom, R. & Harmsen, B. 2017. *Panthera onca*. The IUCN Red List of Threatened Species 2017: e.T15953A50658693. http://dx.doi.org/10.2305/IUCN.UK.2017-3.RLTS.T15953A50658693.en.

Roques S, Furtado M, Jácomo ATA, Silveira L, Sollmann R Torres N Godoy J, Palomares F (2014) Monitoring jaguar populations *Panthera onca* with non-invasive genetics: A pilot study in Brazilian ecosystems. Oryx 48(3): 361-369

Rueda-Zozaya P, Mendoza-Martínez GD, Martínez-Gómez D et al (2016) Genetic variability and structure of jaguar (*Panthera onca*) in Mexican zoos. Genetica 144:59-69

Ruiz-Garcia M, Payán E, Murillo A, Alvarez D (2006) DNA microsatellite characterization of the jaguar (*Panthera onca*) in Colombia. Genes & Genetic Systems 81(2):115-127

Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. Nat Biotechnol 18:233-234

Selkoe KA, Toonen RJ (2006) Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. Ecol Lett 9(5):615-629

Sharma R, Stuckas H, Moll K, Khan I, Bhaskar R, Goyal SP, Tiedemann R (2008) Fourteen new di- and tetranucleotide microsatellite loci for the critically endangered Indian tiger (*Panthera tigris tigris*). Mol Ecol Resour 8(6):1480-1482

Simons ND, Wagner RS, Lorenz JG (2012) Genetic diversity of North American captive-born gorillas (*Gorilla gorilla gorilla*). Ecol Evol 3(1):80-88

Soares TN, Telles MPC, Resende LV, Silveira L, Jácomo ATA, Morato RG, Diniz-Filho JAF, Eizirik E, Brondani RPV, Brondani C (2006) Paternity testing and behavioral ecology: a case study of jaguars (*Panthera onca*) in Emas National Park, Central Brazil. Genet Mol Biol 29(4):735-740

Souza ASM de Castro, Saranholi, BH, Crawshaw Jr. PG, Paviolo AJ, Rampim LE, Sartorello L, Galetti Jr. PM (2017) Re-discovering jaguar in remaining coastal Atlantic Forest in southeastern Brazil by non-invasive DNA analysis. Biota Neotropica 17(2):e20170358. https://dx.doi.org/10.1590/1676-0611-bn-2017-0358

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG (2012) Primer3 - new capabilities and interfaces. Nucleic Acids Res 40(15):e115. https://doi.org/10.1093/nar/gks596

Weber J, Broman K (2001) Genotyping for human whole-genome scans: past, present, and future. In: Demerec M (ed) Advances in Genetics, Vol. 2. Academic Press, New York. pp 77-96.

Wultsch C, Caragiulo A, Dias-Freedman I, Quigley H, Rabinowitz S, Amato G (2016) Genetic diversity and population structure of Mesoamerican Jaguars (*Panthera onca*): Implications for conservation and management. PLoS ONE 11(10):e0162377. https://doi.org/10.1371/journal.pone.0162377

Wultsch C, Waits LP, Kelly MJ (2014) Noninvasive individual and species identification of jaguars (*Panthera onca*), pumas (*Puma concolor*) and ocelots (*Leopardus pardalis*) in Belize, Central America using cross-species microsatellites and faecal DNA. Mol Ecol Resour 14(6):1171-82

Wultsch C, Waits LP, Kelly MJ (2016) A comparative analysis of genetic diversity and structure in jaguars (*Panthera onca*), pumas (*Puma concolor*), and ocelots (*Leopardus pardalis*) in fragmented landscapes of a critical mesoamerican linkage zone. PLoS ONE 11(3): e0151043. doi:10.1371/journal.pone.0151043

Yuan B, Vaske D, Weber J, Beck J, Sheffield VC (1997) Improved set of short-tandem-repeat polymorphisms for screening the human genome. Am J Hum Genet 60:459-460

Zanin M, Adrados B, González N, Roques S, Brito D, Chávez C, Rubio Y, Palomares F (2016) Gene flow and genetic structure of the puma and jaguar in Mexico. Eur J Wildl Res 62(4):461-469

Zou ZT, Uphyrkina OV, Fomenko P, Luo SJ (2015) The development and application of a multiplex short tandem repeat (STR) system for identifying subspecies, individuals and sex in tigers. Integr Zool 10(4):376-388

CHAPTER 5

Investigating the pathogenesis of ovarian carcinoma in North American zoo jaguars (*Panthera onca*) by identification of candidate germline variants using a custom next-generation sequencing cancer gene panel

Abstract

Jaguars (*Panthera onca*) are near threatened throughout their entire geographic range and endangered in the United States. Female jaguars in North American zoological institutions have high prevalence of ovarian carcinomas that cause morbidity and mortality in adult females, including those of breeding age. In women, in addition to breast cancer, 5-10% of all epithelial ovarian cancer and are associated with an inherited germline mutation in tumor suppressor genes, most commonly BRCA1 and BRCA2. Ovarian carcinoma in jaguars occurs in an autosomal dominant pattern based on pedigree analysis, and an inherited germline mutation is proposed as a potential mechanism of carcinogenesis. Using a targeted next-generation sequencing approach, we sequenced the coding regions of 276 cancer genes in jaguars to identify candidate germline variants that may play a role in the pathogenesis of ovarian carcinoma, using the domestic cat as a reference genome. A paired sample approach was used, and most samples were of formalin-fixed paraffin embedded (FFPE) origin. In high-quality DNA samples (frozen tissues, whole blood), 99% of target base pairs were covered. Two sequencing platforms were used; the Illumina HiSeq 2500 with sample multiplexing during library preparation, and the NextSeq-Mid with single sample amplification. Overall, approximately 40% of samples were successfully sequenced at a depth of at least 13x. On the HiSeq platform, mean depth for all samples (n=12) was 258x. Mean depth for frozen samples (n=10; tissue, blood, or cell lines) was 297x, and for FFPE samples (n=2) was 114x. On the NextSeq-Mid platform, mean coverage for all samples (n=13) was 61x. Mean coverage for frozen samples (n=4; whole blood, cell line, frozen ovary) was 84x, and for FFPE samples (n=9) was 51x. Single-sample library amplification resulted in successful sequencing of more FFPE-derived samples than multiplex library amplification.

Increasing time in formalin of FFPE samples had a significant negative correlation with number of raw reads and starting DNA concentration. No frameshift, stop lost, or stop gained variants were found in any genes (n=276). Two heterozygous, missense germline variants were detected in BRCA1 exon 9 (c.1173G>T_p.Ala312Glu) and BRCA2 exon 11 (c.3732C>G_p.Ser1248Arg) in the jaguar, and were predicted to be functionally deleterious. The BRCA1 variant was in 20% of jaguars with ovarian carcinoma, while at least one copy of the BRCA2 variant was in all jaguars with ovarian carcinoma. Loss of heterozygosity in the tumor tissue was not detected for either variant. The BRCA2 variant has also been reported in a human patient with hereditary breast and ovarian cancer syndrome, but the functional significance is unknown. We performed a functional study on the BRCA2 missense variant using a CRISPR/CAS strategy and evaluated cell viability using a cisplatin sensitivity assay. We successfully introduced the BRCA2 S1248R variant into human embryonic kidney (HEK) 293T cells. We also created BRCA2 -/- cells by inserting a large deletion into BRCA2 exon 11. BRCA2 -/- cells had increased cisplatin sensitivity (P=0.002) compared to the S1248R cells and wild type (WT) cells. S1248R cells had intermediate cisplatin sensitivity compared to WT and BRCA2 -/- cells at the highest cisplatin concentration (P=0.01). This variant is being analyzed further by homology-directed repair assays and Western blot. In conclusion, we have identified a missense germline variant in BRCA2 that is in all jaguars with ovarian carcinoma and in some jaguars in the unaffected zoo population that demonstrates increased cisplatin sensitivity compared to WT using a HEK293T cell background.

Introduction

Jaguars (*Panthera onca*) are near threatened throughout their entire geographic range and endangered in the United States (Quigley et al. 2017). Female jaguars in North American zoological institutions have high prevalence of ovarian carcinomas that cause morbidity and mortality by implantation throughout the peritoneal cavity (Maryamma KI et al. 1974; Bossart and Hubbell 1983; Munson 1994; Kazensky 1998; Hope and Deem 2006; Bryan et al. 2015). Ovarian carcinoma is extremely rare in domestic cats and has not been reported in other wild or zoo-managed felids (Gelberg and McEntee 1985). The use of exogenous progestins, such as melengestrol acetate (MGA), in zoo felids, including jaguars, is associated with endometrial hyperplasia, endometrial carcinoma, and mammary carcinoma (Munson et al. 1995; Harrenstein et al. 1996; Munson et al. 2002; Munson 2006; McAloose et al. 2007). In contrast, we have found that ovarian carcinoma in jaguars is not significantly associated with MGA exposure, and the disease occurs in an autosomal dominant pattern based on pedigree analysis of affected animals. Because of these findings, as well as the high prevalence in jaguars, a genetic predisposition is proposed as a potential mechanism of carcinogenesis.

In women, in addition to breast cancer, 5-10% of all epithelial ovarian cancer and are associated with an inherited germline mutation in tumor suppressor genes, most commonly BRCA1 and BRCA2 (Raymus and Gayther 2009; Petrucelli et al. 2010; Meindl et al. 2011). Specific germline mutations in BRCA1 or BRCA2 confer high risk of breast and/or ovarian cancer and are classified as highly penetrant (Meindl et al. 2011).

The most common BRCA1 and BRCA2 mutations associated with ovarian and breast cancer are small insertions and deletions resulting in complete loss of protein function (Petrucelli et al. 2010).

BRCA1 and BRCA2 proteins play critical roles in the maintenance of genomic stability by regulating repair of DNA double-strand breaks through the homologous recombination (HR) pathway (Welsch et al. 2000; Roy et al. 2012). In heterozygous germline mutation carriers, loss of the wild-type allele in tumor tissue along with additional somatic mutations in genes involved in cell-cycle control, such as p53, are associated with ovarian cancer development (Maxwell et al. 2017). Tumor cells deficient in BRCA1 or BRCA2 function are extremely sensitive to DNA damaging agents that are also used as chemotherapeutics in the treatment of ovarian cancer, such as cisplatin and poly (ADP-ribose) polymerase (PARP) inhibitors (Bhattacharyya et al. 2000; Bryant et al. 2005). Germline mutations in additional tumor suppressor genes that function in DNA repair and interact directly with BRCA1 and BRCA2 are also associated with hereditary ovarian cancer and other cancers in humans with variable penetrance (Zhang et al. 2009; Pelltari et al. 2011; Rafnar et al. 2011; Roy et al. 2012; Lawrenson et al. 2015). The overall goal of this study was to identify candidate germline variants in key tumor suppressor genes that may be associated with ovarian carcinoma in this population of North American zoo jaguars. If a germline mutation is found, knowledge of carrier status in individual animals could inform future breeding, medical and management decisions.

The aims of this study were to (1) develop and utilize a custom next-generation sequencing panel of cancer genes based on the domestic cat reference sequence for cross-species use in the jaguar, (2) identify candidate germline variants and assess loss of heterozygosity in ovarian carcinoma tissue of zoo jaguars using a paired sample approach, and (3) assess the functional significance of a BRCA2 missense variant using a CRISPR-CAS strategy and cisplatin sensitivity assay.

Materials and Methods

Sample origin and genomic DNA extraction for next-generation sequencing

All jaguar samples originated from tissues or blood submitted by zoological institutions to the Association of Zoos & Aquariums Reproductive Management Center's Reproductive Health Surveillance Program at Michigan State University and were approved for use under a MSU IACUC exemption. In total, 36 samples were sequenced on the Illumina HiSeq 2500 platform, and 30 samples on the NextSeq 500 platform. The type and origin of samples used for both sequencing runs are summarized in Appendix Tables 5.2 and 5.3. Two jaguar ovarian carcinoma cell lines that were sequenced were initiated in our lab and originated from fresh jaguar ovaries submitted to the RHSP. Domestic cat control DNA samples (mesenchymal cell lines and frozen ovary) were obtained from other studies in our laboratory. Genomic DNA was extracted from FFPE tissue, frozen tissue, cultured cells, and whole blood for sequencing. For FFPE tissues, isolation of tumor tissue was confirmed by visualization on hematoxylin & eosin stained slides. Necrosis, hemorrhage, cystic, and poorly cellular areas were avoided. Tissue was then removed from the paraffin block using a scalpel blade. Corresponding normal tissues were chosen based on availability from each case (usually uterine endometrium and myometrium). FFPE DNA was extracted using the QIAmp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) and Qiagen deparaffinization solution protocol.

Whole blood or cell culture genomic DNA was extracted using the Qiagen DNEasy Blood & Tissue Kit using the manufacturer's protocol. DNA was quantified using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA).

Design of a targeted next-generation sequencing cancer gene panel

A targeted library preparation and next-generation sequencing approach was used to evaluate exon sequences of multiple cancer genes (n=276) in paired tumor and normal jaguar samples. The NuGEN Ovation Targeted Enrichment System (NuGEN, San Carlos CA) was chosen due to the compatibility and specific design for low quality FFPE samples. This platform also has low input DNA requirements (10-500 ng DNA). Custom probes were designed by NuGEN for the exon sequences of 276 genes that we selected using the domestic cat reference genome (*Felis catus* GCA_000181335.2), with a total target size of 1.8 million base pairs (Mb). Other *Panthera* sp. genomes are not well-annotated, and the jaguar genome, which is not assembled or annotated, was not released until after initiation of this project (Figueiró et al. 2017). Genes involved in the pathogenesis of epithelial cancers were selected from Catalogue of Somatic Mutations in Cancer (COSMIC: http://cancer.sanger.ac.uk/cosmic) and current literature to include more recently discovered genes implicated in inherited ovarian and breast cancer in humans (Futreal PA et al. 2004; Cybulski et al. 2015).

Genes with known germline and somatic mutations in epithelial cancer, including ovarian and breast cancer, were included (Appendix Table 5.1). Broad classifications of the gene list include oncogenes (growth factors, growth factor receptors, signal transduction and nuclear regulatory proteins, cell cycle regulators), tumor suppressor genes (cell cycle regulators, DNA repair, apoptotic pathways) and other hormone receptors, such as estrogen, progesterone and androgen receptors.

Library preparation, sequencing, and analysis

Two sequencing runs were performed using different library preparation and sequencing methods. In the first round of sequencing, library preparation of 36 samples was performed using a sample multiplex step prior to library amplification. In the second round of sequencing, 30 samples, including those FFPE samples that did not perform well in the initial sequencing run, were sequenced using single-sample library preparation and amplification.

The workflow summary for library preparation is as follows: 10-500 ng of input genomic DNA is fragmented to 500 base pairs by sonication (Covaris, Woburn MA). DNA ends are repaired, followed by ligation of index forward adaptors for Illumina sequencing with barcode at both ends of randomly fragmented DNA. In a multiplex step (or singleplex), adaptor-ligated DNA is mixed with targeting probes and denatured to allow annealing of probes 3' of the target regions on both strands. The probes have the Illumina reverse adaptor attached to the 5' end. Hybridized probes are extended through the target region on both strands, continuing to the end of the forward adaptor. This results in a library containing the target region as well as both the forward and reverse adaptor sequences. After denaturation, PCR primers are annealed and the target-enriched library is amplified by PCR.

After library preparation, sample quality control was performed by MSU Research Technology Support Facility (RTSF), including quantification of libraries on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA). Libraries were normalized by diluting to equal concentrations before sample pooling and sequencing. The pool was then quantified using the Kapa Biosystems Illumina Library Quantification qPCR kit (Illumina, San Diego CA). Sequencing was performed by the MSU RTSF Genomics Core.

For the first round of sequencing, 100 bp paired end reads were generated on the Illumina HiSeq 2500 platform. For the second round of sequencing, 150 bp single end reads were generated on the Illumina NextSeq 500 Mid platform.

After sequencing, FASTQ files were processed and analyzed similarly for both sequencing runs, as follows: Quality control of raw reads (FASTQC http://www.bioinformatics.babraham.ac.uk/projects/fastqc/); Pre-processing, removal of adaptor sequences and low quality base pairs (CutAdapt - DOI:10.14806/ej.17.1.200 and BBduk - http://www.geneious.com/plugins/bbduk); Alignment to the reference genome (*Felis catus* GCA_000181335.2): Bowtie2 (Langmead and Salzberg, 2012); Generation of SAM and BAM files: SAMtools (Li et al. 2009); Post-alignment processing and removal of duplicate reads: NuDup (ttps://github.com/nugentechnologies/nudup) and Picard tools (http://broadinstitute.github.io/picard/); Variant calling: Freebayes (Garrison and Marth 2012) and GATK (McKenna et al. 2010); Variant annotation Snpeff (http://snpeff.sourceforge.net/) and VARSCAN2 (Koboldt et al. 2012).

Variant filtration and selection of candidate germline variants

The domestic cat was used as the reference genome due to availability of the assembled and annotated genome (*Felis catus* GCA_000181335.2). Other felids from the genus *Panthera* are more closely related to the jaguar and are expected to share more sequence similarity with the jaguar in coding regions (Cho et al. 2013; Figueiró et al. 2017). To narrow down candidate germline variants of interest with potential functional significance associated with ovarian carcinoma in the jaguar, all variants called using prediction software in jaguar candidate genes were compared to the *Panthera tigris altaica* genome (Cho et al. 2013).

All variants shared between the jaguar and tiger were removed as candidates, and were inferred to be the jaguar wild type sequence compared to the domestic cat. Next, all synonymous variants (i.e. those variants without a predicted change in the protein sequence) were removed from further analysis. All nonsynonymous germline variants for each candidate gene were analyzed with the following protein functional analysis programs: SIFT (Kumar et al 2009), PolyPhen-2 (Adzhubei et al. 2010**)** and PROVEAN (Choi et al. 2012). Functional predictions were determined by assessing the predicted amino acid change in the domestic cat and human protein sequences. Those variant sequences predicted to be deleterious/possibly damaging were further validated by restriction enzyme digestion and/or Sanger sequencing, resulting in genotyping of the entire zoo jaguar study population.

Statistical analysis

The presence of candidate germline variants and genotypes in the jaguars with ovarian carcinoma compared to the overall zoo jaguar population were analyzed using Fisher's exact tests or Chi-square analysis. Association between input DNA concentration and time in formalin to number of raw reads was examined by the nonparametric, two-tailed, Spearman's rank correlation coefficient. Data were tested for normality using D'Agostino & Pearson test. Differences in cell viability (based on percentage of MTT reduction) between cell lines with the S1248R variant, BRCA2 -/-, or wild type cells were compared using one-way ANOVA and Student's unpaired, two-tailed *t*-test. Significance was set at $p=<0.05$. Statistical analyses were performed using GraphPad Prism 7.0.

Evaluation of allelic imbalance in candidate genes using microsatellites

Eleven candidate genes hypothesized to be associated with ovarian carcinoma in jaguars were evaluated for allelic imbalance using newly identified microsatellites in the jaguar (Appendix Table 5.8). STK11/LKB1 was not evaluated due to lack of identification of suitable microsatellite markers in this region. Paired samples (frozen ovarian carcinoma and blood) from two jaguars were assessed. All other paired ovarian carcinoma and normal tissue samples were FFPE, and PCR amplification for most microsatellite markers was not successful in these samples, including after redesign for shorter amplicon sizes. Most amplicons greater than 100 bp failed to amplify in these FFPE tissues. Tetranucleotide microsatellite repeats were identified at the 5' and 3' end of each gene using the RepeatMasker track of the UCSC Genome Browser (https://genome.ucsc.edu) domestic cat genome (*Felis catus*_6.2). Primer design for jaguars was completed using the Amur tiger (*Panthera tigris altaica*) genome. Locus-specific primers were designed using Primer3 v.0.4.0 (Koressaar and Remm 2007; Untergasser et al. 2012). Primer sequences for each microsatellite locus are listed in Appendix Table 5.8. A universal primer labeling method was used. Microsatellites were first tested for amplification in the jaguar, then arranged into four multiplexes based on universal primer label and amplicon size.
Each forward primer was 5' labeled with an identical sequence tag to one of four fluorescent dye-labeled universal primers (FAM, PET, NED, or VIC). Universal primer sequences are listed in Appendix Table 5.9.

PCR reactions were prepared in a final volume of 12.5 µl, and included 10x PCR Buffer (20 mM Tris HCL [pH 8.4] and 50 mM KCl), 0.2 mM dNTPs, 2 mM $MgCl_2$, 0.08 uM primer, 0.04 U/µl AmpliTaq Gold (Applied Biosystems, Foster City, CA), and 2-10 ng DNA.

PCR amplification was performed on a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA) under the following cycling conditions: An initial denaturation step of 10 minutes at 94°C, followed by 50 cycles of 1 minute at 94°C, 2 minutes at 57°C, and 3 minutes at 72°C. A final extension step was performed for 60 minutes at 72°C, followed by 20 minutes at 20°C.

PCR amplicons were submitted to the MSU RTSF Genomics Core for high-resolution genotyping using a 3730xl DNA Analyzer with a Genescan 500 LIZ dye size standard (Applied Biosystems, Foster City, CA). Fragment analysis was performed using Peak Scanner Software Version 2.0 (Applied Biosystems, Foster City, CA). Allele calls were confirmed manually, and peak heights were recorded for each microsatellite locus and sample.

To assess allelic imbalance, peak height ratios were calculated using a previously described formula (Paulson et al. 1999): T1/T2 = Peak height of the smaller tumor allele (T1) divided by the peak height of the larger tumor allele (T2), and N1/N2 = Peak height of the smaller normal tissue allele (N1) divided by peak height of the larger normal tissue allele (N2). The presence of allelic imbalance indicative of loss of heterozygosity was interpreted as the peak height ratio of the tumor tissue decreased by 50% or more than the peak height ratio of the normal tissue. Loci that were homozygous or that did not amplify in any sample were considered non-informative.

Assessment of select BRCA1 and BRCA2 germline variants in the zoo jaguar population

Two candidate variants were selected for validation based on the filtration parameters described above. A single nucleotide change (G>T) in exon 9 of the jaguar BRCA1 gene was assessed in genomic DNA samples from 15 paired jaguar tissues (ovarian carcinoma and normal), 12 domestic cats, and 32 additional male and female jaguars. Animals were genotyped using Sanger sequencing. A single nucleotide change (C>G) in exon 11 of the jaguar BRCA2 gene was assessed in genomic DNA samples from 15 paired jaguar tissues (ovarian carcinoma and normal), 12 domestic cats, and 29 additional male and female jaguars. Animals were genotyped using Sanger sequencing and restriction enzyme digestion (HPY99I; New England Biolabs, Ipswitch MA). Primer sequences are listed in Appendix Table 5.9.

Functional analysis of a germline BRCA2 S1248R missense substitution discovered in the jaguar using a CRISPR-CAS strategy and cisplatin sensitivity assay

Human embryonic kidney (HEK293T) cells (ATCC, Manassas, VA) were used in a CRISPR/CAS9-based method for introduction of a single nucleotide change that encodes a S1248R missense substitution in BRCA2 exon 11. This germline variant was discovered that is unique to jaguars and is present in the general zoo jaguar population as well as at least one allele in all jaguars with ovarian carcinoma. HEK 293T cells were maintained in general media consisting of DMEM supplemented with L-glutamine and sodium pyruvate, non-essential amino acids (NEAA), 10% fetal bovine serum, antibiotic-antimycotic, and 10 ug/ml ciprofloxacin, and were maintained at 37 °C in 5% CO_2. A CRISPR-CAS method was used to introduce a ~150 bp deletion into BRCA2 exon 11 to create BRCA2 null cells, or to introduce the single S1248R amino acid substitution to assess functional significance of this variant compared to wild type 293T cells and cells with a large deletion in exon 11 (BRCA2 -/-).

Cas9 gene targeting in 293T cells was performed according to established protocols (Mali et al. 2013). In summary, a targeting guide RNA (gRNA) was designed to cut at the single nucleotide that encodes the S1248R variant. 293T cells were transfected with the targeting gRNA and a homology-directed repair (HDR) oligonucleotide that serves as a template for HDR-based introduction of the nucleotide changes that encode the S1248R substitution. A second gRNA was introduced into some cells to create a large deletion in BRCA2 exon 11 for generation of BRCA2 -/- variants. For transfection, the targeting gRNA or deletion gRNA were ligated into a pCAS2Apuro vector (Addgene, Cambridge, MA) that expresses Cas9 and a puromycin resistant gene for selection. This vector was transformed into DH5 competent cells (Thermo Fisher Scientific) for transfection. 293T cells were transfected with 2 ug of vector containing the targeting gRNA along with the HDR guide oligonucleotide sequence (to create S1248R), or the targeting gRNA and deletion gRNA (to create BRCA2 -/-) in 200 ul Optimem (Life Technologies) and 4 ul polyethylenimine (PEI, 1 ug/ml, Polysciences). After 48 hours, cells were re-plated in general media supplemented with 1 ug/ml puromycin for selection of transfected clones. Clones were selected and DNA was extracted for screening using the Qiagen Blood & Tissue Kit according to the manufacturers protocol. A restriction enzyme (NRUI; New England Biolabs, Ipswitch MA) was used to target the specific nucleotides that were introduced to encode the S>R substitution for screening of clones to confirm the presence of homozygous knock-in variants.

Various clones were isolated that lacked a wild type PCR fragment after NRUI digestion, and others that had the S>R substitution along with a wild type fragment. Two clones harboring the deletion were also generated.

All clones harboring the S>R substitution and BRCA2 -/- deletion were confirmed for presence of the desired substitution or deletion, and lack of a wild type allele with TA cloning (TOPO TA Cloning kit, Invitrogen, Carlsbad CA) and Sanger sequencing. Primer sequences for CRISPR/CAS experiments are listed in Appendix Table 5.9.

Cisplatin sensitivity assays were used to assess function of homologous recombination in the BRCA2 -/- transfected 293T cells, those harboring the S>R substitution of interest, and wild type 293T cells. 10,000 cells were plated in each well of a 24-well plate in media without cisplatin, and in media with increasing concentrations of cisplatin (4, 8, and 12 uM). All experiments were performed in triplicate. Cell viability was assessed after 5 days using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium colorimetric assay (Sigma Aldrich – Millipore Sigma, Burlington MA). Viable cells are measured by the ability to reduce tetrazolium dye to formazan by NADPH-dependent oxidoreductase. Cells were incubated with MTT reagent for 1 hour at 37 °C, followed by dissolution with acidic isopropanol. Formazan product was measured by absorbance on an Envision Multimode plate reader (PerkinElmer, Waltham MA) at a wavelength of 570 nm.

Results

Performance of a targeted next-generation sequencing cancer gene panel

Two library preparation and sequencing methods were used. Summary of samples sequenced, input DNA concentration, number of raw reads, total Gbp sequenced, and overall coverage for each sequencing platform are summarized in Appendix Tables 5.2 and 5.3.

36 samples were first sequenced on the Illumina HiSeq 2500 platform using 100 bp paired end reads. Library preparation for this platform included a multiplex step prior to library amplification. Twelve of 36 samples (33%) had >300,000 raw reads and were included in downstream processing. These included 10 samples derived from frozen tissues, whole blood, or cell lines. Two additional samples were of FFPE origin. The remaining samples that were excluded from further processing had a mean coverage of 0x and were all FFPE (n=24). Mean coverage for all samples (n=12) was 258x. Mean coverage for frozen samples (n=10; tissue, blood, or cell lines) was 297x, and for FFPE samples (n=2) was 114x. Input DNA concentrations (pre-library preparation) were highly variable, and ranged from 50-500 ng.

30 samples were also sequenced on the NextSeq-Mid 500 platform using 150 bp single end reads. This sequencing run was performed using singleplex library preparation; no samples were multiplexed prior to amplification of the libraries to avoid bias of higher quality samples during PCR amplification and probe hybridization. This sequencing platform was chosen based on the number of reads required for each sample to have at least 100x coverage. Thirteen of 30 samples (43%) had >700,000 raw reads and at least 13x depth and were included in downstream processing. Overall, a higher number of FFPE samples had adequate coverage and number of raw reads using singleplex library amplification. Samples included in downstream processing were derived from frozen blood, tissue, or cell lines (n=4), and the remaining were FFPE tissues (n=9). Mean coverage for all samples (n=13) was 61x. Mean coverage for frozen samples (n=4; whole blood, cell line, frozen ovary) was 84x, and for FFPE samples (n=9) was 51x. Input DNA concentrations in this sequencing run were also highly variable, ranging from 53-500 ng.

Total samples from both platforms with adequate sequencing coverage for further analysis included jaguars from the currently healthy population (n=4), domestic cat controls (n=5), paired ovarian carcinoma and normal tissue (n=4), and corresponding cell lines from two jaguar ovarian carcinomas (n=2).

The probes used in this assay targeting 276 cancer genes were designed from the domestic cat reference genome, and captured the majority of target regions in the jaguar. On both platforms, samples with the highest sequencing success had 99% of total base pairs in the target region (1.8 Mbp) covered. In all samples, all regions of the top twelve select candidate genes had at least 5x coverage.

Statistical analysis was performed to assess the effect of sample time in formalin and input DNA concentration (pre-library preparation) with number of raw reads generated and overall coverage per sample. Increasing time in formalin had a significant negative correlation with number of raw reads per sample ($r= -0.57$, $p<0.0001$) and starting DNA concentration ($r= -0.4421$, $p=0.0021$) on the HiSeq 2500 sequencing platform, and no significant correlation on the NextSeq 500 platform. There was also no significant correlation between input DNA concentration or months in formalin and coverage for either sequencing run.

Variant filtration and selection of candidate germline variants

After sequencing, samples were run through a processing pipeline, followed by assessment of two variant callers, GATK and Freebayes. Variants were manually reviewed in the sequencing data for candidate genes in all jaguar samples. GATK was determined to have higher sensitivity, which led to more false positives than Freebayes, predominantly regarding false calling of indels at the ends of target regions. For example, in the jaguar sample with the best

sequencing quality from the HiSEQ 2500 platform, there were 67,296 total variants called using GATK (6682 insertions, 3278 deletions), and 58,952 total variants called using Freebayes (1300 insertions, 1406 deletions). Freebayes was determined to be the most accurate variant calling program, based on visual inspection of the sequencing reads to verify that each variant was present in all reads at that sequence location.

Summary of variants by predicted protein effect (3' or 5' untranslated region, frameshift, missense, synonymous, stop gained, or stop lost) are summarized in Appendix Tables 5.4 (whole blood from healthy jaguars and domestic cat controls) and Appendix Table 5.5 (paired ovarian carcinoma and normal jaguar tissue samples and corresponding cell lines). Taking into account synonymous and missense single nucleotide changes in the coding regions of 276 genes (approximately 1.8 Mb target region), the highest quality jaguar sample had approximately 1 nucleotide difference every 317 base pairs, compared to 1 nucleotide change every 1238 base pairs in the domestic cat sample. For the purposes of this study, we focused on high impact germline variants in the coding regions of candidate cancer genes. In the entire cancer gene panel (n=276), all frameshift, stop gained and stop lost variants were confirmed to be false reads based on visualization in the raw sequence using Integrative Genomics Viewer (Robinson et al. 2011). These false variants were present in <5% of all reads at that nucleotide, or present at the ends of reads with low or no coverage in all samples and were determined to be falls calls. In one of the twelve top candidate genes, all jaguar samples had a homozygous in-frame deletion in BRIP1 that was also present in the Amur tiger genome (p.Ser1061del), and was inferred to be the wild-type sequence in *Panthera* species.

Remaining germline missense variants in the jaguar were further characterized by ruling-out shared variants with other *Panthera* sp. and categorized by predicted functional significance

in the top twelve candidate genes (BRCA1, BRCA2, RAD51C, PALB2, PTEN, STK11/LKB1, TP53, CHEK2, BRIP1, ATM, MLH1 and MSH2). Germline missense variants that were present in all jaguars and absent in other *Panthera* sp. are summarized in Appendix Table 5.6, and further characterized by predicted functional effect in Appendix Table 5.7.

In total, 13 germline nonsynonymous missense single nucleotide variants in 7 of the top 12 candidate genes were found that were unique to the jaguar. Of these, most were predicted to be functionally neutral in the protein. However, two variants, one in BRCA1 and one in BRCA2 were predicted to be deleterious and were selected for further characterization in the population.

Evaluation of allelic imbalance in candidate genes using microsatellites

Tetranucleotide microsatellites (n=22) surrounding 11 candidate genes (BRCA1, BRCA2, RAD51C, PALB2, PTEN, TP53, CHEK2, BRIP1, ATM, MLH1, and MSH2) were identified in the jaguar and evaluated for allelic imbalance. Two jaguars were evaluated, using DNA samples from frozen tumor tissue and blood as the paired normal sample. No microsatellite markers amplified in other paired samples (all FFPE tissues), even after redesign of select primers for smaller amplicon sizes.

Allele calls for each microsatellite locus for the two jaguars are listed in Appendix Table 5.8. Alleles were identical in tumor tissue and blood in each jaguar at all loci. Jaguar DA101783 was homozygous at 6 microsatellite loci, and DA102181 was homozygous at 12 microsatellite loci. Both jaguars had no amplification at 4 loci, which was presumed to be due to primer sequence mismatches between the Amur tiger and jaguar. For the remaining loci that were heterozygous (BRCA1-3', BRCA2-5', RAD51-5', PTEN-5'and 3', CHEK2-5', MSH2-5'and 3', PALB2-5'and 3', ATM-5', MLH1-5', and BRIP1-5'), no allelic imbalance greater than 20% was present in the

tumor tissue compared to the normal tissue. These results were interpreted as no evidence of loss of heterozygosity at these loci.

Assessment of select BRCA1 and BRCA2 germline variants in the zoo jaguar population

Two nonsynonymous, heterozygous missense variants in BRCA1 and BRCA2 were found in all jaguar samples that were not present in the domestic cat or other *Panthera* species, and were predicted to be deleterious based on protein functional analysis programs (SIFT, PolyPhen2, PROVEAN). These variants are present in regions encoding highly conserved protein sequences essential for function. Genotypes in all ovarian carcinoma and paired normal tissue samples were identical, indicating no evidence of loss of heterozygosity in the tumor tissue (Appendix Table 5.10).

In BRCA1, a single nucleotide (G>T) variant was found in exon 9 (c.1173) encoding an alanine to glutamic acid amino acid substitution in jaguars. This variant is in a highly conserved region of BRCA1 in the binding site region of retinoblastoma (RB) protein (Figure 5.1). To further analyze the presence of this variant in the zoo jaguar population, 15 paired jaguar tissues (ovarian carcinoma and normal), 12 domestic cats, and 32 additional zoo jaguars were screened by Sanger sequencing. In the 15 jaguars with ovarian carcinoma, three jaguars (20%) were heterozygous for the variant, and 12 jaguars (80%) were homozygous for the reference allele. No jaguars with ovarian carcinoma were homozygous for the variant allele. In the remaining apparently healthy jaguar population, 14 jaguars were heterozygous (43.7%), one jaguar was homozygous for the variant allele (3.1%), and 17 were homozygous for the reference allele (53.1%). All 12 domestic cats were homozygous for the reference allele. No significant difference was found between jaguars with ovarian carcinoma and the overall zoo population for

the presence of this variant (*P*=0.1), or when compared by genotype (homozygous reference allele, heterozygous, or homozygous variant allele; *P*=0.19).

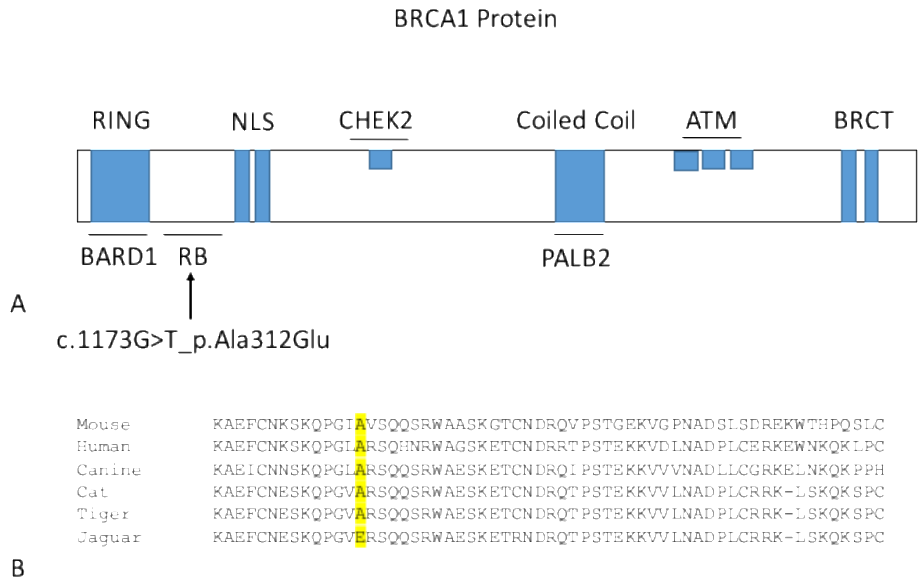

Figure 5.1: Location of the BRCA1 tumor suppressor gene nonsynonymous germline variant found in jaguars and alignment of protein sequences. **A:** Schematic of BRCA1 protein structure. This Ala312Glu variant is predicted to be deleterious/probably damaging and is in a location important for binding of the RB tumor suppressor gene that interacts with BRCA1 in cell cycle checkpoint control. Other important functional domains of BRCA1 include the RING domain, nuclear localization signal (NLS), CHEK2 phosphorylation site, a coiled coil domain and site of PALB2 binding, three ATM phosphorylation sites, and a BRCT domain. **B:** BRCA1 protein multiple species alignment of mouse, human, canine, domestic cat, tiger (*Panthera tigris altaica*), and jaguar (*Panthera onca*). This variant is in a highly conserved region across multiple mammalian species. (Clustal Omega: https://www.ebi.ac.uk/Tools/msa/clustalo/).

In BRCA2, a single nucleotide (C>G) variant was found in exon 11 (c.3732) encoding a nonconservative serine to arginine amino acid substitution. This variant is in a region of BRCA2 that contains eight highly conserved amino acid repeats (BRC repeats) that are essential for RAD51 binding (Figure 5.2). This missense variant occurs in BRC repeat 2. To further analyze the presence of this variant in the zoo jaguar population, 17 paired jaguar tissues (ovarian carcinoma and normal), 12 domestic cats, and 29 additional male and female jaguars were genotyped by Sanger sequencing and restriction enzyme digestion. In 17 jaguars with ovarian carcinoma, 16 jaguars (94%) were heterozygous for the variant, and 1 jaguar (5%) was homozygous for the variant allele. No jaguars with ovarian carcinoma were homozygous for the reference allele, and all affected animals had at least one copy of the variant allele. In 12 domestic cats, 6 were heterozygous for the variant allele, and 6 were homozygous for the reference allele. Interestingly, the 6 domestic cats heterozygous for the variant allele had mammary carcinoma. In the remaining jaguar population without a current diagnosis of ovarian carcinoma, 16 jaguars were heterozygous (55.1%), 7 jaguars were homozygous for the variant allele (24.1%), and 6 jaguars (20.6%) were homozygous for the reference allele. Of the 8 jaguars homozygous for the variant allele, 5 were males. Of the remaining three female jaguars homozygous for the variant allele, one had mammary and endometrial carcinoma.

No significant difference was found between jaguars with ovarian carcinoma and the overall zoo population for the presence of this variant (P=0.9). When compared by genotype (homozygous reference allele, heterozygous, or homozygous variant allele), the jaguar population with ovarian carcinoma had a significantly different genotype than the remaining zoo jaguar population (P=0.01), likely due to the absence of any jaguars with a homozygous reference allele in animals with ovarian carcinoma, and the high number of heterozygous

animals. Because of the location of this variant in the protein, the predicted deleterious effects, and the presence of at least one copy of this variant in all affected jaguars, we chose to evaluate the function of this variant using a CRISPR/CAS strategy.

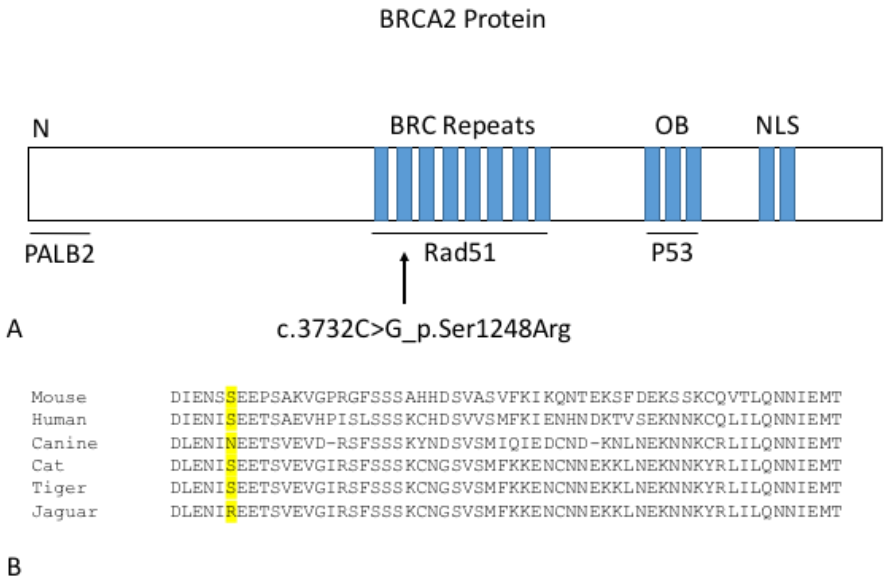

Figure 5.2: Location of the BRCA2 tumor suppressor gene nonsynonymous germline variant found in jaguars and alignment of protein sequences. **A:** Schematic of BRCA2 protein structure. This Ser1248Arg variant is predicted to be deleterious/probably damaging and is located in BRC repeat 2 of a highly conserved series of 8 amino acid repeats essential for RAD51 binding. Other important functional domains of BRCA2 include a PALB2 binding site at the N terminus, three oligonucleotide binding (OB) folds and site of p53 interaction, and a nuclear localization signal (NLS). **B:** BRCA2 protein multiple species alignment of mouse, human, canine, domestic cat, tiger (*Panthera tigris altaica*), and jaguar (*Panthera onca*). This variant is in a highly conserved region across multiple mammalian species. (Clustal Omega: https://www.ebi.ac.uk/Tools/msa/clustalo/).

Functional analysis of a germline BRCA2 S1248R missense substitution discovered in the jaguar using a CRISPR-CAS strategy and cisplatin sensitivity assay

A CRISPR-CAS strategy was used to introduce the BRCA2 S1248R missense substitution discovered in the zoo jaguar population that is present in all jaguars with ovarian carcinoma. This variant was introduced into two clones of HEK293T cells and was confirmed to

be homozygous with no evidence of the wild type allele by restriction enzyme digestion, TOPO cloning and Sanger sequencing (Figure 5.3). To create BRCA2 null cells for comparison to wild type and those harboring the missense variant, a 150 bp deletion was introduced in BRCA2 exon 11, which was also confirmed in two clones that harbor both copies of the deleted gene based on TOPO cloning and Sanger sequencing (Figure 5.3).

To assess the effect of the S1248R variant on cell viability when exposed to a DNA cross-linking agent (cisplatin), viability of wild type HEK293T cells, homozygous S1248R cells, and BRCA2 -/- cells were compared when exposed to various concentrations of cisplatin.

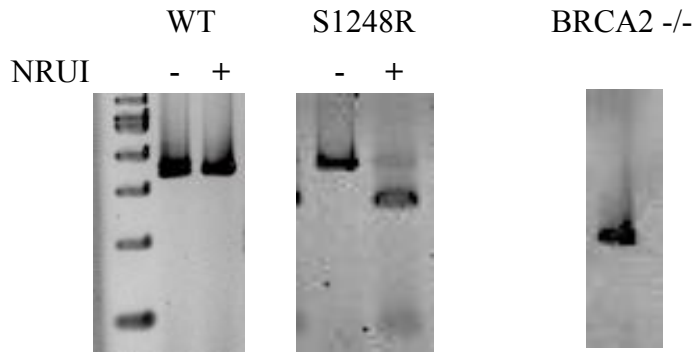

Figure 5.3: CRISPR/CAS strategy to introduce a S1248R missense substitution of BRCA2 exon 11 in 293T HEK cells. NRUI restriction enzyme digestion was used to target only cells with the knock-in substitution for screening. **Left**: wild type 293T cells with a 334 base pair amplified PCR product of the BRCA2 target region that are NRUI undigested (-) and digested (+) controls demonstrating absence of amplicon digestion with NRUI (shown with 100 base pair ladder). **Middle**: 293T cells with knock-in of a three base pair (cgc) nucleotide sequence resulting in S1248R missense and introduction of a unique NRUI restriction site. NRU1 undigested (-) and digested (+) PCR amplicons, demonstrating the successful knock-in with presence of ~260 bp and ~80 bp products and absence of the wild type allele (confirmed by TA cloning and Sanger sequencing of multiple clones). **Right**: CRISPR/CAS strategy to introduce a BRCA2 homozygous deletion of ~150 bp with absence of wild type allele.

Based on percentage of cell viability (measured by reduction of MTT) at increasing concentrations of cisplatin (0, 4,8, and 12 uM), BRCA2 -/- cells were significantly more sensitive to cisplatin than S1248R cells (P=0.034) and WT cells (P=0.005) at 4 uM cisplatin concentrations using unpaired *t*-tests (Figure 5.4). There was also a significant difference in viability at 4 uM concentration when comparing all three cell lines using a one-way ANOVA (P=0.002). S1248R cells had similar cisplatin sensitivity to WT cells at low cisplatin concentrations (4 and 8 uM). There was no significant difference in cell viability between all three cell lines at intermediate cisplatin concentrations. At high cisplatin concentrations (12 uM), S1248R cells had intermediate cisplatin sensitivity compared to WT and BRCA2-/- cells. There was a significant difference in mean cell viability when comparing all three cell lines using one-way ANOVA (P=0.01). Although there was a visible decrease in cell viability of S1248R cells compared to WT at high concentrations, this difference was not significant using an unpaired *t*-test (P=0.08).

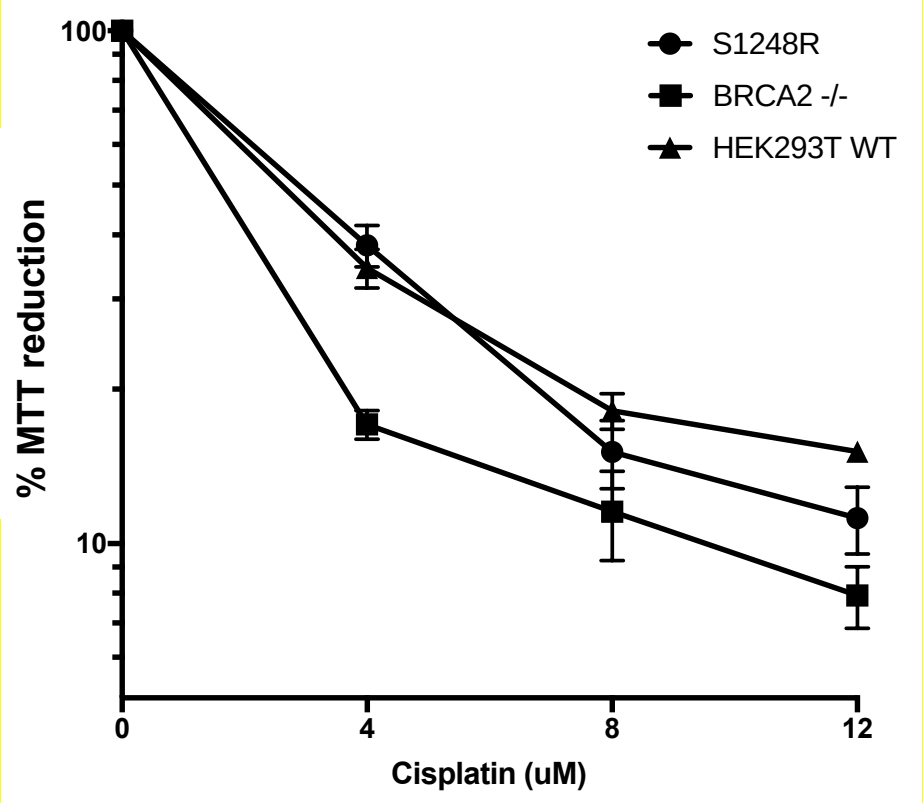

Figure 5.4: Cell viability measured by % MTT reduction (log10 scale) at increasing concentrations of cisplatin as an indicator of sensitivity to a DNA damaging agent. Cell viability of BRCA2 -/- and homozygous BRCA2 S1248R HEK29T cells were compared to wild type cells (HEK 293T WT). Each line represents three independent experiments (mean with SE). BRCA2 -/- cells had significantly increased sensitivity to cisplatin compared to S1248R and WT cells at 4 uM. S1248R cells had intermediate cisplatin sensitivity compared to WT and BRCA2 -/- cells at 12 uM concentrations.

Discussion

Study overview

This study was undertaken to investigate the genetic mechanisms of ovarian carcinoma in jaguars by sequencing the coding regions of candidate genes to first identify the wild type sequences, then identify candidate germline variants that may play a role in the pathogenesis of ovarian carcinoma in this species. Identifying a germline mutation in jaguars associated with ovarian carcinoma could not only provide insight into the mechanisms of tumorigenesis, but could also inform and improve breeding decisions, disease monitoring, and intervention to

protect the valuable breeding population of female jaguars. In this study, we report the first use of a targeted, custom designed next-generation sequencing panel on a wild animal species, to our knowledge. We used various methods for investigating the genetic pathogenesis of ovarian carcinoma in the jaguar without the availability of an assembled or annotated reference genome for this species. We also tested the performance of a targeted next-generation sequencing panel on archived FFPE tissues, and identified candidate germline variants in key tumor suppressor genes that may be associated with ovarian carcinoma in jaguars. We then performed a functional study on one of the discovered missense variants in BRCA2 using a CRISPR/CAS strategy, to determine if the presence of this BRCA2 variant has an impact on DNA repair by measuring cell viability when exposed to of a DNA damaging agent.

Performance of a targeted next-generation sequencing cancer gene panel and selection of candidate germline variants

The next-generation sequencing panel of 276 cancer genes designed using the domestic cat reference genome covered the majority of target regions in the jaguar, which is a more distantly related species (Figueiró et al. 2017). The highest quality jaguar DNA sample had 99% of target regions covered of the total target size of 1.8Mb, indicating that this panel has sufficient cross-species utility for use in the jaguar and likely other felid species. This targeted approach allowed for higher coverage in select genes of interest in multiple samples, which significantly reduced cost compared to whole genome or whole exome approaches. However, this targeted panel using a candidate gene approach covered only ~3% of the coding region of the domestic cat genome, and important genes involved in carcinogenesis may have been missed.

Targeted next-generation sequencing gene panels are commonly used to detect cancer-associated germline mutations in breast and ovarian cancer, focusing on genes known to be

involved in the pathogenesis of hereditary breast and ovarian cancer (Castéra L et al. 2014; Crawford et al. 2017). This panel was designed with an expanded list of genes with known loss of function mutations or driver mutations involved in the pathogenesis of many epithelial cancers, however twelve candidate genes with reported germline mutations in human ovarian and breast cancer were the primary focus of this study. BRCA1 and BRCA2 are the most common tumor suppressor genes harboring germline mutations associated with breast and ovarian cancer with high penetrance and have diverse functions, including cell cycle regulation and DNA repair by homologous recombination (Welcsh et al. 2000; Roy et al. 2012).
In addition to BRCA1 and BRCA2, we chose 10 additional candidate genes with known germline mutations associated with hereditary breast and ovarian cancer and other familial syndromes involving ovarian cancer with moderate to high penetrance.

RAD51C, PALB2, CHEK2, and BRIP1 all have direct interaction with BRCA1 or 2 during homology-based DNA repair and harbor germline mutations associated with ovarian cancer by disrupting DNA repair functions (Meindl et al. 2011; Toss A et al. 2015). In addition to somatic mutations, germline mutations in P53 are associated with inherited ovarian cancer (Malkin 2011, Walsh et al. 2011). Other top candidate genes included in targeted sequencing with germline mutations involved in ovarian and other cancers in humans were STK11/LKB1, PTEN, ATM, MLH1 and MSH2 (Liaw et al. 1997; Thorstenson et al. 2003; Hearle et al. 2006; Bonadona et al. 2011). No indels or other variants were found in any genes, including the top 12 candidate genes that would cause a frameshift or premature stop codon, potentially resulting in protein truncation (Appendix Tables 5.4 and 5.5). Interestingly, two germline, heterozygous missense variants found in the jaguar BRCA1 and BRCA2 genes were predicted to be deleterious. The missense variant in BRCA2 was present in all jaguars with ovarian carcinoma

was further analyzed for functional significance. Of the few missense variants found in the remaining top candidate genes, none were predicted to be deleterious and thus were inferred to be the jaguar wild-type sequence (Appendix table 5.7).

Overall, less than 50% of samples sequenced on both platforms had adequate number of raw reads for downstream processing. Of the 12 samples that had adequate read depth on the HiSeq platform using multiplex library preparation, 10 were frozen blood or tissue samples, and the remaining 2 were FFPE samples. All other FFPE samples (n=24) did not have adequate raw reads for downstream processing. We found that increasing time in formalin of FFPE samples had a significant negative correlation with number of raw reads and starting DNA concentration on the HiSeq platform.

The jaguar samples ranged from 6 to 30 years in formalin, and older samples (>5 years in formalin) had lower DNA quantity after DNA extraction, and lower number of raw reads than the frozen samples. Sample multiplexing prior to library amplification also likely resulted in bias toward amplification of the higher quality samples on this platform. Sample multiplexing prior to PCR amplification of libraries can introduce sample bias and high number of duplicate reads in certain regions, especially if samples are of variable quality and quantity, such as the jaguar FFPE samples (Kebschull and Zador 2015).

In the jaguar samples, frozen tissue and blood on the HiSeq platform had approximately 200x greater depth than FFPE tissues. One study tested the performance of archived FFPE samples of human serous ovarian adenocarcinomas between 3-32 years old, and found that specimens >10 years old had significantly lower depth and coverage of target regions (Carrick et al. 2015). Formalin-induced DNA damage occurs via multiple mechanisms, including interstrand

DNA crosslinking resulting in denaturation, direct DNA fragmentation, and deamination of cytosine bases resulting in inaccurate variant calling (Do and Dobrovic 2015).
DNA fragmentation can reduce amplification of certain regions, resulting in loss of sequencing coverage and overall variability in depth across regions, which we observed in the jaguar FFPE samples.

The same negative correlation between time in formalin number of raw reads and sequencing depth on the HiSeq platform was not observed when evaluating samples from the NextSeq platform using singleplex library amplification. Interestingly, 9 of the 13 samples that had adequate number of raw reads and depth on this platform were FFPE samples.

Library preparation did not require sample multiplexing, so each sample went through a singleplex amplification step, which likely reduced bias between the higher quality and lower quality FFPE samples. The frozen tissues had approximately 30x greater coverage than the FFPE samples on this platform. Overall, singleplex amplification seemed to improve sequencing success on some of the lower quality FFPE samples. Samples on the HiSeq platform had greater depth overall than the NextSeq platform. This was expected due to the differences in output capabilities (9Gb-1Tb; >300 million reads for Hiseq 2500, and 16-19 Gb; 130 million reads for NextSeq; https://www.illumina.com). The NextSeq platform was chosen for the second sequencing round based on availability at the MSU RTSF Genomics Core and the calculated number of reads needed for each sample to have at least 100x coverage. Overall, the NextSeq platform using singleplex library amplification had more even coverage across samples, regardless of genomic DNA origin (FFPE, frozen tissue, whole blood).

Variant filtration

Additional steps in variant filtration were required because of the use of the domestic cat as a reference genome to sequence the target regions in the jaguar. One pitfall of this approach is that the majority of variants present represented the species differences between the wild-type gene sequences, rather than potential germline variants that could be associated with ovarian carcinoma. To filter out these variants, we used the Amur tiger (*Panthera tigris altaica*) sequence to exclude variants present in the jaguar that were also shared with a more closely related species in the same genus (Appendix Table 5.6). Initially, two variant calling software programs were used, GATK and Freebayes. There were a high number of false variants in the GATK program resulting from numerous false positive indels at the ends of reads. Because of the high number of false variants in the GATK program, Freebayes was used for this study.

Overall, most variants detected in jaguar tissues were classified as synonymous or nonsynonymous single nucleotide variants, and nonsynonymous variants were further characterized in the top candidate genes by predicted functional effect, and most were predicted to be functionally neutral, and were inferred to be the wild type jaguar sequence (Appendix Table 5.7). All variants in the 276 genes analyzed that were predicted to be frameshift, stop gained, or stop lost were confirmed as false reads in the Freebayes program. These were found commonly at the ends of read fragments, or were at low frequency at that locus (less than 5% of reads had this variant). Despite the high number of false variants, this platform does have the ability to detect small indels, as demonstrated by an in-frame amino acid insertion confirmed to be present in the jaguar and tiger genomes in the BRIP1 gene. However, larger insertions, deletions, copy number variation and other structural rearrangements involving multiple genes or

large portions of chromosomes that rely on sequence depth for detection may be more difficult to assess on this platform with the high depth variability in low quality FFPE samples.

Assessment of select BRCA1 and BRCA2 germline variants in the zoo jaguar population

The BRCA1 and BRCA2 genes encode proteins with highly conserved regions of functional significance across many species (Szabo et al. 1996; Bignell et al. 1997). Most mutations associated with hereditary breast and ovarian cancer in humans are indels, resulting in protein truncation and complete loss of function (Ramus and Gayther 2009).

In the Ashkenazi Jewish population, which has a high prevalence of hereditary ovarian cancer, there are three common founder mutations (BRCA1*185delAG, BRCA1 *5382insC, and BRCA2*6174delT) that are all classified as indels (Robles-Díaz L et al. 2004). Additionally, large numbers of missense variants discovered in BRCA1 and BRCA2 are associated with variable risk of breast and ovarian cancer, and are classified as variants of uncertain significance (VUS), because the effects on protein function and clinical significance are unknown (Hofstra et al. 2008; Couch et al. 2008; Guidugli et al. 2014; Shimelis H et al. 2017).

In the top candidate genes, two nonsynonymous, heterozygous, missense germline variants, one in BRCA1 and one in BRCA2, were found in all jaguars (n=8) using next-generation sequencing data, and were not present in the domestic cat or Amur tiger sequences. The BRCA1 variant has not been reported in humans, to our knowledge. The BRCA2 variant found in jaguars has been reported in a human patient with hereditary breast and ovarian cancer as a variant of unknown significance in the NIH Breast Cancer Information Core (Szabo et al. 2000). Both the BRCA1 and BRCA2 jaguar variants were predicted to be functionally deleterious at the protein level, and were further analyzed to determine the allele frequency in the

entire jaguar study population, and to determine whether or not these variants were associated with ovarian carcinoma. The BRCA1 missense variant in exon 9 (c.1173G>T_p.Ala312Glu) found in jaguars is in a location essential for BRCA1 interaction with retinoblastoma (RB) protein, which is required for the cell cycle suppression functions of BRCA1, and numerous mutations are present in this region in human ovarian and breast cancer (Aprelikova ON et al. 1999; Clark SL et al. 2014). Despite the important location of this variant and the predicted deleterious effect on protein function, this variant was only present in 20% of jaguars with ovarian carcinoma, and there was no significant association of this variant in jaguars with ovarian carcinoma compared to the rest of the jaguar study population. There was also no evidence for loss of the wild-type allele in the ovarian tumor tissue compared to paired normal tissues or blood (Appendix Table 5.10). Based on these results, we inferred this to be a rare variant specific to the jaguar species.

The other missense variant found in jaguars was in BRCA2 (c.3732C>G_p.Ser1248Arg) and was also predicted to be functionally deleterious. This variant is in Exon 11, within a 3.3 Kb region known as the ovarian cancer cluster region (OCCR). In humans, germline mutations in this region are associated more frequently with ovarian cancer than breast cancer (Thompson and Easton 2001). This variant is located in a region that encodes eight highly conserved amino acid repeats (BRC repeats), and is specifically located in BRC repeat two, which binds with high affinity to RAD51 protein (Carreira and Kowalczykowski 2011). RAD51 is an essential protein in DNA repair by homologous recombination, and BRCA2 is required to guide RAD51 to sites of double-stranded DNA breaks (Carreira et al. 2009). This variant was present in all jaguars with ovarian carcinoma; most were heterozygous, and one jaguar was homozygous. There was also no evidence for loss of the wild-type allele in the ovarian tumor tissue compared to paired

normal tissues or blood (Appendix Table 5.10). This variant was also common in the jaguar study population without ovarian carcinoma (55.1% were heterozygous).

Interestingly, 7 jaguars were also homozygous for the BRCA2 missense variant, and 5 of these were males. There is no known cancer predisposition or other diseases reported with high frequency in male zoo jaguars. In humans, BRCA1 and BRCA2 germline mutations are associated with breast cancer in males, as well as pancreatic and prostate cancer (van Asperen et al. 2005). Mutations in BRCA1 and BRCA2 in women associated with breast and ovarian cancer are usually in heterozygous form, with eventual loss of the wild type allele in the tumor tissue (Kobayashi et al. 2013; Maxwell et al. 2017). Although there is no overt phenotype in heterozygous mutation carriers, in-vitro studies have shown that heterozygous mutations in BRCA1 and BRCA2 are associated with decreased cell viability when exposed to DNA damaging agents, and have defective DNA repair (Warren et al. 2003; Vaclová et al. 2015).

If this variant caused complete loss of BRCA2 function, we would not expect animals to be homozygous for this variant, as it is known that BRCA2-null status is embryonic lethal in mice (Hakem et al. 1998). One exception is that some biallelic loss of function mutations in BRCA1 and BRCA2 are associated with Fanconi anemia in humans, which causes early onset pancytopenia, acute myeloid leukemia, and other cancers (Nalepa and Clapp 2018). If this jaguar BRCA2 variant is functionally deleterious, it may be hypomorphic, resulting in only partial loss of protein function, which could explain the lack of a disease phenotype in some animals homozygous for this variant. Although the frequency of the variant allele was not significantly different between jaguars with ovarian carcinoma and the remaining study population, the genotype frequencies were significantly different. This was likely due to the high number of jaguars with ovarian carcinoma that were heterozygous for this variant, and the absence of any

jaguars that were homozygous for the wild type allele compared to the rest of the study population. Unlike the BRCA1 variant, the BRCA2 variant was also present in domestic cats. 12 domestic cats were genotyped for this variant, and 6 were heterozygous. All of those domestic cats with one copy of this variant had a history of mammary carcinoma. Although the effect of this variant on BRCA2 function remains unknown, this is an interesting finding, as BRCA2 mutations have not been reported in domestic cats associated with mammary carcinoma development, to our knowledge.

Functional analysis of a germline BRCA2 S1248R missense substitution discovered in the jaguar using a CRISPR-CAS strategy and cisplatin sensitivity assay

Because the BRCA2 missense variant was present in all jaguars with ovarian carcinoma, and was predicted to be deleterious to protein function, this variant was further analyzed using a functional assay. This variant has also been reported in a human patient with hereditary breast and ovarian cancer, but the functional significance is unknown (Szabo et al. 2000). To test the effect of this jaguar missense variant on BRCA2 protein function, we used a CRISPR-CAS strategy to knock-in the S1248R missense variant and assess relative effects on DNA repair ability by comparing cell viability to BRCA2 -/- and wild type cells when exposed to a DNA-damaging agent. We successfully used this CRISPR/CAS strategy to introduce the S1248R homozygous missense variant into BRCA2 of HEK293T cells. We also created BRCA2 -/- homozygous cells by introducing a large deletion in exon 11. In a study of murine BRCA2, a deletion in exon 11 that encodes the region of BRC repeats, such as the deletion that we designed, caused disruption of RAD51 binding in cells transfected with the BRCA2 exon 11-deleted expression vector (Sarkisian et al. 2001).

After introduction of the S1248R cells, the BRCA2 -/- cells, and the wild type HEK 293T cells to increasing concentrations of cisplatin, we found that the BRCA2 -/- cells were significantly more sensitive to cisplatin than S1248R cells at low cisplatin concentrations. S1248R and WT cells had similar cisplatin sensitivity at low and intermediate cisplatin concentrations, however at high concentrations, S1248R cells were more sensitive to cisplatin than WT cells. From these findings, we can conclude that the presence of this variant may be associated with a diminished capacity for DNA repair when exposed to high concentrations of a DNA-damaging agent using a HEK293T cell background. Additional studies are underway to more directly assess homologous recombination ability by a homology-directed repair assay, and the S1248R and BRCA2-/- cells are also being analyzed at the protein level by Western blot.

In addition to CRISPR-based and other knock-in methods to assess sensitivity to DNA-damaging agents such as cisplatin, PARP-inhibitors, or mitomycin C survival assays, other assays have been used to assess the functional significance of VUS in BRCA1 and BRCA2 (Guidugli et al. 2014). Some common assays involve homology-directed repair, yeast recombination, centrosome amplification, and protein-protein interaction (Couch et al. 2008; Guidugli et al. 2014). A combination of functional and predictive computational or evolutionary modeling approaches have also been used to assess the significance of VUS (Fleming et al. 2003; Woods et al. 2016; Guidugli et al. 2018). The advantage of a CRISPR/CAS method is that the exact variant can be introduced into cells with high efficiency via homology-directed repair, and the functional significance can then be assessed (Ann Ran F et al. 2013; Yang L et al. 2014). Mutations in BRCA1 and BRCA2 that confer loss of protein function render tumor cells, such as ovarian cancer cells, highly sensitive to DNA-damaging agents, including cisplatin (Foulkes 2006). Among other mechanisms, such as induction of reactive oxygen species, cisplatin directly

damages DNA by binding to purine residues, resulting in intrastrand cross-linking, ultimately leading to cessation of cell division and apoptosis (Dasari and Tchounwou, 2014). As BRCA2 is essential for homology-directed repair, introducing a variant that results in partial or complete loss of function should impair cell viability when exposed to cisplatin. BRCA2 function in-vitro has been assessed by using cisplatin and other DNA-damaging agent survival assays in various cell types, including HEK cells and ovarian cancer cells (Freeburg et al. 2009; Ghosh et al. 2013; Rytelewski et al. 2014).

There are limitations to this method of assessing the significance of VUS. First, we are testing the effect of this variant on BRCA2 function in a human cell line rather than our species or cell type of interest. Although this variant is in a highly conserved region of the protein, the functional significance could potentially change depending on species-specific protein sequences. We are also indirectly measuring BRCA2 function by cell viability when exposed to a DNA-damaging agent, rather than directly measuring homologous recombination ability. Additionally, while this may give insight into the effects of this variant on BRCA2 function, the relationship of this variant with ovarian cancer risk remains speculative. Using this approach in the combination of other functional assays, such as the homology-directed repair assay in cells from our species of interest would be beneficial.

Evaluation of allelic imbalance in candidate genes using microsatellites

There was no evidence of loss of heterozygosity in jaguar ovarian carcinoma samples in the top 12 candidate genes compared to the constitutive normal DNA (blood or normal tissue) based on next-generation sequencing data. To further assess regions of the jaguar genome surrounding top candidate genes, an additional method using microsatellite markers was used to

evaluate for allelic imbalance in ovarian carcinoma tissues compared to corresponding normal DNA. Allelic imbalance has been reported in many cancers, including ovarian cancer, and results in an alteration of the normal 1:1 ratio of allelic expression at heterozygous loci, from mechanisms such as copy number variation or allelic loss (Ryland et al. 2015). For example, ovarian carcinomas commonly have locus-specific loss of heterozygosity (LOH) in the region of important tumor suppressor genes, such as BRCA1 and BRCA2, or loss of larger chromosomal regions (Hansen et al. 2002; Bozzetti et al. 2004; Brozek I et al. 2009; Skirnisdottir et al. 2012).

In humans, those with germline mutations in BRCA1 or BRCA2 commonly have loss of the wild type allele in tumor tissue due to the accumulation of additional somatic mutations, resulting in complete loss of gene function (Rzepecka IK et al. 2012). More recent studies have also found that retention of the wild-type allele in breast and ovarian cancers of germline BRCA mutation carriers is an important mechanism of resistance to platinum-based chemotherapeutics and PARP-inhibitors (Maxwell et al. 2017). Identifying allelic loss or imbalance in regions where important tumor suppressor genes are located may give insight into the genetic pathogenesis of ovarian cancer in jaguars, especially in the presence of a germline variant that could affect protein function. Allelic imbalance was assessed in jaguar ovarian carcinomas and paired normal tissues by identifying microsatellite markers on both ends of each candidate tumor suppressor gene. Only two neoplasms and paired normal tissues could be assessed due to poor amplification of PCR products in the remaining FFPE tissues. In those markers that were heterozygous and thus characterized as informative, no evidence of allelic imbalance, including LOH, were identified in the two jaguar ovarian carcinomas compared to normal tissue samples, including in BRCA2, where one missense variant of interest is located. In jaguar paired samples, one microsatellite locus was analyzed surrounding each end of the gene, which does not

completely rule-out possible allelic imbalance in these regions. Contamination of the tumor sample with normal DNA may have masked allelic imbalance in the jaguar samples, although previous studies have reported detection of allelic loss in FFPE ovarian carcinoma samples using PCR-based microsatellite methods even with some degree of contamination with normal DNA (Gruis NA et al. 1993). More robust methods, such as genome-wide allelic imbalance detection would be useful, and could possibly help identify regions of the jaguar ovarian carcinoma genome where an important tumor suppressor gene may be located.

The role of somatic variants in jaguar ovarian cancer

In addition to germline variants, the causal role of somatic variants in jaguar ovarian carcinoma should also be assessed. Though not the focus of this study, the three most common genes harboring somatic mutations in human ovarian carcinoma, p53, KRAS, and BRAF, were evaluated for somatic mutations in jaguar ovarian carcinomas (Kurman et al. 2014). In the four jaguar ovarian carcinomas with paired normal tissue for comparison, no somatic mutations were observed in ovarian carcinoma samples compared to constitutive DNA (normal tissue or blood) samples. Serous ovarian carcinomas in women are classified as low-grade and high-grade based on histologic features and mutation profiles. High-grade carcinomas are the most common morphology associated with inherited germline mutations, and most also harbor somatic mutations in p53 (Buller et al. 2001).

Low-grade serous carcinomas are associated with somatic KRAS and BRAF activating mutations (Singer et al. 2003; Ho et al. 2004). KRAS and BRAF have key functions in the mitogen activated protein kinase (MAPK) pathway involved in cell proliferation and differentiation (Oikonomou et al. 2014). Limitations of finding somatic mutations in this study

were the low number of paired samples with adequate sequencing depth (n=4). Variation in depth of sequencing, tumor heterogeneity and contamination of the tumor sample with normal DNA (stroma, blood, etc.) can also lower the somatic variant allele frequency, making these mutations difficult to detect.

Conclusions

In conclusion, we sequenced the coding regions of 276 cancer genes in jaguars to identify candidate germline variants that may play a role in the pathogenesis of ovarian carcinoma. Identifying a germline mutation in jaguars associated with ovarian carcinoma would not only provide insight into the mechanisms of tumorigenesis, but could also inform breeding decisions, disease monitoring, and intervention to protect the valuable breeding population of female jaguars. We successfully used a targeted, custom designed next-generation sequencing panel to sequence 276 cancer genes in the jaguar using the domestic cat reference genome, with high depth (>100x) and 99% coverage of target regions in the highest quality samples. Approximately 40% of FFPE samples were successfully sequenced using two different platforms and library preparation methods. Single-sample library amplification seemed to produce less sample bias and allowed for successful sequencing of more FFPE-derived samples than multiplex library amplification. FFPE tissues had good coverage of target regions, but had decreased and variable depth compared to frozen samples. Increasing time in formalin of FFPE samples had a significant negative correlation with number of raw reads and starting DNA concentration.

Two heterozygous, missense germline variants were detected in BRCA1 and BRCA2 in the jaguar, and were predicted to be functionally deleterious. Population studies of the prevalence of these variants were performed, and the BRCA1 variant was only in 20% of jaguars

with ovarian carcinoma, while at least one copy of the BRCA2 variant was in all jaguars with ovarian carcinoma. No loss of the wild type allele was detected in tumor tissue. No frameshift, stop gained, or stop lost variants that could potentially cause protein truncation and loss of function were observed in any of the genes sequenced (n=276).

We performed a functional study on the BRCA2 missense variant discovered in jaguars using a CRISPR/CAS strategy and cisplatin sensitivity assay. This missense variant has also been reported in humans with hereditary breast and ovarian cancer, and the functional significance has not been assessed. We successfully introduced the S1248R variant, as well as a deletion creating BRCA2 -/- cells for comparison of cell viability at varying cisplatin concentrations. BRCA2 -/- cells were significantly more sensitive to cisplatin than the S1248 variant cells. Additionally, S1248R cells had intermediate cisplatin sensitivity compared to WT and BRCA2-/- cells. The presence of this variant may be associated with a diminished capacity for DNA repair when exposed to high concentrations of a DNA-damaging agent using a HEK293T cell background. Additional studies are underway to more directly assess homologous recombination ability by a homology-directed repair assay, and the S1248R and BRCA2-/- cells are also being analyzed at the protein level by Western blot. Determining the functional significance of this variant in-vitro could contribute to knowledge about the role of this missense variant in human ovarian and breast cancer as well.

New germline mutations and susceptibility loci in chromosomal regions are reported frequently that are associated with increased risk of breast or ovarian cancer, especially with the onset of high-throughput sequencing technologies (Phelan et al. 2017). Additional germline mutations in jaguars associated with ovarian carcinoma susceptibility may be within a yet undiscovered gene with tumor suppressor function in this species. Additionally, based on other

studies in our laboratory showing that the genetic diversity of the North American zoo population is similar to that of wild jaguars, a founder mutation specific to the zoo population may be less likely. It is possible that ovarian carcinoma also occurs in wild jaguars, and there is a species-specific predisposition involving certain gene(s) in the jaguar species as a whole. The prevalence of disease in wild jaguars is difficult to determine due to inaccessibility for necropsy and death at a younger age (11-12 years) in wild jaguars compared to zoo jaguars (mean 16.2 years) based on data from our 56 RHSP female study jaguars (Fanti and Nowakowski 2009). Whole genome or whole exome studies, as well as gene expression assays such as RNA-seq in both wild and zoo jaguars would provide a more robust assessment of genes or entire chromosomal regions that may be involved in the genetic pathogenesis of ovarian carcinoma.

APPENDIX

Table 5.1: List of genes (n=276) chosen for targeted next-generation sequencing of exons in jaguar samples, using the domestic cat (*Felis catus* GCA_000181335.2) as a reference sequence for probe design. Genes were selected from the Catalogue of Somatic Mutations in Cancer (COSMIC: http://cancer.sanger.ac.uk/cosmic), derived from the census of human cancer genes, and the current literature to include more recently discovered genes with implicated in inherited ovarian or breast cancer in humans.

ABCG2	BLM	CTNNB1	FGFR3	IGF1R	MDM4	PARP1	PTCH1	STAG2
ACSL3	BRAF	CXCR4	FGFR4	IGF2R	MED12	PAX3	PTEN	STK11 (LKB1)
ADM	BRCA1	DDB2	FH	IGFBP3	MEN1	PAX7	PTK1	SUZ12
AKAP9	BRCA2	DDIT3	FIGF	IL2	MET	PAX8	RAC1	TCEA1
AKT1	BRIP1	DDX5	FLT1	IL6ST	MINPP1	PBRM1	RAD21	TCF1 HNF1A
AKT2	CACNA1D	DICER1	FLT4	INSR	MITF	PBX1	RAD50	TCF12
APC	CALR	E2FL	FOS	IRS1	MLH1	PCM1	RAD51	TCF7L2
AR	CANT1	EBF1	FOXA1	JAK2	MRE11A	PCNA	RAD51C	TERT
ARID1A	CASP8	EGF	FUS	JAK3	MSH2	PDGFA	RAD51B	TFE3
ARID1B	CCNB1IP1	EGFR	GATA3	JAZF1	MSH6	PDGFB	RAF1	TFEB
ARID2	CCND1	ELK4	GNA11	KCNJ5	MST1R	PDGFRA	RB1	TFG
ASPSCR1	CCNE1	ELKS (ERC1)	GNAQ	KDM5C	MTOR	PDGFRB	RECQL4	TGFB3
ATAD2	CD82	EP300	GNAS	KDM6A	MUC1	PIK3C3	RET	TGFBR1
ATF1	CDC73	ERBB2	GOLGA5	KDR	MYB	PIK3CA	RINT1	TGFBR2
ATM	CDH1	ERG	GOPC	KIT	MYC	PIK3CB	RNF43	THBS1
ATP1A1	CDK12	ESR2	GSTT1	KLF4	MYCN	PIK3CD	SDHA	TMPRSS2
ATP2B3	CDKN1A	ESR1	H3F3B	KLK4	NBN	PIK3CG	SDHB	TP53
ATRX	CDKN1B	ETV1	HERPUD1	KMT2D	NCOA1	PIK3R1	SDHC	TP73
AXIN1	CDKN2A	ETV4	HEY1	KRAS	NCOA2	PIM1	SDHD	TPM3
BAP1	CHEK2	ETV5	HGF	KTN1	NCOA4	PLAG1	SETD2	TPO
BARD1	CHN1	ETV6	HIF1A	LHFP	NDRG1	PMS1	SFPQ	TPR
BCL10	CIC	EWSR1	HMGA1	LIFR	NF1	PMS2	SHC1	TRIM27
BCL11A	COL1A1	EXT1	HNRNPA2B1	LPPR4	NF2	POU5F1	SLC45A3	TRIM33

Table 5.1 (Cont'd)

BCL11B	COPEB	EXT2	HOOK3	MADH4	NFIB	PPARA	SMAD2	TSC1
BCL2	CREB1	FAS	HRAS	MAP2K1	NOTCH1	PPARG	SNCG	TSC2
BCL3	CREB3L1	FBXW7	ICAM1	MAP2K2	NR4A3	PPP2R1A	SP1	VDR
BCL6	CREB3L2	FES	IDH1	MAP2K4	NRAS	PRCC	SRC	VEGFA
BCL7A	CREBBP	FGF5	IDH2	MAP3K1	NTRK1	PRKAR1A	SRGAP3	VHL
BCL9	CRTC3	FGFR1	IFNG	MAX	NTRK3	PRKCA	SS18	VTI1A
ABHD15	CSFR1	FGFR2	IGF1	MDM2	PALB2	PGR	SS18L1	WIF1
WRN	WT1	XRCC1	XRCC2	YWHAE	ZNF331			

Table 5.2: Sample characteristics and performance summary of exon sequencing of 276 cancer genes covering a target region of 1.8 Mb. Sequencing was performed on an Illumina HiSeq 2500 platform using 100 bp paired end reads. Samples were multiplexed prior to library amplification.

Sample ID	Sample Description[a]	Input DNA (ng)	Number of Raw Reads (Pre-processing)	Total Gbp	Coverage (Depth)[b] x
DA101994 NWB	Whole blood	109	5,537,859	1.66	550
CatControl4	Domestic cat cell line	138	3,461,016	1.04	367
DA101506 NWB	Whole blood	106	3,348,111	1.0	356
CatControl2	Domestic cat cell line	139	2,656,602	0.8	274
DA100751OT	FFPE	205	2,126,755	0.64	216
DA101583 NWB	Whole blood	152	2,086,912	0.63	302
DA101783CL	Ovarian carcinoma cell line	570	1,944,294	0.58	283
DA101603 NWB	Whole blood	240	1,931,865	0.58	182
CatControl1	Domestic cat cell line	218	1,884,713	0.57	180
CatControl3	Domestic cat cell line	483	1,844,559	0.55	269
DA101783 N	FFPE	50	832,253	0.25	101
DA101783 OT	FFPE	254	360,733	0.11	26
Z01-225 N	FFPE	245	221,487	0.07	NA
TCP8670 MT	FFPE	172	210,044	0.06	NA
DA100751 N	FFPE	157	139,047	0.04	NA
92-698 OT	FFPE	85	133,973	0.04	NA
96-2088 N	FFPE	48	106,931	0.03	NA
94-927 N	FFPE	226	70,746	0.02	NA
96-2088 MT	FFPE	213	64,641	0.02	NA
94-2809 MT	FFPE	355	56,338	0.02	NA
Z04-21 MT	FFPE	26	40,323	0.01	NA
Z01-225 OT	FFPE	245	32,818	0.01	NA
95-1551 N	FFPE	135	30,179	0.01	NA
Z01-165 OT	FFPE	57	29,843	0.01	NA
Z01-132 MT	FFPE	62	26,724	0.01	NA
94-927 OT	FFPE	349	24,520	0.01	NA
95-1551 OT	FFPE	302	24,404	0.01	NA
Z04-21 N	FFPE	119	24,373	0.01	NA
92-698 N	FFPE	44	23,849	0.01	NA
DA101955 N	FFPE	135	6,979	0	NA
94-927 MT	FFPE	213	6,184	0	NA
Z08-04 OT	FFPE	153	5,985	0	NA
92-862 OT	FFPE	36	5,113	0	NA
DA101955 OT	FFPE	157	4,143	0	NA
Z01-132 N	FFPE	32	3,737	0	NA
Z04-21 OT	FFPE	107	3,187	0	NA

[a] FFPE = Formalin fixed paraffin embedded. All other samples were stored at -80 °C prior to sequencing.
[b] NA= Not applicable. These samples were not processed further due to the low number of raw reads.

Table 5.3: Sample characteristics and performance summary of exon sequencing of 276 cancer genes covering a target region of 1.8 Mb. Sequencing was performed on an Illumina NextSeq-Mid platform using 150 bp single end reads. Singleplex library preparation and amplification was used.

Sample ID	Sample Description[a]	Input DNA (ng)	Number of Raw Reads (Pre-Processing)	Total Gbp	Coverage (Depth)[b] x
DA101700NWB	Whole blood	264	16,100,263	2.42	219
DA100751N	FFPE	296	12,359,555	1.85	108
DA102181OT	FFPE	500	11,512,020	1.73	189
DA102181CL2	Ovarian carcinoma cell line	500	4,939,324	0.74	58
951551OT	FFPE	500	3,216,501	0.48	50
DomCatControl	Ovary	366	2,824,942	0.42	38
DA101783OT	FFPE	460	2,371,371	0.36	29
DA101504NWB	Whole blood	58.4	1,883,091	0.28	21
DA102181N	FFPE	500	1,535,402	0.23	18
951551N	FFPE	178	1,458,897	0.22	20
TCP8670MT	FFPE	500	1,146,669	0.17	17
942809N	FFPE	53.4	1,030,852	0.15	13
TCP8670N	FFPE	500	773,225	0.12	18
942809MT	FFPE	53.4	757,962	0.11	NA
94927OT	FFPE	298	756,076	0.11	NA
DA102181WB	Whole blood	174	752,832	0.11	NA
Z01225OT	FFPE	198	565,139	0.08	NA
DA102181FrOT	Ovarian tumor	361	524,218	0.08	NA
Z01132MT	FFPE	191	156,995	0.02	NA
92698OT	FFPE	408	130,130	0.02	NA
92698N	FFPE	183	124,377	0.02	NA
DA101783FrOT	Ovarian tumor	421	123,693	0.02	NA
Z01225N	FFPE	85	95,744	0.01	NA
Z01132N	FFPE	91.8	83,451	0.01	NA
DA101783WB	Whole blood	416	54,367	0.01	NA
Z01132OT	FFPE	51.6	45,210	0.01	NA
94927MT	FFPE	324	22,600	0	NA
DA101955OT	FFPE	69.2	19,644	0	NA
DA100751OT	FFPE	153	12,928	0	NA
DA101523WB	Whole blood	213	4,503	0	NA

[a] FFPE = Formalin fixed paraffin embedded. All other samples were stored at -80 °C prior to sequencing.
[b] NA= Not applicable. These samples were not processed further due to the low number of raw reads.

Table 5.4: Variant effect summary for jaguar whole blood and domestic cat control samples. Reads were aligned to the domestic cat genome (*Felis catus*_6.2) and variant calls were made using Freebayes, VARSCAN2 and Snpeff.
[a] All frameshift, stop gained, and stop lost variants were confirmed to be false after visualization of reads using Integrative Genomics Viewer V2.3.61.

Sample ID	**Jaguar DA101700**	**Jaguar DA101504**	**Jaguar DA101506**	**Jaguar DA101994**	**Domestic Cat**	**Domestic Cat**
Sample Type	whole blood	whole blood	whole blood	whole blood	cell line	ovary
Sample Storage	-80C	-80C	-80C	-80C	-80C	-80C
Sequencing Platform	NextSeq	NextSeq	HiSeq 2500	HiSeq 2500	HiSeq 2500	NextSeq
Average Depth (x)	219	21	356	550	367	38
3' UTR	2993	2395	3890	3693	1092	820
5' UTR	311	236	443	453	147	89
Frameshift[a]	22 (0)	27 (0)	16 (0)	23 (0)	11 (0)	34 (0)
Missense	1306	1133	1748	2185	594	530
Synonymous	3385	2985	3403	3490	859	705
Stop gained[a]	10 (0)	8 (0)	13 (0)	31 (0)	10 (0)	16 (0)
Stop lost[a]	0	0	1 (0)	1 (0)	1 (0)	0 (0)

[a] All frameshift, stop gained, and stop lost variants were confirmed to be false after visualization of reads using Integrative Genomics Viewer V2.3.61.

Table 5.5: Variant effect summary for the jaguar paired normal tissue and ovarian carcinoma FFPE samples, along with corresponding cell lines from two ovarian carcinomas. Reads were aligned to the domestic cat genome (*Felis catus*_6.2) and variant calls were made using Freebayes, VARSCAN2 and Snpeff.

Sample ID	**Jaguar DA100751**	**Jaguar DA100751**	**Jaguar DA102181**	**Jaguar DA102181**	**Jaguar DA102181**	**Jaguar DA101783**	**Jaguar DA101783**	**Jaguar 951551**	**Jaguar 951551**
Sample Type	normal tissue	ovarian carcinoma	normal tissue	ovarian carcinoma	ovarian carcinoma cell line	ovarian carcinoma	ovarian carcinoma cell line	normal tissue	ovarian carcinoma
Sample Storage	FFPE	FFPE	FFPE	FFPE	-80C	FFPE	-80C	FFPE	FFPE
Sequencing Platform	NextSeq	HiSeq 2500	NextSeq	NextSeq	NextSeq	HiSeq 2500	HiSeq 2500	NextSeq	NextSeq
Average Depth (x)	108	216	18	189	58	26	283	20	50
3 prime UTR variant	2582	2792	2365	2769	2762	2766	2984	2416	2638
5 prime UTR variant	326	380	232	289	290	364	415	232	265
frameshift variant[a]	48 (0)	5 (0)	23 (0)	21 (0)	34 (0)	32 (0)	16 (0)	23 (0)	18 (0)
missense variant	1939	2672	1132	1249	1485	1928	1911	974	1030
synonymous variant	3410	3118	2892	3236	3221	2951	3191	2955	3049
stop gained[a]	41 (0)	54 (0)	15 (0)	11 (0)	21 (0)	38 (0)	36 (0)	3 (0)	5 (0)
stop lost[a]	2 (0)	5 (0)	0	0	0	1 (0)	1 (0)	0	0

[a] All frameshift, stop gained, and stop lost variants were confirmed to be false after visualization of reads using Integrative Genomics Viewer V2.3.61.

Table 5.6: Next-generation sequencing results of germline variants found in constitutive DNA (whole blood or normal tissue) of top candidate genes in all jaguars (n=8) data compared to the domestic cat reference sequence (*Felis catus*_6.2). Variants are categorized by predicted protein sequence effect (synonymous or nonsynonymous), and genotype in all jaguars (heterozygous or homozygous). All nonsynonymous variants that are unique to the jaguar in each candidate gene and that were not present in the domestic cat or Amur tiger (*Panthera tigris altaica*) were selected as candidate germline variants for further analysis (last column).

Gene	**Reference (*Felis catus*) coding region (bp)**	**Synonymous variants**			**Nonsynonymous variants**			**Nonsynonymous variants unique to jaguar compared to *Panthera tigris altaica.***
		Total (n)	**Heterozygous (n)**	**Homozygous (n)**	**Total (n)**	**Heterozygous (n)**	**Homozygous (n)**	**(n)**
BRCA1	6918	14	3	11	36	12	24	2
BRCA2	10083	47	5	42	64	6	58	5
RAD51C	3458	1	1	0	1	0	1	0
PALB2	3537	16	7	9	21	7	14	2
PTEN	1303	1	1	0	0	0	0	0
STK11/LKB1	1629	9	2	7	2	2	0	0
TP53	1161	10	4	6	1	1	0	0
CHEK2	2502	6	4	2	5	1	4	1
BRIP1	4320	16	7	9	18	5	13	1
ATM	9322	56	22	34	20	6	14	1
MLH1	2529	13	6	7	7	2	5	1
MSH2	3075	10	3	7	1	1	0	0

Table 5.7: Next-generation sequencing results of nonsynonymous germline variants in all jaguars (n=8) discovered in candidate genes that are unique to the jaguar and were not present in the domestic cat or Amur tiger. Germline variants are all missense single nucleotide changes. Two variants were predicted to be deleterious/potentially damaging based on protein function prediction software (SIFT, PolyPhen2, Provean).

Gene	Chromosome	Position	Ref[a]	Var[b]	Genotype[c]	Protein Change[d]	Predicted Functional Significance
BRCA1	E1	43512575	T	C	1/1	p.Glu398Gly	Neutral
BRCA1	E1	43512833	G	T	0/1	p.Ala312Glu	Deleterious
BRCA2	A1	11410690	A	G	0/1	p.Glu522Gly	Neutral
BRCA2	A1	11412660	A	G	1/1	p.Lys706Glu	Neutral
BRCA2	A1	11414145	G	C	1/1	p.Gly1201Arg	Neutral
BRCA2	A1	11414273	C	G	0/1	p.Ser1243Arg	Deleterious
BRCA2	A1	11454542	A	G	1/1	p.Lys3183Glu	Neutral
PALB2	E3	24692867	C	T	1/1	p.Thr447Ile	Neutral
PALB2	E3	24695781	A	C	0/1	p.His760Pro	Neutral
CHEK2	D3	20137836	T	G	1/1	p.Gln361Pro	Neutral
BRIP1	E1	28708702	C	T	1/1	p.Ala678Thr	Neutral
ATM	D1	6761222	G	A	0/1	p.Ala1142Thr	Neutral
MLH1	C2	152531608	T	C	1/1	p.Ser332Gly	Neutral

[a] Ref = Reference allele in the domestic cat genome
[b] Var = Variant allele in jaguar samples
[c] 1= Reference allele; 0=Variant allele; 1/1 = Homozygous reference; 0/1 = Heterozygous
[d] Protein position based on the domestic cat reference genome (*Felis catus*_6.2).

Table 5.8: Tetranucleotide microsatellite repeats identified at the 5' and 3'ends of eleven tumor suppressor genes hypothesized to be candidates for germline mutations associated with with ovarian carcinoma in jaguars. The fluorescent label, microsatellite motif, primer sequences, reference sequence amplicon size, and results of allele calls in two paired jaguar samples (frozen ovarian carcinoma and blood) are listed.

Gene[a]	Fluorescent Label	Motif	Forward Primer Sequence[b]	Reverse Primer Sequence[c]	Reference amplicon size (bp) *Panthera tigris altaica*	Alleles in DA101783 ovarian carcinoma and blood[d]	Alleles in DA102101 ovarian carcinoma and blood[e]
BRCA1 A	NED	TTTC	CCCCCACGTGAAATGGAA	CTCTCAGGTCACTGGGTCAA	238	266	266
BRCA1 B	FAM	TTCC	AACTGGAGCAGTTTGGGAAA	GGTAGGCAGGGAGAGAGGAA	163	174/190	178/190
BRCA2 A	FAM	GAAA	GAGAGGAGCAGCCAGGAAA	ACGGAGAATGCTCCTATGAA	258	234/242	242
BRCA2 B	PET	GAAA	ACAAACCAGAGGAAGGAA	ACTGTATAACAAGCGACTTCAA	221	233	229
RAD51C A	FAM	TTTC	AAGAGGTGAGAACAGGGTGAAA	AAACCCCAAACCACAATATAA	120	133/137	137
RAD51C B	PET	TTTC	AAATAATCTCCAAGAAGGAA	GGCACAGATACTATGCCTTCAA	243	NA	NA
PALB2 A	VIC	GAAA	GTCTCTGTGTAGTCTTACAAA	GAGACCATGACCTGAGCCGAA	171	141/154	141
PALB2 B	PET	TCTA	TGTGTGCCTCGTTCCTTCTA	TATTATCTTCTGTCTTAGGAA	205	220	208/224
CHEK2 A	VIC	TTCC	TTGCTACTGACTGTGCCGTAA	GCAGGTGCTGCATAGATAA	139	130/142	130/142
CHEK2 B	FAM	TTCC	CTGCTGAAAGCAACAGCAAA	CAGGCTTGCCAAAGACAGAA	235	NA	NA
BRIP1 A	NED	TTTC	CCTGAGGTGTCTTCCCTGAA	AAGAGGTGAGAACAGGGTGAAA	288	298/302	302/372
BRIP1 B	FAM	GAAA	CCACCCTGGTGAAACTTAGAA	TTAGTCAGGCTGAATGTCACAA	248	NA	NA
MLH1 A	VIC	TTCC	CATCCCAGCATGTATATAAA	CTCTCACCCTCTCAAGTTGAA	208	193/209	193
MLH1 B	PET	TTTC	TATGGGAGCACCAGAAGGAA	CAGCATGGAGACTGCTTGCAA	169	191	195
MSH2 A	FAM	TTCC	TGTACACACTAATATTCCAA	GCCTTCTCCACTAGCAGAGAA	204	209/217	213
MSH2 B	PET	TTTC	TTAAGCCAGCACATTTCTTGAA	TGACAAGAAAGGAAGGAAGGAA	236	230/234	240
PTEN A	NED	TTTC	GCAGGGTCTCTTTTGTTTCTAA	GTTCCCCTCCTGAGACCAA	135	164/168	164/168
PTEN B	PET	GGAA	ACTCAGGGTTGTGCGTAA	CTTGCTGGCATGAGGTGAA	127	155/159	151/155
p53 A	VIC	GGAA	AAACCCAGTATCCACACTGAA	CAAACAGCCAACTCCATGTAA	239	NA	NA
p53 B	VIC	TTCC	TTCTTGCTGGTTCCCATGGAA	GCCTCAATTTATCCCCACAA	269	292	288
ATM A	VIC	GGAA	CTGAGACATGAGCGGAGAAA	TGGATTCACAGTTTCAAGAA	178	254/269	262
ATM B	NED	TTTC	CGTTGTTAAGCTTCAGTGCAA	TGTGGATGCTCATGTTTAGGAA	207	260	264

Table 5.8 (Cont'd)

[a] Each microsatellite is named according to gene and location (A=5' end; B=3' end)
[b,c] Primers were designed using the Panthera tigris altaica PanTig 1.0 assembly (Cho et al. 2013)
[d,e] Results from high resolution genotyping for paired samples. Two alleles are listed for heterozygous loci, and one allele is listed for homozygous loci. Alleles in ovarian carcinoma and paired normal samples were identical. NA = No amplification

Table 5.9: Primer sequences for candidate gene allelic imbalance (universal primers), BRCA1 and BRCA2 variant validation, and CRISPR/CAS experiments.

Primer Description	Primer ID	Sequence
Universal Primers for Candidate Gene Allelic Imbalance	Uni 1 - FAM Uni 2 – PET[a] Uni 3 – NED[a] Uni 4 – VIC[a]	CTCCAACTCACCTCCAACAAA AAACCTCTCTCCACACCCAAA CTCACCTCCCACTCCACAAA AACTCCACCACTCCCACAAA
BRCA1 Variant	F R	CCTACACAGGGGATCAGCAT CAAATACTCGTGCCAGCTCA
BRCA2 Variant	F R	GCTGTAACAAAAGTGCTTCTGG ACCTTCAGTATCTTCAGCAACA
CRISPR/CAS: Targeting Site for S1248R Knock-in	Targeting gRNA top Targeting gRNA bottom	CACCGTGATATTGAGAATATTAGTG AAACCACTAATATTCTCAATATCAC
CRISPR/CAS: Introduction of CGC (S1248R) nucleotide sequence by Homology Directed Repair	HDR Guide Oligo	AGAATCATGACATTTACTTGAAGAT AAACTTATTGGATGTACCTCTGCAG AAGTTTCCTCGCGAATATTCTCAATA TCACTAAACAGTTTCACAGCTT
CRISPR/CAS: Generation of BRCA2 -/- Deletion	Downstream targeting gRNA top Downstream targeting gRNA bottom	CACCGAATAATATTGAAATGACTAC AAACGTAGTCATTTCAATATTATTC
CRISPR/CAS: Screening of BRCA2 Exon 11 Target Region	F R	TCTGCTCATGGCACAAAACTGA GAGAATTTCTACTGGCAGCAGT

[a] Primer sequences were from Applied Biosystems, Foster City, CA. All other primer sequences are from Integrated DNA Technologies, San Jose CA.

Table 5.10: Genotyping results from Sanger sequencing of the BRCA1 (Exon 9, C.1173G>T, p.Ala312Glu) and BRCA2 (Exon 11, c.3732C>G, p.Ser1248Arg) candidate missense variants in jaguar ovarian carcinomas and paired normal DNA samples (A) and whole blood from the zoo jaguar population (B). Loss of heterozygosity (loss of wild-type allele) was not detected in any ovarian carcinoma tumor DNA compared to constitutive DNA from that animal.

A

Jaguar ID	Sex	BRCA1 genotype[c]		BRCA2 genotype[c]	
		Normal tissue	Ovarian carcinoma	Normal tissue	Ovarian carcinoma
DA100751[a]	F	0/1	0/1	0/1	0/1
DA102181[ab]	F	0/1	0/1	0/0	0/0
DA101783[ab]	F	1/1	1/1	0/1	0/1
951551[a]	F	1/1	1/1	0/1	0/1
981006	F	1/1	1/1	0/1	0/1
94927	F	1/1	1/1	0/1	0/1
92698	F	1/1	1/1	0/1	0/1
DA101995	F	0/1	0/1	0/1	0/1
Z0421	F	1/1	1/1	0/1	0/1
952312	F	1/1	1/1	0/1	0/1
97N2357	F	1/1	1/1	0/1	0/1
z00109	F	1/1	1/1	0/1	0/1
92862	F	1/1	1/1	0/1	0/1
Z0804	F	1/1	1/1	0/1	0/1
942106	F	1/1	1/1	0/1	0/1

[a] Genotype also confirmed by next-generation sequencing; [b] Genotype also confirmed in corresponding ovarian carcinoma cell lines; [c] 1= Reference allele; 0=Variant allele; 1/1 = Homozygous reference; 0/1 = Heterozygous; 0/0 Homozygous variant; NA = Not genotyped

Table 5.10 (cont'd)

B

Jaguar ID	Sex	BRCA1 genotype[c] Whole blood	BRCA2 genotype[c] Whole blood
da101669	M	0/1	0/0
da101025	M	1/1	0/1
da101026	M	0/1	0/1
da101693	M	1/1	0/0
da101964	M	1/1	0/1
da101700[a]	M	0/0	0/1
1134	M	0/1	0/1
da101505	M	0/1	0/0
da101738	M	1/1	NA
da101524	M	0/1	0/1
da101604	M	0/1	0/0
da101503	M	1/1	1/1
da101668	M	1/1	1/1
103359	M	1/1	0/0
413009	M	0/1	0/1
DA101583	F	0/1	0/1
DA101506[a]	F	1/1	1/1
DA101603	F	1/1	0/1
DA101504[a]	F	0/1	0/1
da101523	F	0/1	NA
da101540	F	1/1	0/1
da102179	F	0/1	1/1
da101699	F	1/1	0/1
113901	F	1/1	0/0
da101027[d]	F	0/1	0/0
da101694	F	1/1	1/1
da100510	F	0/1	0/0
da101703	F	1/1	NA
da100946	F	0/1	0/1
da101739	F	1/1	1/1
SB1106	F	1/1	0/1
DA101994[a]	F	1/1	0/1

[a] Genotype also confirmed by next-generation sequencing; [b] Genotype also confirmed in corresponding ovarian carcinoma cell lines; [c] 1= Reference allele; 0=Variant allele; 1/1 = Homozygous reference; 0/1 = Heterozygous; 0/0 Homozygous variant; NA = Not genotyped; [d] This jaguar had mammary carcinoma

REFERENCES

REFERENCES

Adzhubei IA, Schmidt S, Peshkin L et al. (2010) A method and server for predicting damaging missense mutations. Nat Methods 7(4): 248-249

Ann Ran F, Hsu PD, Wright J, et al. (2013) Genome engineering using the CRISPR-Cas9 system. Nature Protocols 8(11):2281-2308

Aprelikova ON, Fang BS, Meissner EG et al. (1999) BRCA-1 associated growth arrest is RB-dependent. PNAS 96(21):11866-11871

Bhattacharyya A, Ear US, Koller BH, Weichselbaum RR, Bishop DK (2000) The breast cancer susceptibility gene BRCA1 is required for subnuclear assembly of RAD51 and survival following treatment with the DNA cross-linking agent cisplatin. The Journal of Biological Chemistry 275(31): 23899-23903

Bignell G, Micklem G, Stratton MR, et al. (1997) The BRC repeats are conserved in mammalian BRCA2 proteins. Human Molecular Genetics 6(1):53-58

Bonadona V, et al. (2011) Cancer risk associated with germline mutations in MLH1, MSH2 and MSH6 Genes in Lynch Syndrome. JAMA. 305(22): 2304-2310

Bossart GD, Hubbell G (1986) Ovarian papillary cystadenocarcinoma in a jaguar. J Zoo Anim Med 14(1):73-76

Bozzetti C, Bortesi B, Merisio C (2004) Loss of heterozygosity (LOH) in ovarian cancer. International Journal of Gynecology & Obstetrics 85:294-295

Brozek I, Ochman K, Debniak J et al. (2009) Loss of heterozygosity at BRCA1/2 loci in hereditary and sporadic ovarian cancers. Journal of Applied Genetics 50(4):379-384

Bryan LK, Edwards JF, Hoppes SM (2015) Pathology in Practice. JAVMA 247(10):1117-1119

Bryant HE, Schultz N, Thomas HD et al. (2005) Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. Nature 434(14): 913-916

Buller RE, Lallas TA, Shahin MS, et al. (2001) The p53 mutational spectrum associated with BRCA1 mutant ovarian cancer. Clinical Cancer Research 7:831-838

Carreira A, Kowalczykowski SC (2011) Two classes of BRC repeats in BRCA2 promote RAD51 nucleoprotein filament function by distinct mechanisms. PNAS 108(26):10448-10453

Carreira A, Hilario J, Amitani I, et al. (2009) The BRC repeats of BRCA2 modulate the DNA-binding selectivity of RAD51. Cell 136:1032-1043

Carrick DM, Mehaffey MG, Sachs MC et al. (2015) Robustness of next generation sequencing on older formalin-fixed paraffin-embedded tissue. Plos One DOI:10.1371/journal.pone.0127353

Castéra L, Krieger S, Rousselin A et al. (2014) Next-generation sequencing for the diagnosis of hereditary breast and ovarian cancer using genomic capture targeting multiple candidate genes. European Journal of Human Genetics 22:1305-1313

Cho YS, Hu L, Hou H et al. (2013) The tiger genome and comparative analysis with lion and snow leopard genomes. Nat Commun 4:2433. doi:10.1038/ncomms3433

Choi Y, Sims GE, Murphy S, Miller JR, Chan AP (2012) Predicting the Functional Effect of Amino Acid Substitutions and Indels. PLoS ONE 7(10): e46688

Clark SL, Rodriguez AM, Snyder RR, et al. (2012) Structure-function of the tumor suppressor BRCA1. Computational and Structural Biotechnology Journal 1(1): e201204005, http://dx.doi.org/10.5936/csbj.201204005

Couch FJ, Rasmussen LJ, Hofstra R, et al. (2008) Assessment of functional effects of unclassified genetic variants Hum Mutat 29(11):1314-1326

Crawford B, Adams SB, Sittler T, et al. (2017) Multi-gene panel testing for hereditary cancer predisposition in unsolved high-risk breast and ovarian cancer patients. Breast Cancer Res Treat 163:383-390

Cybulski C, et al. (2015) Germline RECQL mutations are associated with breast cancer susceptibility. Nat Genet 47: 643-646

Dasari S, Tchounwou PB (2014) Cisplatin in cancer therapy: molecular mechanisms of action. Eur J Pharmacol 0:364-378

Do H, Dobrovic A (2015) Sequence artifacts in DNA from formalin-fixed tissues: causes and strategies for minimization. Clinical Chemistry 61(1):64-71.

Figueiró HV, Li G, Trindade FJ et al. (2017) Genome-wide signatures of complex introgression and adaptive evolution in the big cats. Sci Adv 3(7):e1700299

Fleming MA, Potter JD, Ramirez CJ, et al. (2003) Understanding missense mutations in the BRCA1 gene: an evolutionary approach. PNAS 100(3):1151-1156

Foulkes WD (2006) BRCA1 and BRCA2: chemosensitivity, treatment outcomes and prognosis. Familial Cancer 5:135-142

Freeburg EM, Goyeneche AA, Seidel EE et al. (2009) Resistance to cisplatin does not affect sensitivity of human ovarian cancer cell lines to mifepristone cytotoxicity. Cancer Cell International 9:4 doi:10.1186/1475-2867-9-4

Futreal PA, et al. (2004) A census of human cancer genes. Nat Rev Cancer 4(3):177-183

Garrison E, Marth G (2012) Haplotype-based variant detection from short-read sequencing. arXiv preprint arXiv:1207.3907 [q-bio.GN]

Gelberg HB, McEntee K (1985) Feline ovarian neoplasms. Vet Pathol 22:572-576

Ghosh S, Sur S, Yerram SR, et al. (2013) Hypersensitivities for acetaldehyde and other agents among cancer cells null for clinically relevant Fanconi anemia genes. The American Journal of Pathology 184(1):260-270

Gruis NA, Abeln ECA, Bardoel AFJ, et al. (1993) PCR-based microsatellite polymorphisms in the detection of loss of heterozygosity in fresh and archival tumour tissue. Br J Cancer 68:308-313

Guidugli L, Carreira A, Caputo SM et al. (2014) Functional assays for analysis of variants of uncertain significance in BRCA2. Hum Mutat 35(2):151-164

Guidugli L, Shimelis H, Masica DL, et al. (2018) Assessment of the clinical relevance of BRCA2 missense variants by functional and computational approaches. The American Journal of Human Genetics 102:233-248

Hakem R, Luis de la Pompa J, Mak TW (1998) Developmental studies of BRCA1 and BRCA2 knock-out mice. Journal of Mammary Gland Biology and Neoplasia 3(4):431-432

Hansen LL, Jensen LL, Dimitrakakis C et al. (2002) Allelic imbalance in selected chromosomal regions in ovarian cancer. Cancer Genet Cytogenet 139(1):1-8

Harrenstein LA, Munson L, Seal US (1996) Mammary cancer in captive wild felids and risk factors for its development: a retrospective study of the clinical behavior of 31 cases. J Zoo Wildl Med 27(4): 468-476

Hearle N, et al. (2006) Frequency and spectrum of cancers in the Peutz-Jeghers syndrome. Clin Canc Res 12(10):3209-3215

Ho CL, Kurman RJ, Delhari R, et al. (2004) Mutations of BRAF and KRAS precede the development of ovarian serous borderline tumors. Cancer Research 64:6915-6918

Hofstra RMW, Spurdle AB, Eccles D et al. (2008) Tumor characteristics as an analytic tool for classifying genetic variants of uncertain clinical significance. Hum Mutat 29(11):1292-1303

Hope K, Deem SL (2006) Retrospective study of morbidity and mortality of captive jaguars (*Panthera onca*) in North America, 1982-2002. Zoo Biol 25:501-512

Kazensky CA, Munson L, Seal US (1998) The effects of melengestrol acetate on the ovaries of captive wild felids. J Zoo Wildl Med 29(1): 1-5

Kebschull JM, Zador AM (2015) Sources of PCR-induced distortions in high-throughput sequencing data sets. Nucl Acid Res 43: e143

Kobayashi H, Ohno S, Sasaki Y, et al. (2013) Hereditary breast and ovarian cancer susceptibility genes (review). Oncology Reports 30:1019-1029

Koboldt D, Zhang Q, Larson D et al. (2012) VarScan2: Somatic mutation and copy number alteration discovery in cancer by exome sequencing. Genome Research DOI: 10.1101/gr.129684.111

Koressaar T, Remm M (2007) Enhancements and modifications of primer design program Primer3. Bioinformatics 23(10):1289-91. https://doi.org/10.1093/bioinformatics/btm091

Kumar P, Henikoff S, Ng PC (2009) Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc 4(7):1073-81

Kurman RJ, Carcangiu ML, Herrington CS and Young RH (2014) WHO Classification of Tumours of Female Reproductive Organs. International Agency for Research on Cancer (IARC), Lyon, France

Langmead B, Salzberg S (2012) Fast gapped-read alignment with Bowtie2. Nature Methods 9:357-359

Lawrenson K, et al. Common variants at the CHEK2 gene locus and risk of epithelial ovarian cancer. Cacinogenesis 36(11):1341-1353. 2015

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R and 1000 Genome Project Data Processing Subgroup (2009) The Sequence alignment/map (SAM) format and SAMtools. Bioinformatics 25:2078-9

Liaw D, et al. (1997) Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nature Genet 16:64-67

Mali P, Yang L, Esvelt KM et al. (2013) RNA-guided human genome engineering via Cas9 Science 339(6121):823-826

Malkin D (2011) Li-Fraumeni syndrome. Genes Cancer 2(4):475-484

Maryamma KI, Sivadas CG, Krishnan Nair M, Rajan A (1974) Cystadenocarcinoma of ovary with leiomyoma of uterus in a jaguar (*Panthera onca*). Indian Vet J 51:269-270

Maxwell KN, Wubbenhorst B, Wenz BM et al. (2017) BRCA locus-specific loss of heterozygosity in germline BRCA1 and BRCA2 carriers. Nature Communications 319: doi:10.1038/s41467-017-00388-9

McAloose D, Munson L, Naydan DK (2007) Histologic features of mammary carcinomas in zoo felids treated with melengestrol acetate (MGA) contraceptives. Vet Pathol 44:320-326

McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo MA (2010) The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data**.** Genome Res 20:1297-303

Meindl A, Ditsch N, Kast K, Rhiem K, Schmutzler R (2011) Hereditary breast and ovarian cancer. Med Dtsch Arztebl Int 108(19): 323-330

Munson L (1994) A high prevalence of ovarian papillary cystadenocarcinomas in jaguars (*Panthera onca*). Vet Pathol (Abstract) 31:5

Munson L, Stokes JE, Harrenstein LA (1995) Uterine cancer in zoo felids on progestin contraceptives. Vet Pathol (Abstract) 32: 578

Munson L, Gardner IA, Mason RJ, Chassy LM, Seal US (2002) Endometrial hyperplasia and mineralization in zoo felids treated with melengestrol acetate contraceptives. Vet Pathol 39:419-427

Nalepa G, Clapp DW (2018) Fanconi anaemia and cancer: an intricate relationship. Nature Reviews Cancer 18:168-185

Oikonomou E, Koustas E, Goulielmaki M, et al. (2014) BRAF vs RAS oncogenes: are mutations of the same pathway equal? Differential signaling and therapeutic implications. Oncotarget 5(23): 11752-11777

Paulson TG, Galipeau PC, Reid BJ (1999) Loss of heterozygosity analysis using whole genome amplification, cell sorting, and fluorescence-based PCR. Genome Research 9:482-491

Pelttari LM, et al. (2011) RAD51C is a susceptibility gene for ovarian cancer. Hum Mol Gen 20(16): 3278-3288.

Petrucelli N, Daly MB, Feldman GL (2010) Hereditary breast and ovarian cancer due to mutations in BRCA1 and BRCA2. Genet Med 12(5):245-256

Phelan CM, Kuchenbaecker KB, Tyrer JP et al. (2017) Identification of 12 new susceptibility loci for different histotypes of epithelial ovarian cancer. Nature Genetics 49(5): 680-691

Quigley H, Foster R, Petracca L, Payan E, Salom R, Harmsen B (2017) *Panthera onca*. The IUCN Red List of Threatened Species. e.T15953A50658693

Rafnar T, et al. (2011) Mutations in BRIP1 confer high risk of ovarian cancer. Nat Genet 43(11):1104-1109

Ramus SJ, Gayther SA (2009) The contribution of BRCA1 and BRCA2 to ovarian cancer. Molecular Oncology 3:138-150

Robinson JT, Thorvaldsdóttir, Winckler W, Guttman M, Lander ES, Getz G, Mesirov JP (2011) Integrative Genomics Viewer. Nature Biotechnology 29:24-26

Robles-Díaz L, Goldfrank DJ, Kauff ND et al. (2004) Hereditary ovarian cancer in Ashkenazi Jews. Familial Cancer 3:259-264

Roy R, Chun J, Powell SN (2012) BRCA1 and BRCA2: different roles in a common pathway of genome protection. Nat Rev Cancer 12(1):68-78

Ryland GL, et al. (2015) Loss of heterozygosity: what is it good for? BMC Med Genomics. 8(45):1-12.

Rytelewski M, Tong JG, Buensuceso A, et al. (2014) BRCA2 inhibition enhances cisplatin-mediated alterations in tumor cell proliferation, metabolism, and metastasis. Molecular Oncology 8:1429-1440

Rzepecka IK, Szafron L, Stys A, et al. (2012) High frequency of allelic loss at the BRCA1 locus in ovarian cancers: clinicopathologic and molecular associations. Cancer Genetics 205:94-100

Sarkisian CJ, Master SR, Huber J, et al. (2003) Analysis of murine BRCA2 reveals conservation of protein-protein interactions but differences in nuclear localization signals. The Journal of Biological Chemistry 276(40):37640-37648

Shimelis H, Mesman RLS, Von Nicolai C, et al. (2017) BRCA2 hypomorphic missense variants confer moderate risks of breast cancer. Cancer Research 77(11):2789-2799

Singer G, Oldt III R, Cohen Y, et al. (2003) Mutations in BRAF and KRAS characterize development of low-grade ovarian serous carcinoma. Journal of the National Cancer Institute 95(6):484-486

Skirnisdottir I, Mayrhofer M, et al. (2012) Loss-of-heterozygosity on chromosome 19q in early-stage serous ovarian cancer is associated with recurrent disease. BMC Cancer 12:407-f

Szabo C, Masiello A, Ryan JF, Brody LC (2000) The breast cancer information core: database design, structure, and scope. Hum Mutat 16(2):123-131

Szabo CI, Wagner LA, Francisco LV et al. (1996) Human, canine and murine BRCA1 genes: sequence comparison among species. Human Molecular Genetics 5(9):1289-1298

Thompson D, Easton D (2001) Variation in cancer risks, by mutation position, in BRCA2 mutation carriers. Am J Hum Genet 68:410-419

Thorstenson YR, et al. (2003) Contributions of ATM mutations to familial breast and ovarian cancer. Cancer Res 63:3325-3333

Toss A, Tomasello C, Razzaboni E et al. (2015) Hereditary ovarian cancer: not only BRCA1 and 2 genes. Biomed Res Int V:2015, ID 341723 http://dx.doi.org/10.1155/2015/341723

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG (2012) Primer3 - new capabilities and interfaces. Nucleic Acids Res 40(15):e115. https://doi.org/10.1093/nar/gks596

van Asperen CJ, Brohet RM, Meijers-Heijboer EJ, Hoogerbrugge N, Verhoef S, Vasen HF, Ausems MG, Menko FH, Gomez Garcia EB, Klijn JG, Hogervorst FB, van Houwelingen JC, et al. (2005) Cancer risks in BRCA2 families: estimates for sites other than breast and ovary. J Med Genet 42(9):711–719

Vaclová T et al. (2015) DNA repair capacity is impaired in healthy BRCA1 heterozygous mutation carriers. Breast Cancer Res Treat. 152:271-282

Walsh T, et al. (2011) Mutations in 12 genes for inherited ovarian, fallopian tube and peritoneal carcinoma identified by massively parallel sequencing. PNAS 108(44):10832-10837

Warren M, Lord CJ, Masabanda J, et al. (2003) Phenotypic effects of heterozygosity for a BRCA2 mutation. Human Molecular Genetics 12(20):2645-2656

Welcsh PL, Owens KN, King MC (2000) Insights into the functions of BRCA1 and BRCA2. TIG 16(2): 69-74

Woods NT, Baskin R, Golubeva V, et al. (2016) Functional assays provide a robust tool for the clinical annotation of genetic variants of uncertain significance. NPJ Genomic Medicine 1: doi:10.1038/npjgenmed.2016.1

Yang L, Yang JL, Byrne S, et al. (2014) CRISPR/Cas9-directed genome editing of cultured cells. Current Protocols in Molecular Biology 107:31.1.1-31.1.17

Zhang F, et al. (2009) PALB2 links BRCA1 and BRCA2 in the DNA-damage response. Curr Biol 19:524-529

CHAPTER 6

Future Directions

Cancer in wild and zoo-managed species contributes to population decline and has negative impacts on conservation (McAloose and Newton 2009). With continued habitat loss and other human impacts on species survival in the wild, zoo populations are becoming more important for maintenance of healthy breeding populations. Understanding the pathogenesis of diseases in wild and zoo animal species is not only important for endangered species survival, but also contributes to the understanding of human disease. Conducting research on endangered species can be challenging due to the lack of species-specific resources, such as genomic information, in-vitro, and in-vivo models of disease. However, lack of resources should not be a deterrent to studying wildlife disease, as new tools continue to be developed. For example, the first whole genome sequence of the jaguar has just been reported (Figueiró et al. 2017).

In addition to the jaguar, there is a high prevalence of certain cancers in other zoo felids that are significant causes of mortality, such as pheochromocytomas in clouded leopards and transitional cell carcinomas in fishing cats (Landolfi and Terio 2006; Corner et al. 2017). A species-specific genetic predisposition is also suspected, but the underlying mechanisms have not been assessed. In our study, we developed tools using a comparative genetics approach to study the genetic pathogenesis of ovarian carcinoma in jaguars and the genetic diversity of the zoo jaguar population using the domestic cat and Amur tiger as reference sequences. We also found that valuable genetic information could be derived from some archived formalin-fixed specimens. We report the first use of a targeted next-generation sequencing platform on a wild animal species, and discovered a germline missense variant in the BRCA2 gene that may be associated with ovarian carcinoma in jaguars. We confirmed by pedigree analysis that jaguar ovarian carcinoma occurs in an autosomal pattern of inheritance in some cases, and also found no association of ovarian carcinoma with the use of exogenous progestin contraceptives.

These are important findings that further support a possible inherited genetic mechanism for ovarian cancer development in jaguars. We tested the functional significance of the BRCA2 germline missense variant discovered in jaguars that has also been reported as a variant of unknown significance in humans, and found a subtle increase in cisplatin sensitivity compared to wild type cells, that may indicate an association with impaired DNA repair function. This warrants further investigation, and we are continuing to evaluate this missense variant by homology-directed repair assay, and at the protein level by Western blot. Knowledge of the functional significance also contributes to the understanding of BRCA2 protein function in general, as well as the potential role in human and jaguar ovarian carcinoma.

One limitation of this study was that only a small number of genes were sequenced relative to the entire genome, and important genes or chromosomal regions involved in the pathogenesis of ovarian carcinoma may have been missed. Furthermore, a hallmark of hereditary ovarian cancer in humans is widespread genomic instability, with structural variations such as copy number variation and larger indels that contribute to the pathogenesis of disease (Bowtell et al. 2015). The use of targeted next-generation sequencing can limit the ability to detect larger structural variations (Abel and Duncavage 2013). Whole genome sequencing of paired tumor and normal tissue would be an important next step in discovering chromosomal regions that may contain other tumor suppressor genes with important germline variants in jaguars that confer susceptibility to ovarian carcinoma. The role of somatic driver mutations in jaguar ovarian carcinoma pathogenesis should also be studied in more detail.

We also developed a complete multiplex of twelve new microsatellite markers to evaluate genetic diversity of the zoo jaguar population, which has not been reported. We found that the North American zoo jaguar population has high genetic diversity, comparable to wild jaguar

populations. Based on this set of microsatellite markers, there was no evidence that the zoo jaguar population has become genetically isolated from wild populations. This leads us to speculate that ovarian carcinoma may be a species-wide problem, and not solely affect zoo jaguars. The disease may not be apparent in wild females due to death at a younger age than zoo-managed jaguars. This multiplex was also designed using primers in conserved regions flanking microsatellite markers, and can be used as a population management tool to evaluate genetic diversity. Pilot studies in our laboratory confirm that this multiplex can be used in other *Panthera* species as well as more distantly related felids. This multiplex may also be of potential use on noninvasive samples for field studies in the survey of wild felid populations.

REFERENCES

REFERENCES

Abel HJ, Duncavage E (2013) Detection of structural DNA variation from next generation sequencing data: a review of informatics approaches. Cancer Genet 206(12):432-440

Bowtell D et al. (2015) Rethinking ovarian cancer II: reducing mortality from high-grade serous ovarian cancer. Nature Reviews Cancer 15: 668-679

Corner S, Walsh T, Padilla L, MacNeill A, Wallig M, Kiupel M, Terio K (2017) Histologic and immunohistochemical characterization of pheochromocytomas in twenty clouded leopards (*Neofelis nebulosa*). Vet Pathol 54(2):269-276

Figueiró HV, Li G, Trindade FJ et al (2017) Genome-wide signatures of complex introgression and adaptive evolution in the big cats. Sci Adv 3(7):e1700299

Landolfi JA, Terio KA (2006) Transitional cell carcinoma in fishing cats (*Prionailurus vivverinus*): pathology and expression of cyclooxygenase-1, -2 and p53. Vet Pathol 43(5):674-681

McAloose DM and Newton AL (2009) Wildlife cancer: a conservation perspective. Nature Reviews Cancer 9: 517-526