

ABSTRACT

JACKSON, EMILY DAWN. CA125 Expression on Spontaneous Ovarian Adenocarcinomas from Geriatric Laying Hens. (Under the direction of Paul Mozdziak).

Despite years of research ovarian cancer remains the most lethal of all gynecological malignancies. Ovarian cancer is the fourth leading cause of cancer death among women in the United States. It is estimated that in the United States in 2007 there will be 22,430 new cases of ovarian cancer diagnosed and 15,280 deaths. The high mortality rate is due to the fact that ovarian cancer typically presents itself as a late stage disease. There is currently no method to detect ovarian cancer in the early stages when the disease is 90% treatable. Progress toward understanding the ovarian cancer disease process is hindered by the absence of a biologically adequate animal model. Domestic hens maintained under intensive egg-laying conditions spontaneously develop ovarian adenocarcinomas. A typical hen will produce 280 or more eggs in 50 weeks. Just as in older females, fertility in hens decreases after 2 years of age.

The first objective in the current study was to further establish the chicken as an acceptable animal model for human disease. This was done by determining the presence of a known ovarian cancer tumor marker, CA125, in avian ovarian cancer tissue samples and cells isolated from solid tumors. CA125 is a glycoprotein expressed on the cell surface of ovarian cancer cells and has a molecular weight above 200 kDa. In the avian species CA125 is represented by a band of molecular weight 25-50 kDa.

The objective of the second experiment was to determine if stage or level of metastasis has an affect on CA125 expression in avian ovarian cancer samples. CA125 has been established as a tool in the clinical setting for following progression of disease and

response to treatment because CA125 increases with recurrence of disease after remission. The level of metastasis is significant because ovarian cancer typically is not detected until late disease stage when tumors have spread beyond the ovary to other body organs. Increased levels of CA125 would therefore be correlated with increased levels of disease spread throughout the body cavity. Despite the evidence seen in human clinical results, this experiment did not show any correlation between CA125 expression and disease stage or level of metastasis.

The third objective was to evaluate the presence of CA125 in oviductal tumors. CA125 production may play a significant role in determining the primary site of tumor formation, which aids in disease management. This experiment tested whether avian primary oviductal tumors express CA125. It also tested whether oviductal tumors secondary to ovarian cancer expressed CA125. It has been shown that CA125 can be shed from a primary tumor and attach to mesothelin on the mesothelium in the peritoneal cavity to allow metastasis to occur. The results suggest that primary oviductal tumors do not express CA125 and secondary oviductal tumors also do not express CA125.

The objective of the fourth experiment was to compare CA125 expressed in human ovarian cancer with samples taken from avian ovarian tumors. Objective 1 resulted in a protein of ~50 kDa while the reported CA125 protein detected in humans is 200 kDa. The second aim in the current study was to determine if there is a correlation between E-cadherin and the tumor marker, CA125. There is controversy about the status of E-cadherin expression in human ovarian disease. Finding a link between the cell adhesion molecule and CA125 may shed light onto the role of CA125 in ovarian cancer. We found that CA125 is

expressed in a human ovarian tumor, while there was no E-cadherin expression in any avian sample.

CA125 Expression on Spontaneous Ovarian Adenocarcinomas from Geriatric Laying Hens

by

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BIOGRAPHY

Emily Jackson was born May 12, 1981 in Thomasville, North Carolina. She was the daughter of a United Methodist minister which caused her to move every several years throughout western North Carolina, however spending the majority of her youth in the Charlotte area. She graduated with honors from East Gaston High School in Mt. Holly, NC in 1999. During her 4 years of high school she was placed on the National Deans List. She attended North Carolina State University and graduated with honors with a Bachelor of Science degree in Biological Sciences with a Minor in Genetics in 2002. During her 3.5 years of undergraduate work she received The Outstanding Freshman Award and was also a member of Phi Sigma Pi National Honor Fraternity. She then did a year of post-baccalaureate studies before entering into a graduate program in 2004. She has been fulfilling the requirements for a Master of Science in Physiology with a Minor in Biotechnology under the supervision of Dr. Paul Mozdziak in the department of Poultry Science at North Carolina State University. During her graduate work she was placed on the National Deans List for graduate students.

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CA125 Expression from Spontaneous Ovarian Adenocarcinomas from Geriatric Laying Hens

1. LITERATURE REVIEW

1.1 THEORIES BEHIND OVARIAN CANCER FORMATION

1.1.1 INCESSANT OVULATION THEORY

Ovarian cancer is the fourth most common cancer death among women in the United States. It was estimated that in the year 2005 there would be 22,220 new cases of ovarian cancer in the United States and 16,210 women would succumb to their disease within the same year (15). The risk of developing ovarian cancer during the lifetime of a woman is approximately 2 percent (12,15). The fact that ovarian cancer is a relatively uncommon disease does not make it an insignificant one. It ranks as the most lethal gynecological malignancy (1,2). Only 50% of women who are diagnosed with ovarian cancer survive to the 5 year point. One of the reasons for the high mortality data is that ovarian cancer is mostly asymptomatic and rarely presents itself at early, curable stages. It has been shown that 90% of tumors can be cured if diagnosed at early stage I disease, compared to only 30% of tumors being successfully treated when diagnosed at stage III disease. Only 25% of tumors are diagnosed at stage I in the United States when treatment is most effective (3,4). Greater than 90% of all ovarian tumors seem to originate from a thin layer of epithelial cells that surrounds the ovary. The ovarian surface epithelium (OSE) originates during embryonic development from the coelomic epithelia (8). These tumors can be further divided into histological subgroups including serous, mucinous, endometrioid, clear cell, Brenner undifferentiated, or a mixture of the various subtypes (12). The heterogeneity of tumors further complicates the search for better management plans of this lethal disease.

To date, little is known about the etiology of epithelial ovarian cancer. It is known that using oral contraceptives, pluriparity, hysterectomy, and tubal ligation decreases a woman's risk of developing ovarian cancer (12). However, early menstruation, nulliparity, prediagnosis of polycystic ovarian syndrome, and late onset of menopause increase a woman's risk of ovarian cancer (3). Taken together the literature suggests that ovarian cancer may be caused by reproductive and/or hormonal factors.

The formation of inclusion cysts is important when studying the causes of ovarian cancer because it is thought that epithelial cells found in inclusion cysts in the ovaries are more likely to undergo malignant transformation than the actual epithelial cells on the surface of the ovary. The OSE has the ability to differentiate into stromal or epithelial phenotypes (12). The reason that OSE cells have the ability to convert from epithelial to stromal may be to accommodate OSE cells that become trapped within the ovary's stroma. If the cells are unable to convert than the result is epithelial cell aggregations in the stoma and ultimately inclusion cyst formation (12). There is controversy about the reason for inclusion cyst formation. Some suggest that the cysts form as a result of ovulation as clefts formed by regression of a corpus albicans or by direct spread of surface epithelial cells into the cavity of a corpus luteum (14). The clefts eventually close off and become inclusion cysts. Others believe they can form from inflammatory adhesions, pinching off of the mesothelium, or OSE cell secretion of proteolytic enzymes, which enable penetration of the underlying stroma. Despite the route of formation of inclusion cysts, the importance is whether or not the epithelial cells are more likely to undergo malignant transformation than on the surface. The epithelial surface of the ovary is avascular, which means its hormonal exposure is limited to paracrine mechanisms. However, OSE cells found in inclusion cysts are inside the ovarian

stroma, which places them in close contact to hormone producing cell types and allows hormonal exposure through endocrine as well as paracrine mechanisms (12). It has also been documented using immunohistochemistry that OSE cells in inclusion cysts stain more positively for CA125 than do the OSE cells surrounding the ovary. The presence of CA125 in inclusion cysts is valuable information because CA125 is the only currently validated marker for ovarian cancer.

Fathalla (23) introduced the concept of the incessant ovulation theory in 1971 as the causative factor of ovarian cancer formation. The incessant ovulation theory suggests that the tearing of the ovarian surface epithelium with each ovulation and the subsequent regeneration of the epithelium allows for a cell containing a genomic defect to be propagated causing clonal expansion of a cancerous cell. The risk of propagating a defect with each ovulation explains why long durations of ovulatory menstrual cycles with no breaks for parity increases the risk of developing ovarian cancer.

Studies have recently been conducted in Taiwan that noted a correlation between the number of children born alive by women and the risk of death from ovarian cancer (5). Yang et al. (5) concluded that the risk of death decreased with each child a woman birthed. The risk decreased from 2.45 with one child, to 1.43 with the second child, and finally a .59 risk with a third child. The thought behind this concept is that during the gestational period of 9 months a woman is not experiencing ovulations due to the effects of placental and ovarian hormones being produced in the mother. The absence of ovulation results in an interruption in the shearing and regeneration of ovarian surface epithelia and decreases the chance of DNA damage done during cellular proliferation. Progesterone is also produced at higher levels during gestation to maintain the pregnancy. It has been speculated that progesterone

may induce apoptosis and prevent ovarian cancer formation (11). Pregnancy ensures a preventative effect after pregnancy but that protection declines with time since the last pregnancy occurred. In a study done by Chiaffarino et al. (11), it was shown that a significantly higher percentage of the women with ovarian cancer had birthed their last child 20 or more years before diagnosis. A smaller group of women had developed ovarian cancer 10 years after birthing their last child. This study demonstrated the protective affect of parity and also the diminishing protection with time since last child birth.

Trends of the past century lend more evidence to the incessant ovulation theory. The role of women in society has shifted from child-rearing to working and contributing to the family income. In the 19th century women were estimated to produce 40-50 oocytes during their reproductive lifespan compared to recent estimations of 400-500 oocytes in the 20th century (6). Studies conducted on the incidence of ovarian cancer show that there was an increase in ovarian cancer risk in women who reached their reproductive age at the beginning of the 20th century when reproduction rates were steadily decreasing (24). The trend continued until the mid-20th century when oral contraceptives were introduced into the population. It was reported in Denmark and Sweden that there was a peak incidence rate of ovarian cancer in 1970, followed by a slight decrease thereafter due to the common use of birth control and an increase in reproduction rates in the 1960's (25). To further support the hypothesis is the fact that it is rare for non-primate animals to develop ovarian cancer. Most large farm animals are seasonal breeders and other small animals will only cycle in the presence of a male or only when mated. None of these species continuously cycle and therefore their reproductive lifespan does not expose them to the risks of developing ovarian cancer. Hens are the only exception to this and they spontaneously develop ovarian cancer similar to humans because

they can be maintained under intense egg laying conditions. A typical hen will produce 280 eggs in a 50 week period (7). Also, exposure of hens to progestin inhibits ovulation and increases the hens' level of protection against ovarian cancer. This puts them at a similar ovulatory age as humans and makes them an acceptable model for ovarian cancer studies.

Another view of the incessant ovulation theory involves exposure of the follicle rupture site to inflammatory mediators and toxic oxidants. Ovulation and luteinization produces excess reactive oxidants which infiltrate the nearby epithelial cells that migrate to the site of rupture to replicate and fill in the void created by ovulation. The reactive oxidative species can bind to DNA, RNA, and/or protein and cause cytotoxicity and carcinogenicity by allowing mutation of tumor suppressor genes initiating carcinogenesis (9). Oxidative stress is the excessive production of reactive oxidative species and a disturbance in the balance of oxidative defense that can counteract these oxidative molecules. Therefore, antioxidants are the obvious preventative agent to protect intracellular molecules against damaging free radicals. The most effective agent is vitamin E due to its contribution to membrane phospholipid stability producing a barrier against free radicals penetrating the cells (8). Women who have lower circulating levels of Vitamin E, as well as vitamin C, are shown to have higher rates of ovarian cancer than women who have sufficient levels of antioxidants (9).

The incessant ovulation theory; however, does not explain other observations found when studying ovarian cancer. For instance, it has been mentioned that twin pregnancies provide more protection against ovarian cancer than single pregnancies. The incessant ovulation theory also does not explain the relationship found between early age at menopause and the increased risk of developing ovarian cancer. The discrepancies in the incessant ovulation

theory have led scientists to believe that there could be a hormonal cause behind the development and formation of ovarian cancer.

1.1.2 GONADOTROPIN HYPOTHESIS

The second hypothesis of ovarian tumor formation is the pituitary gonadotropin hypothesis. The gonadotropin theory suggests that increased gonadotropin (Luteinizing Hormone (LH) and/or Follicle Stimulating Hormone (FSH)) exposure increases estrogenic stimulation of the ovarian surface epithelium either directly or indirectly (6). The direct effect would be caused by activation of gonadotropin-response genes in cells that are undergoing malignant transformation. On the other hand the effect could be indirect by stimulating production of sex steroids by the ovary that could act through paracrine or endocrine mechanisms to cause malignant transformation (12). The highest rates of epithelial ovarian cancer occur in women that have reached menopausal age. The mean age of ovarian cancer incidence is between 57-59 years. Women in advanced age produce more gonadotropins than woman who are premenopausal. Gonadotropins acting as tumor promoters would explain why the increased concentrations of LH and FSH at menopause can cause effects of ovarian carcinogenesis. The gonadotropin phenomenon would exert its greatest effects in age specific cases with incidence peaks in the mid 70's (14). The age specificity also poses a problem for this theory because the expected age of post menopausal gonadotropin caused ovarian cancer is 70 however 75% of cases would already have occurred from age 57-59. The late age onset only supports a role for ovarian cancer formation and late peaks in ovarian cancer incidence. Another problem for this theory is that hormone replacement therapy (HRT) for treatment of post menopausal symptoms has been shown to cause a slight increase in ovarian cancer risk. However, hormone replacement therapy decreases LH and FSH concentrations. The gonadotropin hypothesis cannot explain

why HRT increases ovarian cancer risk. It is thought that this observation might be hormonal, only involving sex steroids not gonadotropins.

To support the research that oral contraceptive use and pluriparity decrease the risk of ovarian cancer, these two situations also decrease the levels of gonadotropins produced by the pituitary and released into circulation. Frequently women who are infertile undergoing infertility treatments are exposed to increased levels of gonadotropins to induce ovulation. These women have 2.8 times the risk of invasive ovarian cancer and 4.0 times the risk of low malignant potential ovarian cancer compared to a control group of women who were infertile and not undergoing therapy (6,12). Also, women with polycystic ovarian syndrome (POS) have an increased risk of developing ovarian cancer. POS is characterized by hypersecretion of LH (13).

It was debated for many years whether or not ovarian surface epithelial OSE cells expressed gonadotropin receptors. In a review by Konishi et al.(13) in 1999 it was stated that OSE cells express the LH/hCG receptor mRNAs (13). They also report that Zheng et al (26) found expression of FSH receptors on the OSE. These results suggest that the ovarian surface epithelium does contain gonadotropin receptors and are therefore responsive to gonadotropin stimulation of cell proliferation and inhibition of apoptosis of the OSE cells during the repair process of the ovary after ovulation has occurred.

1.1.3 ANDROGEN HYPOTHESIS

A third and final approach to looking at ovarian cancer formation also involves hormonal regulation. However, the androgen theory involves the sex steroid hormone androgen. Studies have shown increased cell proliferation of normal OSE cells after exposure to androgen via androgen receptors. Oral contraceptives decreases androgen levels, which supports the observation that they offer a preventative effect against ovarian cancer. Polycystic ovarian syndrome (POS) increases ovarian cancer risk and is known to be a hyperandrogenic disease (12). In one study mentioned by Lukanova et al (12), premenopausal women had 44% more androstenedione than controls. While androstenedione is a weak androgen it is not irrelevant because OSE cells have the enzyme 17 β -hydroxysteroid dehydrogenase, which is necessary to convert this steroid into testosterone, which is 10 times more potent (12,14). Within the ovaries, androgen secretion is higher than estrogen secretion. This causes the epithelial cells, especially those in inclusion cysts, to be exposed to paracrine androgen. Also there are 2-3% free, unbound estrogens and androgens circulating in the body. This allows exposure of the same epithelial cells to circulating levels of androgens via endocrine effects. To lend support to the fact that most ovarian cases are reported peri- or postmenopausal the postmenopausal ovary is also appreciably androgenic.

Animal studies also support these findings. It has been shown that giving guinea pigs testosterone stimulated the growth of OSE cells and caused benign cysts and small adenomas (12). Neonatally thymectomized mice have been observed to produce large amounts of testosterone from androstenedione prior to tumor occurrence (14). The animal models along with epidemiologic evidence mentioned previously allow the androgen hypothesis to be a third plausible mechanism behind ovarian cancer formation.

As with most theories there is evidence for and against all cases and the debate behind ovarian cancer development will continue on until more is understood about the nature of the disease. Although there are several hypotheses for ovarian cancer formation there is still hope in the quest to understand the etiology behind epithelial ovarian cancer. Epithelial ovarian cancer has proven to be a very heterogenous disease, with many different subtypes and intratumor genetic variation. As a result of this heterogeneity, ovarian tumors vary in their characteristics and expression of various markers and proteins, including the main ovarian cancer tumor marker CA125. This allows the formation of tumors from all hypothetical mechanisms to coexist instead of competing with one another (10).

1.2 THE HISTORY OF OVARIAN CANCER AND CA125

1.2.1 THE HISTORY OF OVARIAN CANCER

Ovarian cancer is a great clinical challenge because two-thirds of patients diagnosed with ovarian cancer present at an advanced disease stage. The incidence of ovarian cancer varies in different parts of the world. Ovarian cancer is most common in industrialized nations, in particular the United States, Europe, and Israel (15). The opposite is true for the developing countries with lowest incidence rate, including Japan and Asia. There is strong evidence that environment is responsible for the onset of this disease when one observes that women who migrate from a low-risk country to a high risk country gradually increase their risk of developing ovarian cancer to the same as those in the developed country (15). It is also thought that race plays a role in ovarian cancer risk because the incidence rate is lower for African-American and Asian-American women than it is for Caucasian women (16).

The disease targets mostly peri- and postmenopausal women with a peak incidence rate occurring at 60 years of age. The age of incidence is thought to decrease in women with familial ovarian cancer and women with germ cell tumors versus epithelial tumors (17). To offer more evidence to ovarian cancer being an environmental disease only 5-10% of epithelial ovarian tumors are thought to be genetically linked. Exposure to carcinogenic agents is thought to lead to neoplastic transformation which causes loss of tumor suppressor genes and activation of oncogenes.

1.2.2. THE HISTORY OF CA125

CA125 is a cell membrane glycoprotein expressed on the surface of a variety of epithelial cells, and it is expressed in a variety of benign and malignant conditions, including ovarian cancer. CA125 is not sensitive or specific enough to be the solitary tool for diagnosing ovarian cancer; however, the combined use of CA125 testing, physical and pelvic exam, and transvaginal ultrasonography is 90% accurate at detecting disease progression. Only 50% of early stage tumors express CA125, while 80% of late stage tumors express CA125. Unfortunately there is little benefit to early detection of late stage tumors because disease has usually metastasized beyond the point of treatment. The CA125 antigen is a heterogenous combination of glycoproteins with a molecular weight range from 200-2000 kDa (18). The antigen is heavily serine and threonine-linked (O-) and N-linked glycosylated. Differences in glycosylation result in heterogeneity of the CA125 antigen. Heterogeneity allows for lower molecular weight species of CA125 to exist.

Robert C. Bast (19) was the first person to develop an antibody to detect CA125 in ovarian cancer. OC125 is a murine monoclonal antibody made from immunization with the OVCA 433 cell line (19). The CA125 serum test introduced by Bast et al. in 1983 was the first tumor marker test for epithelial ovarian cancer and used OC125 as both the capture and indicator antibody (20). Recently, more suitable tests have been created that use a different capture, M11, and indicator, OC125, antibody. This test became possible when it was discovered that CA125 has two major epitopes: the OC125-like and M 11-like epitopes (18). Even more recently another epitope has been named as the Ov197-like epitope (21). Changes in the concentration of CA125 in serum samples correlate well with the course of the disease during chemotherapy (22).

Until recently little was known about the molecular biology of CA125. It is now known that CA125 is encoded by the Mucin 16 gene. This gene is localized to chromosome 19 (22). The glycoprotein maintains the characteristics of other mucin molecules, in that its main amino acids are serine, threonine, and proline. The high leucine content, however, is characteristic of only Mucin 16, as are the length of the tandem repeats. Mucin 16 has 9 tandem repeats of 156 amino acids identified thus far. There are also 6 sea-urchin sperm protein, enterokinase, and agrin (SEA) domains in the protein sequence, 5 in the tandem repeat region. These could be potential sites that are susceptible to proteolytic cleavage. There is also a span of 25 hydrophobic amino acids that could represent a membrane-spanning region. There is also a 31 amino acid cytoplasmic tail that contains a putative tyrosine phosphorylation site. This is significant because the secretion of CA125 from a solid tumor into the circulation is stimulated by epidermal growth factor which is a well known tyrosine kinase (22).

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1. OBJECTIVES

AIM 1: TO DETERMINE CA125 EXPRESSION IN TISSUE FROM THE TWO YEAR OLD LAYING HEN

The two year old laying hen is a good model for human ovarian cancer because it is the only currently known species that spontaneously develops ovarian cancer. The ovulatory lifespan of a chicken is similar to the reproductive lifespan of a human. After two years of age, egg production in the hen drastically decreases and the animal is considered unproductive for agricultural purposes. The decrease in reproductive potential mimics menopause in women, and it is the time when ovarian cancer begins to present itself in an observable portion of the bird flock. The long term goal of experimentation is to compare the expression of CA125 in chickens to what is seen in humans with respect to different clinical characteristics.

The first objective is to establish the chicken as an animal model for human ovarian cancer. This will be done by analyzing CA125 expression in chickens to observe whether the established human ovarian cancer tumor marker is present in the avian species.

AIM 2: TO DETERMINE THE SIGNIFICANCE OF DISEASE PROGRESSION ON CA125 EXPRESSION IN THE CHICKEN

CA125 has been well established as a tumor marker in human ovarian cancer. It has its major pitfalls in sensitivity and specificity. These two issues are significant enough to limit the proteins use for diagnosis in the clinical setting. A positive CA125 test is not enough to elicit a diagnosis of ovarian cancer. Transvaginal ultrasonography is employed in conjunction with CA125 and ultimately surgery to determine ovarian cancer in a woman. CA125, however, is useful in monitoring disease progression and response to treatment. This is due to the fact that CA125 level changes after treatment can offer insight into regression or recurrence of disease.

The second objective is to determine how CA125 expression changes in different stages of disease and different levels of metasatasis in the chicken.

AIM 3: TO DETERMINE OVIDUCTAL INVOLVEMENT IN CA125 EXPRESSION

By being able to diagnose the primary tumor an oncologist can better execute a disease management plan and provide tissue-specific treatment to the patient. Primary tumor diagnosis has been shown to significantly improve disease prognosis. The third objective is to determine if CA125 is expressed in oviductal tumors found in the chicken. It is significant to know whether the oviduct is also a source of CA125 in an effort to determine the primary site of tumor formation.

CA125 has also been hypothesized to function in cell adhesion and metastasis in ovarian cancer. The concept is that CA125 is shed from the ovary and adheres to mesothelin present on the peritoneal mesothelium, which allows secondary tumor formation. The next objective is to determine if oviductal tumors that are expressed alongside ovarian tumors express CA125 in an effort to determine if CA125 is shed from the ovary and attaches to the oviduct where it establishes a secondary tumor.

AIM 4: COMPARISON OF HUMAN AND AVIAN OVARIAN TUMORS AND E-CADHERIN CORRELATION TO CA125 IN BOTH SPECIES

E-Cadherin is a transmembrane protein with four tandemly repeated extracellular domains. The first extracellular domain is the site of binding to other cadherins or to catenins. The first extracellular domain is also the location of monoclonal antibody binding on the E-cadherin protein. The cell-adhesion molecule is important for establishing cell polarity, maintaining normal tissue morphology, and cellular differentiation. It is believed to be a tumor suppressor in most cancers because down-regulation of E-cadherin results in cancer invasion and metastasis. It is controversial whether the molecule is present in ovarian cancer, however in studies reporting the expression of E-cadherin it has been shown to act opposite of a tumor suppressor. In these studies, the presence of E-cadherin is shown to be correlated with invasive potential, while down-regulation is associated with a normal ovary state.

The final objective is to compare a human ovarian tumor to an avian ovarian tumor to see how CA125 varies between the two species. To aid in further elucidating the function of CA125 in ovarian cancer, it is significant to know how CA125 correlates to E-cadherin expression in both species.

3. CA125 EXPRESSION IN SPONTANEOUS OVARIAN ADENOCARCINOMAS FROM LAYING HENS

3.1 ABSTRACT

3.1.1 Objectives

Currently, there is not a fully characterized model for human ovarian cancer; however, 2- to 4-year-old laying hens spontaneously develop ovarian tumors. CA125 expression is a hallmark of ovarian cancer in women. The major objective of this study was to characterize the in vitro growth of avian ovarian tumor cells, and CA125 expression in avian ovarian tumors.

3.1.2 Methods

Immunohistochemistry was employed to evaluate CA125 expression in avian ovarian tumor tissue. A high temperature antigen retrieval step was an essential part of the CA125 immunostaining procedure. In vitro growth curves were constructed for avian ovarian cancer cells. Western blotting was used to estimate the size of the CA125 reactive protein and to confirm CA125 expression.

3.1.3 Results

The growth of avian tumors in culture fit a sigmoidal curve for cell growth and suggests a cell cycle time of 28 h. The tumors taken from the chicken stained positive for CA125. Approximately 90% of cells isolated from avian ovarian tumors also stained positive for CA125. Western blots show a band of approximately 25 kDa when immunodetected with CA125.

3.1.4 Conclusions

Similar to human ovarian tumors, chicken ovarian tumors express CA125. Cultured chicken ovarian cancer cells express CA125 and CA125 expression does not appear to change with time in culture.

3.2 INTRODUCTION

Ovarian cancer is the most lethal of all gynecological diseases, and it ranks fourth in cancer deaths among women (1, 2). The American Cancer Society predicted that in 2005 approximately 22,220 new cases of ovarian cancer will be diagnosed in the United States and it is estimated that 16,210 of these women will succumb to the disease (3, 4). The reason for the alarmingly high mortality rate is late diagnosis at advanced stages of the disease, and diagnosis after cancer has metastasized to other organs. Fewer than 30% of women can be successfully treated when the disease is diagnosed at late stage III compared to a 90% cure rate at stage I. However, less than 25% of all cases are diagnosed at stage I in the United States (3, 5).

One factor hindering significant advancement in the study of ovarian cancer is the lack of a fully characterized animal model system. Ovarian tumors arise spontaneously with age in some strains of mice and in Wistar and Sprague-Dawley rats, but such tumors are extremely rare (1, 6). Mice can also be inbred or manipulated by viral vectors to become tumorigenic. Tumor cells can be xenografted into SCID mice (6). However, the majority of the mouse based animal models have genetically induced tumors that are inappropriate for large-scale chemoprevention studies. The mouse model may be inadequate because the tumors are not formed spontaneously, and are biologically different from the naturally occurring tumors (1). A better model system over the current mammalian systems is an animal that spontaneously forms ovarian tumors in a reproducible way.

The domestic hen [*Gallus domesticus*] may be a useful model for studying ovarian cancer because hens maintained under intensive egg-laying conditions spontaneously develop ovarian adenocarcinomas (6, 7). A typical hen will produce 280 or more eggs in 50

weeks (8). Egg production drastically decreases after two years of age when the hens begin to be considered unproductive for agricultural purposes. Likewise, ovulations decrease with age as the period between clutches of eggs increases (7). Currently, the incessant ovulation theory of ovarian cancer formation suggests that the risk of developing ovarian cancer is directly related to the number of ovulations a woman experiences in her lifetime. Women who start menstruating at an early age, women with no children, and women who experience menopause after age 50 are at an increased risk (3). The observed spontaneous tumor formation in two-year-old laying hens led to its consideration as a model for human ovarian cancer. The presence or absence of the human tumor marker CA125 in the domestic fowl would support the utility of the avian model to study ovarian cancer.

The current method of screening for ovarian cancer involves a blood test to detect the cancer antigen CA125, which is a glycoprotein expressed on the cell membrane of ovarian tumors that has a molecular weight above 200,000 (9, 10). CA125's use in the clinical setting makes it an important marker to verify the avian model. The objective of the study was to confirm the presence of CA125 in avian ovarian tumors and cells isolated from the tumors.

3.3 MATERIALS AND METHODS

3.3.1 Tissue Collection

Hens employed in this study were commercial Single Comb White Leghorn [*Gallus domesticus*] that had been maintained for 2 years at the Piedmont Research Station in Salisbury, NC. Each hen ovulated approximately 450 times prior to death, and they had never been subjected to a synchronized molt. Hens ranged in age from 86 weeks to 105 weeks of age. In total, 450 hens were necropsied for tumors, and 15 ovarian tumors were discovered for an ovarian cancer incidence rate of 3%. Necropsies were performed at North Carolina State University. All procedures involving animals were approved by the North Carolina State University Institutional Animal Care and Use Committee. When an ovarian adenocarcinoma was identified, it was aseptically removed and cut into several pieces, which were placed either in 4% paraformaldehyde overnight for immunohistochemistry or into 5 mL of Hank's Balanced Salt Solution for primary cell culture.

For cell culture, tumor tissue was mechanically and enzymatically digested using 0.17% Trypsin/0.085% Collagenase for 30 minutes at 37°C. Tissue was washed with media containing Dulbecco Modified Eagles Medium (DMEM), 10% Fetal Bovine Serum (FBS), 1% Gentamicin, and 1% Non-Essential Amino Acids. The pellet was passed through a 100 µm Nitex filter to separate cells from debris. The cells were cultured with DMEM, 1% Gentamicin, 1% Non-Essential Amino Acids, and 10% FBS.

3.3.2 Growth Data and Immunocytochemistry

Isolated cells were plated into ten wells of ten twenty-four well plates at a density of 11,202 cells per well. Cells were also seeded onto three two-well chamber slides (Fisher Scientific, Chicago, IL) at a density of 22,404 cells per chamber. A plate and slide were fixed at different time intervals to assess different stages of growth. The cells in plates were fixed at 24 h, 72 h, 96 h, 110 h, 122 h, and 134 h after initial plating. At 134 h, the cells were 100% confluent in all wells. The cells in chamber slides were fixed at 24 h, 110 h, and 158 h, at which time the cells were 100% confluent. The cells were fixed using 4% paraformaldehyde and stored at 4°C until processed for immunohistochemistry.

The remaining four plates were allowed to grow to confluency, trypsinized using 0.25% Trypsin:EDTA, and re-inoculated onto seven six-well plates (Fisher Scientific, Chicago, IL) at a density of 375,000 cells per well. Three chamber slides were inoculated at a density of 562,500 cells per chamber. A higher cell number than the initial seeding was employed because it appeared qualitatively that cell growth was beginning to slow. More cells were plated to ensure cells reached a confluent state. Plates were fixed with 4% Paraformaldehyde at 24 h, 48 h, 72 h, 96 h, 144 h, 192 h, and finally 240 h. Slides were fixed at 24 h, 144 h, and 528 h.

To assess growth, the cells on the plates were stained with propidium iodide (PI) and examined under a Leica fluorescence microscope using a 40x objective. PI stains the nucleus of the cell making it possible to visualize each viable cell on the plate. Images of ten fields per well were obtained from each six well plate using a Spot-RT CCD (Diagnostic Instruments, Sterling Heights, MI) camera. The images were collected and used to quantify

growth. The cells per image were enumerated using Image Pro Plus 6.0 imaging software (Media Cybernetics Inc., Silver Springs MD, USA).

The chamber slides were used for immunocytochemistry. The slides were washed three times for 2 min each with distilled H₂O placed into boiling 0.01M citrate buffer pH 6.0 for 15 min and then cooled at room temperature for 20 min. Citrate buffer was removed using two rinses with PBS for 2 min.

A circle was drawn around the cells on the slide using a PAP pen. To block unspecific binding, the outlined portion of the slide was incubated in 1% BSA, PBS pH 7.4 for 30 min. The slides were kept in a humidity chamber to prevent dehydration. The cells were washed twice for 5 min each with PBS and incubated with the primary antibody purchased from Zymed (San Francisco, California) against CA125 (1:50) in 1% BSA, PBS pH 7.4 overnight in a humidity chamber at 4°C. The slides were washed three times for 5 min each with PBS. Subsequently, the slides were incubated with a fluorescein-conjugated secondary antibody (goat anti-mouse IgG 1:50) in 10% Goat serum in PBS pH 7.4 for 2 h in a humidified chamber in the dark and washed three times for 5 min each with PBS. Subsequently, PI (50 µg/ml) was applied to the slides for 10 min in the dark. The specimens were mounted using a glycerol-based mounting media containing *p*-phenyinediamine (PPD) (12). All slides were stored at -20°C until analysis.

3.3.3 Immunohistochemistry

The tumors that were fixed in 4% paraformaldehyde were washed in three exchanges of PBS, dehydrated, cleared, and embedded in paraffin. Ten micron thick sections were cut.

Positive control slides (Fig. 2) of human ovarian tumors, purchased from Zymed (San Francisco CA, USA), were processed concurrently with the chicken samples. The slides were deparaffinized using 3 changes of xylene. For antigen retrieval, sections were placed in boiling 0.01M citrate buffer pH 6.0 for 15 min and then cooled at room temperature for 20 min. The slides were lastly rinsed with PBS for 2 min. The sections were incubated in 1% BSA, PBS pH 7.4 for 30 min to block nonspecific binding. The sections were incubated with the primary antibody against CA125 (1:50) diluted in 1% BSA, PBS pH 7.4 overnight in a humidified chamber at 4°C. Negative control slides (Fig. 2) were generated by omitting the primary antibody. The slides were washed three times for 5 min each with PBS, incubated with a fluorescein-conjugated secondary antibody (goat anti-mouse IgG 1:50) in 10% Goat serum in PBS pH 7.4 for 2 h in a humidified chamber in the dark. The slides were washed three times for 5 min each with PBS. PI (50 µg/ml) was then applied to the slides for 10 min in the dark after which time the slides were rinsed once briefly with PBS and then mounted in glycerol mounting media containing PPD (12).

3.3.4 Western Blot

Normal ovarian tissue was taken from birds not afflicted with ovarian cancer. Tumor tissue was also taken from the ovaries of birds that exhibited ovarian tumors. The tissue was weighed and immersed in lysis buffer (20mM Tris-HCL pH 7.5, 10 mM NaCl, 10 mM KCl, and 3 mM MgCl₂). Additionally, 0.5 mm mini glass beads were added to each tube to dissociate the tissue. The tubes were then placed into a mini bead beater and homogenized for 1 minute. The protein suspension was spun for 4 min at 10,000xg. A BCA assay (Sigma, St. Louis MO, USA) was performed to quantitate the amount of protein per sample. Protein was mixed 1:1 with Laemmli sample buffer (0.0625 M Tris-HCl, 2% SDS, 10% Glycerol, 5% 2-Mercaptoethanol, and 0.001% bromophenol blue) and boiled for 3 min at 100°C.

The protein (100 µg per lane) was fractionated through an SDS-PAGE gel. The resolving gel consisted of 8% total acrylamide, 0.2 M Tris pH 8.8, 0.4% SDS, 0.03% ammonium persulfate, and 0.1% *N,N,N',N'*-tetramethylethylenediamine (TEMED). The stacking gel contained 3% total acrylamide, 0.1 M Tris pH 6.8, 0.1% SDS, 0.05% ammonium persulfate, and 0.5% TEMED to accelerate polymerization. The gel was run at a constant current of 30 mA using the Hoefer Mighty Small II (Hoefer Scientific Instruments, San Francisco CA, USA) with 1x running buffer (0.25M Tris, 1.92M glycine, and 0.5% SDS) until the dye front reached the bottom of the gel.

Protein was transferred onto a PVDF membrane (Immobilon, Millipore Bedford MA) in transfer buffer (25 mM Tris, 192 mM glycine, 15% methanol, and 0.5% SDS) under 400 mA constant current for 1 h and 20 min using the Hoefer Mighty Small Transfer apparatus (Amersham Pharmacia Biotech AB, San Francisco CA). Nonspecific protein binding was blocked by immersing the membranes in 3% non-fat dry milk plus Tris-buffered

saline Tween-20 (TBST) for 1 h at room temperature with gentle agitation. The membranes were immunostained with mouse anti-CA125 (Zymed, San Francisco CA) at a dilution of 1:300 overnight at 4°C. Secondary antibody incubation (2 h) followed using a 1:2000 dilution of goat anti-mouse IgG-HRP. The protein was then detected using the Immuno-Star HRP chemiluminescence kit (Bio-Rad, Hercules CA).

3.3.5 Statistics/Data Analysis

The number of cells counted per plate or slide was analyzed using JMP 5.1 Statistical Analysis software. A one-way ANOVA was performed on the means generated from the cell numbers gathered using Image Pro Plus imaging software. Means were compared using Tukey-Kramer HSD.

3.4 RESULTS

3.4.1 Growth Data

The growth curves fit the typical sigmoidal curve for cell growth (Figure 1).

The growth curves suggest a cell cycle time of 28 h.

3.4.2 Immunocytochemistry of Cultured Cells

Subsamples of the cultured cells taken from the avian ovarian tumors were stained for CA125. Table 1 shows the mean with standard error of cells that were CA125 positive in primary culture. There was no significant difference between any of the treatments of sampling times. Figure 3 illustrates CA125 positive cultured cells.

Growth was monitored in cultured cells in secondary culture. The cells at this time show a flattened and elongated morphology that may suggest the onset of proliferative senescence. Table 2 shows the mean with standard error of cells that stained CA125 positive in secondary culture. There was no significant difference between any of the means reported. Figure 4 shows the staining of the chicken ovarian cancer cells at different levels of confluency.

3.4.3 Immunohistochemistry of Solid Tumors

All ovarian chicken tumors stained with the CA125 antibody were positive for the cell surface glycoprotein. The slides of chicken tissue were compared with a human ovarian cancer positive control slide and a negative control slide where the primary antibody against CA125 was omitted. Figure 5 shows the pattern of staining for the positive control. Nuclei were counterstained with propidium iodide (PI) to show the nuclei of cells in the tissue sections.

Human tumors exhibit a staining pattern that is brightly positive around the epithelial surface of the tumor and within the blood cell population. The staining around the tumor represents CA125 as a cell surface marker, and CA125 in the blood indicates CA125 as a serum marker (5). The avian tumors observed exhibit a pattern of CA125 staining throughout the tumor. The species difference in CA125 expression may be related to tumor morphology. Tumors from the chicken appear to have infoldings rather than the smooth surface characteristic of human ovarian tumors. Despite the differences in the pattern of staining, it is clear that CA125 is expressed in the tissue of ovarian tumors both from the chicken and from the human.

3.4.4 Western Blot

Western blotting was carried out to validate the results obtained using immunohistochemistry and to estimate the size of the CA125 reactive protein. The results of this experiment show that CA125 is present in avian ovarian tumors (Fig. 6A). A 25kDa band is present in the lane prepared with avian ovarian cancer tissue, and no band was detected in the lane containing protein isolated from normal ovarian tissue. Likewise, the same band was present in the western blot performed to demonstrate repeatability across tumors (Fig. 6B).

3.5 DISCUSSION

CA125 is a tumor marker found on the cell surface of human ovarian cancer cells. The marker is a glycoprotein with a molecular weight around 200,000 Da (9). CA125 is significant because rising or falling levels of CA125 in the serum of patients with ovarian cancer appears to be correlated with the progression/regression of the disease (13). Given the importance of CA125 in human cancer, it is important to understand the etiology of CA125 staining in tumors presented by the domestic laying hen (Fig. 5). It was discovered that both ovarian tumor tissue from the chicken and ovarian cancer cells in culture from the chicken express CA125 homolog (Fig. 3-5).

Rodriguez-Burford et al (1) concluded that CA125 was not expressed in ovarian tumors from the chicken (1). A major difference between the previous study and the current study was that Rodriguez-Burford et al (1) used a low temperature antigen retrieval with enzymatic predigestion in contrast to the high temperature antigen retrieval employed in the current study. The current results clearly illustrate CA125 reactive protein in avian ovarian tumors, and the negative results reported in the previous study could have resulted from the choice of antigen retrieval. The antigen retrieval step is crucial because fixation crosslinks proteins and may mask antigenic epitopes on the cell surface. The high temperature antigen retrieval allows these crosslinks to be broken down and allows the antigens to be fully available for staining with a primary antibody. Frost et al. (14) demonstrated that low temperature antigen retrieval works differently than high temperature antigen retrieval, and each responds differently when employed to unmask the same antigen. It is possible that the CA125 marker requires a high temperature antigen recovery and that enzyme predigestion degrades the antigenic epitope inhibiting recognition by the primary antibody.

Western blotting techniques revealed a band in the lane containing ovarian tumor tissue and the absence of a band in the lane containing normal ovarian tissue (Fig. 6). To show that the results are repeatable across tumors, a blot was performed using three different avian ovarian tumors. The presence of the same band in all tumor lanes suggests that chicken ovarian tumors express CA125, while normal chicken ovaries do not express CA125.

The avian CA125 protein was approximately 25 kDa, which is smaller than the expected size based upon the human nucleotide sequence available in Genbank (AF414442), but the chicken western blotting results reported by other investigators who found that human ovarian cancer cells produced a CA125 reactive band at approximately 48 kDa (15). Imai et al (16) as well as Davis et al (17) report that the CA125 antigenic determinant is composed of conformationally dependent peptides. CA125 is composed of heavy O- and N-linked glycosylations. Variations in the structure of the protein could alter the size based upon the extent of disassociation. CA125 also has endogenous protease activity inherent in the 200 kDa protein core (18). A last possible cause of a small protein band could be that CA125 is dissociated under strong denaturing conditions (19).

It was important in this study to not only determine that avian ovarian tumors expressed CA125, but also that the ovarian cancer cells expressed the cell surface tumor marker in vitro. The isolated cells were approximately 90% CA125 positive. Cells can transform in culture to a phenotype that is not characteristic for the donor cell type (20). The CA125 positive results indicate that even with a significant amount of time in culture the cells still express the cell surface marker that is characteristic of ovarian cancer cells. It appears that the proportion of CA125 positive cells does not change over time.

CA125 is expressed throughout cell growth, suggesting that CA125 is not regulating cell proliferation. Although there is no direct evidence in the literature that CA125 expression is cell cycle dependent, it is possible that CA125 expression may vary with a cell's position in the cell cycle because it is localized on the surface of human tumors, which are presumably at a different place in the cell cycle than cells deeper within the tumor. It is tempting to speculate based upon Figure 1 that in the event CA125 was directly regulating cell growth that CA125 expression would change with time in culture or when cells enter into senescence. In this study, we demonstrated the presence of CA125 in chicken ovarian adenocarcinomas and cells, as well as characterized the expression pattern of the tumor marker in chicken versus human disease. This study suggests that chicken ovarian adenocarcinomas have characteristics in common with the human form of the disease. The role of CA125 is not yet well understood (13), but establishing a fully characterized animal model for the human disease will lead to future studies aimed at understanding the mechanisms behind the disease. Future studies should focus on understanding the difference in CA125 localization between avian and human ovarian tumors.

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Table 1. Mean and standard error values for the proportion of cells stained with CA125 primary antibody at different time intervals in primary culture.

	Proportion of cells stained CA125 positive per slide
Time 0	.9313 ±.0421
Time 96 (50% confluency)	.9384±.0312
Time 144 (100% confluency)	.8972±.0315

Table 2. Mean and standard error for the proportion of CA125 staining of avian ovarian adenocarcinoma cells at different time intervals in secondary culture.

	Proportion of cells stained CA125 positive per slide
144 hours	.9048±.0714
696 hours (50% Confluency)-Senescent	.8825±.0249

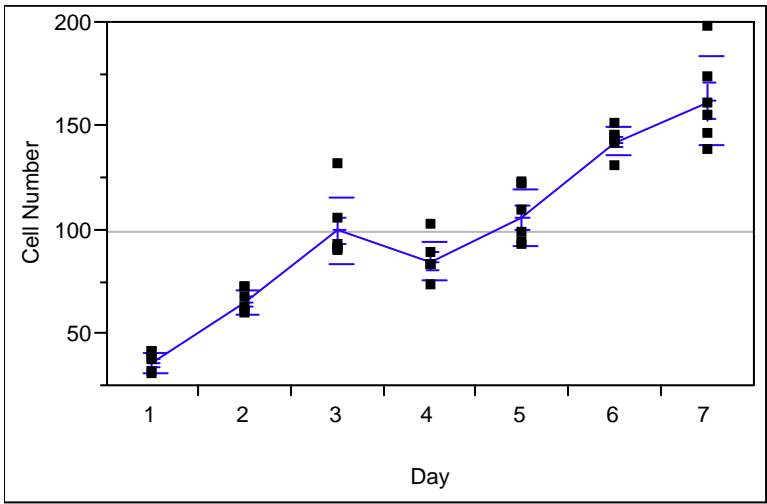
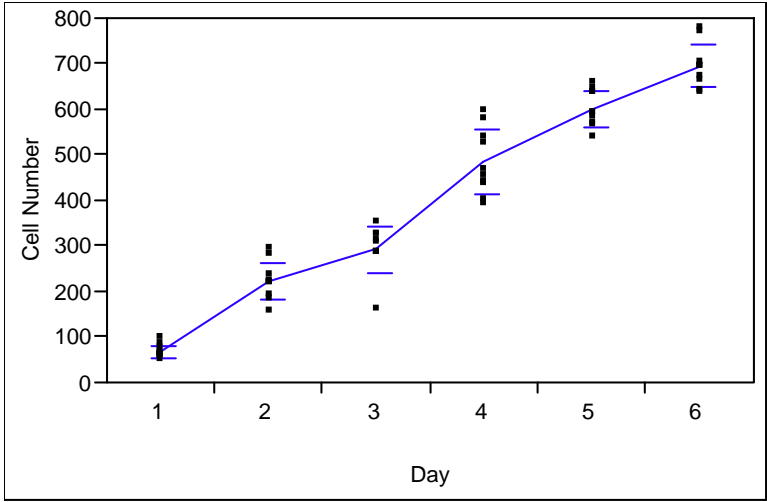


Fig. 1. Growth curve of chicken ovarian cancer cells grown for 7 days. A and B show the results from two sets of cultures. The bars indicate standard error variance from the mean.

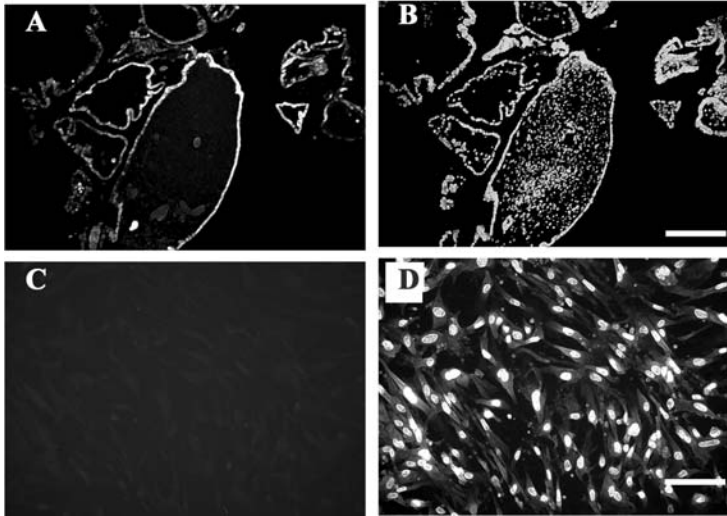


Figure 2. Staining verification. (A) Human positive control slide stained for CA125. (B) Human positive control slide stained with propidium iodide. Scale bar represents 100 μm . (C) Negative avian control slide for CA125 (primary antibody omitted). (D) Negative avian control slide stained with PI. Scale bar represents 25 μm .

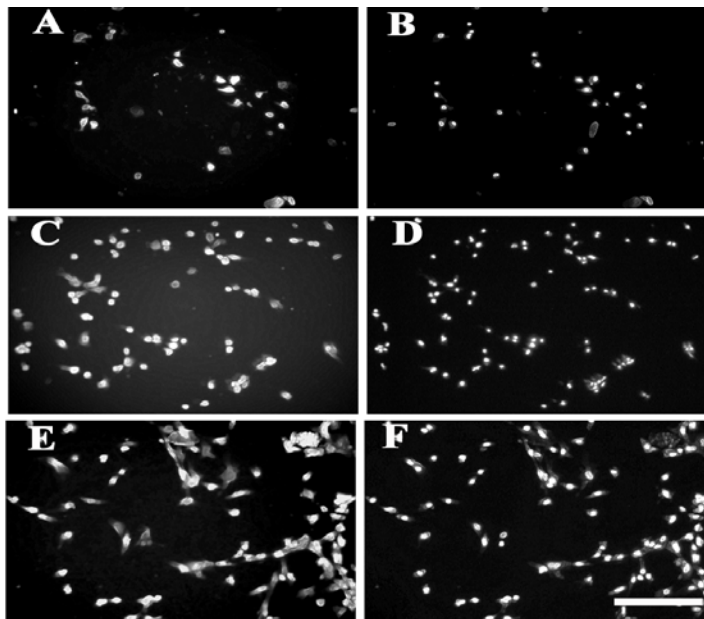


Figure 3. CA125 and propidium iodide (PI) staining of avian ovarian cancer cells during culture using standard immunocytochemistry techniques. (A) CA125 staining of avian ovarian cancer cells at time 0 after 24 h in culture. (B) PI staining of avian ovarian cancer cells at time 0 after 24 h in culture. (C) CA125 staining of avian ovarian cancer cells at 96 h of culture at 50% confluency. (D) PI staining of avian ovarian cancer cells at 96 h. (E) CA125 staining of avian ovarian cancer cells at time 144 and 100% confluency. (F) PI staining of avian ovarian cancer cells at 144 h. Scale bar represents 25 μm .

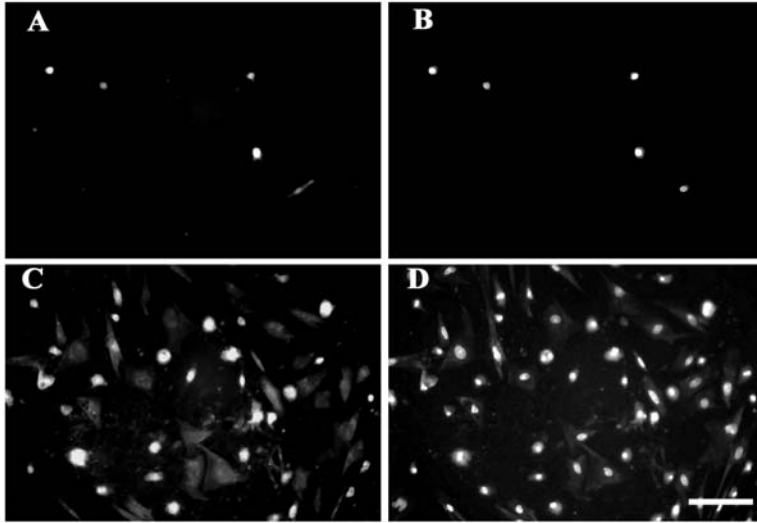


Figure 4. CA125 and propidium iodide staining of avian ovarian cancer cells at different levels of confluency. (A) CA125 staining at time 144. (B) PI staining at time 144. (C) CA125 staining at 50% confluency (696 h). Cells appear to have entered the senescent phase. (D) PI staining at 50% confluency (Time 696). Cells are senescent. Scale bar represents 25 μm .

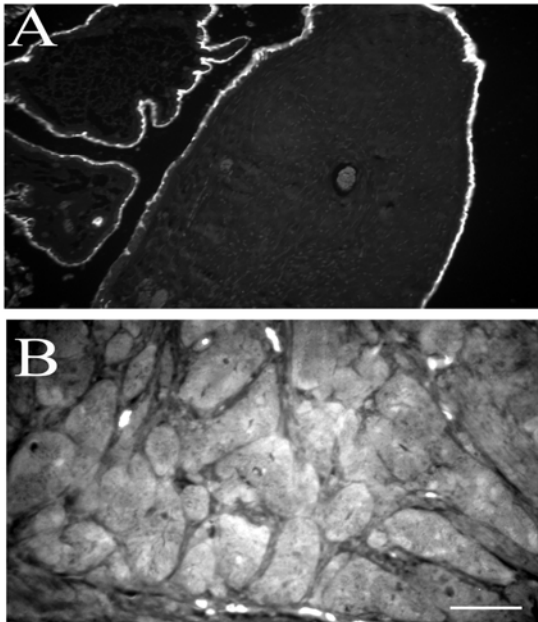


Figure 5. CA125 staining of a positive control slide and an avian ovarian cancer tissue section. (A) CA125 positive control stain. (B) Chicken tissue section stained with CA125. Scale bar represents 50 μm .

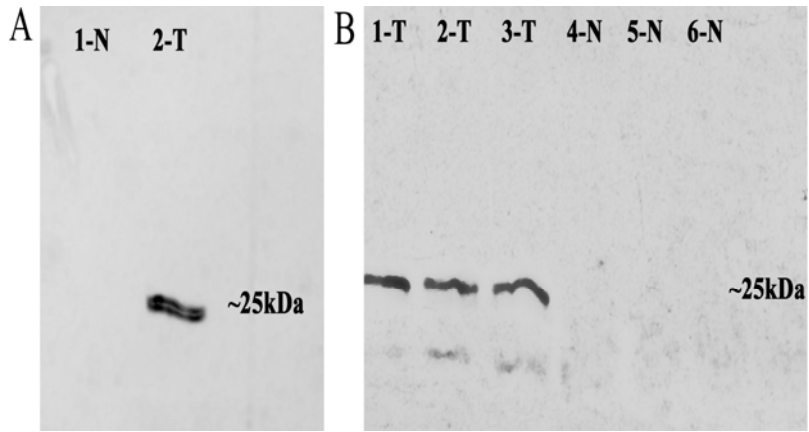


Figure 6. Western blot results with 1 min. exposure. (A) Lane one represents normal avian ovarian tissue. Lane two represents avian ovarian cancer tissue and a 25 kDa band indicating CA125 expression. (B) Lanes one through three represent tissue from an ovarian tumor. Lanes four through six represent normal ovary.

4. The Role of CA125 in Ovarian Cancer Prognosis

4.1 ABSTRACT

4.1.1 Objective

CA125 is most commonly used in monitoring progression of ovarian cancer in the clinical setting. CA125 levels are altered in response to changes in disease state due to treatment or recurrence of the disease. The objective of this study was to assess the presence of CA125 in avian ovarian cancer samples from birds with different stages of disease and different amounts of metastasis.

4.1.2 Methods

Western blotting was employed to determine if CA125 is dependent on stage of disease or level of metastasis in avian ovarian adenocarcinomas. Chemiluminescence was used to detect the protein present on PVDF membranes.

4.1.3 Results

Stage of disease and level of metastasis does not appear to influence CA125 expression in avian ovarian cancer samples.

4.1.4 Conclusion

The lack of CA125 expression can be explained by heterogeneity between and within tumor samples. Experimental conditions also cannot be ruled out as a cause for the absence of CA125 expression.

4.2 INTRODUCTION

CA125 has been an important factor for ovarian cancer in the clinical setting since its discovery in 1981 by the monoclonal antibody OC125 discovered by Bast (1). The pitfalls to CA125 have been well documented throughout the years and remain the same today. There are questions regarding the sensitivity and specificity of CA125. The lack of sensitivity and specificity has hindered CA125's use as a solitary tool in disease diagnosis. CA125 determinations have value as a diagnostic tool in the discrimination of ovarian cancer only when used in conjunction with other tumor markers (8). There is no question that CA125 values can increase the accuracy of diagnosis of ovarian cancer, but only when used in combination with other tests (11). This provides a safeguard against the number of false positive and false negatives that can occur when CA125 alone is used in diagnosis because it does not sufficiently distinguish benign from malignant masses preoperatively (12).

However, the marker finds its primary role in following progression of ovarian cancer and monitoring response to treatment (1). CA125 can be measured at primary diagnosis and compared with samples taken during and after treatment to analyze the effects of treatment on disease response and progression. Increasing or decreasing levels of CA125 correlate with progression or regression of disease in 93% of patients (10). It has been reported to be an accurate early indicator of resistance to treatment during front line therapy (2). It has also been reported that an increase in CA125 measurements precedes recurrence of disease in 75% of all patients (8). A rise in CA125 of 50%, 100%, or to above the cut off level of 35 U/ml has been shown to predict progression of ovarian cancer (9).

Less than 50% of stage I tumors express CA125 and approximately 80% of stage III tumors express CA125 (1). The likelihood of detecting CA125 at primary diagnosis

increases with the stage of disease. The fact that two-thirds of ovarian cancers are diagnosed in late stages of the disease when metastasis has already occurred is what causes it to remain the most lethal of all gynecological malignancies. Stage I disease has a five-year survival rate of 93% and stage II has a survival rate of 70% (3). A diagnosis of disease at stage III or beyond results in a survival rate of only 29% (3, 4). The chance of relapse is correlated with initial stage of disease and tumor grade (3).

The objective of the study is to determine if either disease stage or metastasis has any affect on CA125 expression in avian ovarian cancer samples. This information would allow us to know if CA125 acts in the same manner in both human and chicken ovarian tumors. Knowing the impact that disease state has on CA125 expression would lend further insight into the true role of the tumor marker in ovarian cancer etiology.

4.3 MATERIALS AND METHODS

4.3.1 Tissue Collection

Hens employed in this study were commercial Single Comb White Leghorn [*Gallus domesticus*] that had been maintained for 2 years at the Piedmont Research Station in Salisbury, NC. Each hen ovulated approximately 450 times prior to death, and they had never been subjected to a synchronized molt. Hens ranged in age from 86 weeks to 105 weeks of age. Necropsies were performed at North Carolina State University and also at the Piedmont Research Station. All procedures involving animals were approved by the North Carolina State University Institutional Animal Care and Use Committee. When an ovarian adenocarcinoma was identified, it was aseptically removed and cut into several pieces, which were placed in RNA later, formaldehyde, or snap frozen in liquid nitrogen for further analysis. Stages of disease were classified based upon visual inspection of the ovary at the time of necropsy. A stage 1 tumor is an approximately 1 cm tumor nodule on the ovarian surface. Stage 2 is a large tumor of the ovary, approximately 1-3 cm, but without total ovarian involvement. Stage 3 exhibits multiple large tumors on the surface of approximately 3 cm or larger or total involvement of the ovary.

4.3.2 Western Blot

Tumor tissue was taken from the ovaries of birds that exhibited ovarian tumors. The tissue was weighed and immersed in lysis buffer (20mM Tris-HCL pH 7.5, 10 mM NaCl, 10 mM KCl, and 3 mM MgCl₂). Additionally, 0.5 mm mini glass beads were added to each tube to dissociate the tissue. The tubes were then placed into a mini bead beater and homogenized for 1 minute. The protein suspension was spun for 4 min at 10,000xg. A Bradford assay (Sigma, St. Louis MO, USA) was performed to quantitate the amount of protein per sample.

Protein was mixed 1:1 with Laemmli sample buffer (0.0625 M Tris-HCl, 2% SDS, 10% Glycerol, 5% 2-Mercaptoethanol, and 0.001% bromophenol blue) and boiled for 3 min at 100°C.

The protein (100 µg per lane) was fractionated through an SDS-PAGE gel. The resolving gel consisted of 8% total acrylamide, 0.2 M Tris pH 8.8, 0.4% SDS, 0.03% ammonium persulfate, and 0.1% *N,N,N',N'*-tetramethylethylenediamine (TEMED). The stacking gel contained 3% total acrylamide, 0.1 M Tris pH 6.8, 0.1% SDS, 0.05% ammonium persulfate, and 0.5% TEMED to accelerate polymerization. The gel was run at a constant current of 30 mA using the Hoefer Mighty Small II (Hoefer Scientific Instruments, San Francisco CA, USA) with 1x running buffer (0.25M Tris, 1.92M glycine, and 0.5% SDS) until the dye front reached the bottom of the gel.

Protein was transferred onto a PVDF membrane (Immobilon, Millipore Bedford MA) in transfer buffer (25 mM Tris, 192 mM glycine, 15% methanol, and 0.5% SDS) under 400 mA constant current for 1 h and 20 min using the Hoefer Mighty Small Transfer apparatus (Amersham Pharmacia Biotech AB, San Francisco CA). Nonspecific protein binding was blocked by immersing the membranes in 3% non-fat dry milk plus Tris-buffered saline Tween-20 (TBST) for 1 h at room temperature with gentle agitation. The membranes were immunostained with mouse anti-CA125 (Zymed, San Francisco CA) at a dilution of 1:300 overnight at 4°C. Secondary antibody incubation (2 h) followed using a 1:3000 dilution of goat anti-mouse IgG-HRP. The protein was then detected using the Immuno-Star HRP chemiluminescence kit (Bio-Rad, Hercules CA).

4.4 RESULTS

Avian ovarian cancer samples from birds at different stages of disease were analyzed for CA125 expression. The stages included in the study were as follows: stage I, stage II, stage III. There is no evidence that the stage of ovarian cancer progression has an affect on CA125 expression in the avian model (Fig. 1).

Avian ovarian cancer samples were also taken from birds with different levels of metastasis. The degree of metastasis was measured as follows: 1 other organ involved, 2 other organs involved, and 3 other organs involved. There were no results consistent with the hypothesis that level of metastasis has an affect on CA125 levels present in the avian model of ovarian cancer (Fig. 2).

4.5 DISCUSSION

The hypothesis in the current study was that stage of ovarian cancer disease and level of metastasis would have an impact on CA125 expression in the avian model. In the human model it is known that such factors are contributors to CA125 levels. The correlation is seen when women with advanced stage disease are more likely to have an increased level of CA125 in serum samples. 80% of women with advanced stage disease are expected to have increased CA125 above baseline levels, compared to only 50% of women with stage I disease. CA125 also increases with disease progression to levels higher than what would have been seen at initial diagnosis (10). This is what makes this tumor marker an appropriate tool for monitoring disease progression and response to treatment (1).

The avian model of ovarian cancer did not show results consistent with the human model with respect to the two factors in question. The results from this experiment combined with results from previous experiments give a CA125 positive occurrence rate of ~20%. This leads one to suspect that the low level of CA125 expression in avian ovarian cancer samples is due to another factor beyond disease progression. Heterogeneity in ovarian cancer has been well documented and may be the culprit behind the results seen in the current experience.

In animal species, tumor cell populations have been reported to be heterogeneous in different biochemical and biologic properties, one of those being antigenic profile. Antigen-antibody studies have shed light onto three different forms of heterogeneity. There is heterogeneity among cell lines originating in different patients, among cell lines derived from different metastases, and lastly among cells within a cell line (intratumor heterogeneity) (5). Tumor characteristics change with cell origin and numerous host variables, such as age and

hormonal status to name a few, introduce differences amongst tumors from different individuals (6). Even in distinctively similar patients, tumor characteristics can vary. It is not unrealistic to believe that tumors vary amongst individuals when one considers that tumors vary within themselves.

Cellular phenotypes, including antigenic expression, are functions of the cell cycle. Cells within a tumor can be found to be at all stages of cell division leading to regions within a single tumor that vary in histological characteristics. Heppner (6) went as far as to note that an isolated tumor subpopulation is unimportant except for highlighting the diversity of the cell society it came from. With the presence of subpopulations being well established, it is now evident that surface determinants are not uniformly distributed in a single tumor, however, individual subpopulations express antigens in different regions of a tumor (7). Considering that the samples analyzed in the current study were only a subset of the total tumor it is possible that the portion of tumor chosen for CA125 study may not have contained the tumor marker, whereas it may have been present in another region of the tumor. It would have been ideal to have taken the entire tumor instead of only a portion of the tumor for western blot analysis.

Different tumor types also express different histological characteristics. It has been documented that mucinous tumors may be unrelated to the other epithelial ovarian cancers (3). It has also been documented that mucinous ovarian tumors do not express CA125. In the current study, it is unknown what subtype the ovarian tumors were and it is possible that the tumors are of a histological type that does not express CA125 or expresses at a lower frequency than epithelial ovarian tumors.

Besides heterogeneity, there are other potential explanations for the observed results. The first potential explanation is that most initial antigenic studies were done on chemically or virally induced tumors. It has since been found that tumors formed spontaneously in a host species are seldom able to be detected by antigenic analysis (7). Demonstration of antigenicity is reliant on the test being performed and the sensitivity of the parameters of such tests. It is plausible that western blot analysis for CA125 expression used in the current study was not sensitive enough to detect the antigen in the samples tested. In the clinical setting serum CA125 is tested using ELISA with double capture antibody. The double capture antibody would allow two different sites of the antigen to be bound, therefore increasing the chance that the antigen will be captured. It is not impossible to believe that western blotting does not have such sensitivity and perhaps is not the appropriate test to be performed when analyzing CA125 status. It is also possible that the conditions of the experiment are too harsh for an already sensitive antigen. Homogenization and sample preparation cannot be eliminated as reasons for the low CA125 positive test results.

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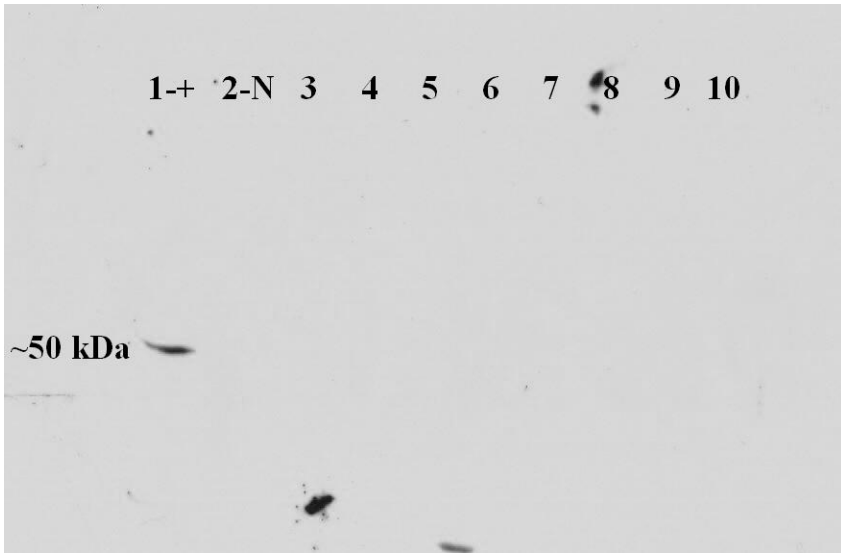


Figure 1. Western blot results of avian ovarian tumors at various stages. Lane 1 represents an avian ovarian tumor previously shown to be CA125 positive. Lane 2 represents a normal ovary.

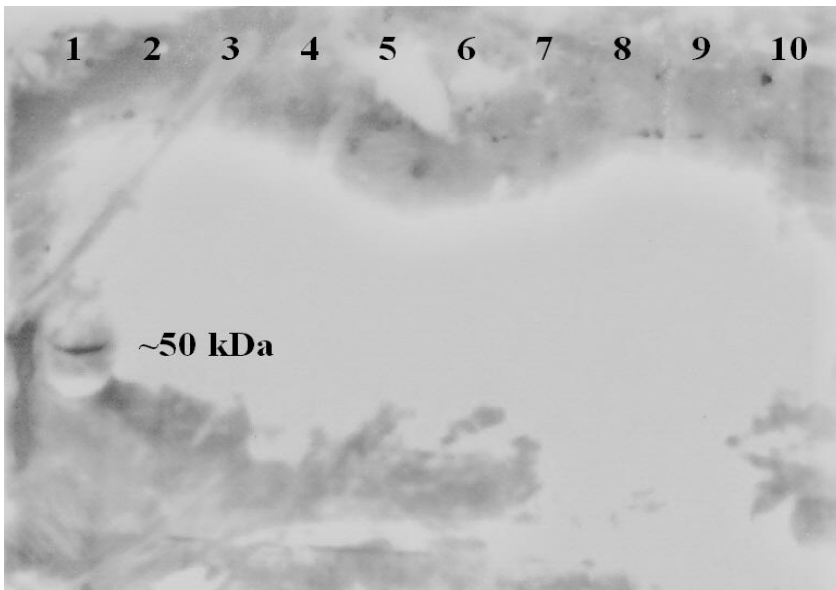


Figure 2. Western blot results of avian ovarian tumors at various levels of metastasis. Lane 1 represents an avian ovarian tumor known to be CA125 positive. Lane 2 represents a normal ovary.

5. CA125 in Avian Oviductal Cancer

5.1 ABSTRACT

5.1.1 Objective

The site of primary tumor formation is important when treating cancer that has spread to other organs. The objective of the current study is to attempt to determine the primary tumor site in chickens afflicted with cancer in both the ovary and oviduct. It is useful to know whether the oviduct is also a source of CA125 expression or if cells shed from the ovary during primary cancer formation result in the presence of CA125 in cancers of the oviduct. If tumors originating in the ovary release CA125 that is later found in cancers of other organs than this lends evidence to CA125 as a possible culprit behind metastatic potential and cellular adhesion.

5.1.2 Methods

Western blotting techniques were used to analyze avian oviductal tumor samples for the presence of CA125. Chemiluminescence was employed to detect the presence of protein on a PVDF membrane.

5.1.3 Results

CA125 does not appear to be present in avian oviductal tumor samples. This includes oviductal tumors with no ovarian involvement and tumors that were thought to be secondary to the ovarian adenocarcinoma.

5.1.4 Conclusion

There were no oviductal tumors that expressed CA125 in the current study. The negative results in primary oviductal tumors indicate that the oviduct is not the original

source of CA125. The negative results also present in secondary ovarian tumors indicate that CA125 is not shed to the oviduct from the primary ovarian site of tumor formation.

5.2 INTRODUCTION

In treating cancer, knowing the tissue of origin allows for cancer-specific treatments that can be administered and followed. The survival duration is greater in patients that have a diagnosed primary tumor in contrast to patients where the primary site of tumor formation is unknown. This is particularly true in patients diagnosed with lymphoma, primary breast and primary ovarian cancer (1). Metastasis of adenocarcinomas is described by cells leaving the primary tumor via lymphatics or blood transport to the target organ resulting in growth of metastases in that organ (2).

CA125 has been thought to play a role in cell adhesion and metastases formation in ovarian cancer via its interaction with another tumor marker, mesothelin. By blocking the mesothelin antigen with an anti-mesothelin antibody, CA125 positive cells could not bind to mesothelin positive cells (3). The inhibitory effect of the anti-mesothelin antibody indicates that the binding potential between CA125 and mesothelin mediates cellular adhesion.

Ovarian cancer metastasis usually remains localized to the peritoneal cavity. Secondary cancers are typically attached to the peritoneal mesothelium lining the abdominal cavity. The peritoneal mesothelium is the site of mesothelin expression, which suggests that CA125 is shed from the primary ovarian tumor site and attaches to mesothelin on the peritoneal mesothelium, which causes secondary cancer formation. In the laying hen, it is not atypical to observe an oviductal tumor presented alongside an ovarian tumor. The objective of the current study is to determine whether CA125 is shed from the ovary and attaches to the oviduct to form a secondary metastatic mass. Ovarian samples that were shown to be CA125 negative were also tested to determine whether CA125 is present in oviductal tumor samples

from the same bird. This result would offer evidence to the oviduct being the primary site of tumor formation.

CA125 has not been the solitary tool in diagnosis of ovarian cancer because it is not specific to ovarian cancer. CA125 has also been reported in other adenocarcinomas, including those of the fallopian tube, endometrium, and endocervix (4). The fallopian tube in humans can be compared to the oviduct in birds. Both are structures that pass an egg from the ovary into the next structure of the reproductive system. The fallopian tube has been shown to express CA125 when becoming metastatic. It is therefore reasonable to expect that the oviduct in laying hens may also express CA125. In the current study, we immunostained primary oviductal tumors in the laying hen with anti-CA125 to determine whether the oviduct expresses CA125 when becoming metastatic.

5.3 MATERIALS AND METHODS

5.3.1 Tissue Collection

Hens employed in this study were commercial Single Comb White Leghorn [*Gallus domesticus*] that had been maintained for 2 years at the Piedmont Research Station in Salisbury, NC. Each hen ovulated approximately 450 times prior to death, and they had never been subjected to a synchronized molt. Hens ranged in age from 86 weeks to 105 weeks of age. Necropsies were performed at North Carolina State University and also at the Piedmont Research Station. All procedures involving animals were approved by the North Carolina State University Institutional Animal Care and Use Committee. When an ovarian and/or oviductal adenocarcinoma was identified, it was aseptically removed and cut into several pieces, which were placed in RNA later, formaldehyde, or snap frozen in liquid nitrogen for further analysis.

5.3.2 Western Blot

Tumor tissue was taken from the ovaries and/or oviducts of birds that exhibited ovarian and/or oviductal tumors. The tissue was weighed and immersed in lysis buffer (20mM Tris-HCL pH 7.5, 10 mM NaCl, 10 mM KCl, and 3 mM MgCl₂). Additionally, 0.5 mm mini glass beads were added to each tube to dissociate the tissue. The tubes were then placed into a mini bead beater and homogenized for 1 minute. The protein suspension was spun for 4 min at 10,000xg. A Bradford assay (Sigma, St. Louis MO, USA) was performed to quantitate the amount of protein per sample. Protein was mixed 1:1 with Laemmli sample buffer (0.0625 M Tris-HCl, 2% SDS, 10% Glycerol, 5% 2-Mercaptoethanol, and 0.001% bromophenol blue) and boiled for 3 min at 100°C.

The protein (100 µg per lane) was fractionated through an SDS-PAGE gel. The resolving gel consisted of 8% total acrylamide, 0.2 M Tris pH 8.8, 0.4% SDS, 0.03% ammonium persulfate, and 0.1% *N,N,N',N'*-tetramethylethylenediamine (TEMED). The stacking gel contained 3% total acrylamide, 0.1 M Tris pH 6.8, 0.1% SDS, 0.05% ammonium persulfate, and 0.5% TEMED to accelerate polymerization. The gel was run at a constant current of 30 mA using the Hoefer Mighty Small II (Hoefer Scientific Instruments, San Francisco CA, USA) with 1x running buffer (0.25M Tris, 1.92M glycine, and 0.5% SDS) until the dye front reached the bottom of the gel.

Protein was transferred onto a PVDF membrane (Immobilon, Millipore Bedford MA) in transfer buffer (25 mM Tris, 192 mM glycine, 15% methanol, and 0.5% SDS) under 400 mA constant current for 1 h and 20 min using the Hoefer Mighty Small Transfer apparatus (Amersham Pharmacia Biotech AB, San Francisco CA). Nonspecific protein binding was blocked by immersing the membranes in 3% non-fat dry milk plus Tris-buffered saline Tween-20 (TBST) for 1 h at room temperature with gentle agitation. The membranes were immunostained with mouse anti-CA125 (Zymed, San Francisco CA) at a dilution of 1:300 overnight at 4°C. Secondary antibody incubation (2 h) followed using a 1:3000 dilution of goat anti-mouse IgG-HRP. The protein was then detected using the Immuno-Star HRP chemiluminescence kit (Bio-Rad, Hercules CA).

5.4 RESULTS

Oviductal tumor samples taken from birds with no other diseased organs display no expression of CA125 (Fig. 1). Avian oviductal tumors that were present alongside ovarian tumors do not express CA125.

Figure 2 represents a panel of normal organs taken from a bird with no signs of disease that were screened for CA125 expression. Included in this panel is a sample from a healthy oviduct. There is no CA125 expression seen in the normal oviduct, just as there is no CA125 expression seen in the normal ovary.

5.5 DISCUSSION

CA125 has been shown to be elevated in many body conditions, including ovulation, pregnancy, endometriosis, benign ovarian tumors, liver disease, kidney disease, and many other cancers. The most important of the other conditions for the current study is fallopian tube adenocarcinoma because it shares similar histology with ovarian cancer (5). It is even thought that in some cases primary fallopian tube cancer is misdiagnosed as disseminated ovarian cancer because of the similarities between the two cancers (5,6). The fallopian tube in mammals is similar to the oviduct in the laying hen. In both structures an egg is transported from the ovary to the next reproductive structure, which is the shell gland in chickens versus the uterus in humans. In necropsies performed for this study it was observed that the most common cancer seen in addition to ovarian cancer is cancer of the oviduct. The presence of another tumor raised questions as to the primary origin of the tumor. In some instances the primary site of tumor formation could be inferred because of the size of the nodule and the advancement of disease in that particular organ.

The hypothesis in this study was that oviductal tumors, whether primary or secondary to an ovarian tumor, would not express CA125. In fact this is what we observed. This is significant because the cases of ovarian cancer that were observed to be CA125 positive in previous studies can be attributed solely to the ovarian mass present in the bird. Knowing the primary site of tumor formation would allow for studies that investigate the etiology of the disease and characteristics associated with ovarian cancer.

It cannot be ruled out that the results were due to experimental conditions as previously mentioned. The results were detected using western blotting techniques. It is possible that this method of testing is not sensitive enough to detect the antigen in the

samples generated from the tumors used in this study. CA125 has been reported to be a heat and proteolytic sensitive molecule. While it is not thought that heat played a role in the negative results it is possible that another form of cleavage occurred, whether proteolytic or harsh sample preparation. All samples were fractionated on an SDS-PAGE gel both with and without boiling conditions. Both sets of results came out the same with no protein band present representing CA125.

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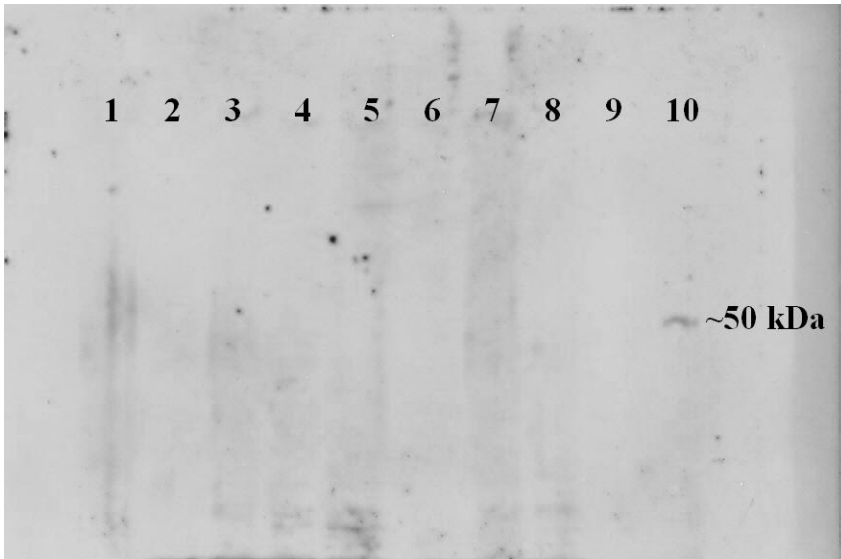


Figure 1. Western blot results of avian oviductal tumor samples stained with CA125. Lanes 1 -9 represent oviductal tumors. Lane 10 represents a CA125 positive avian ovarian cancer sample.

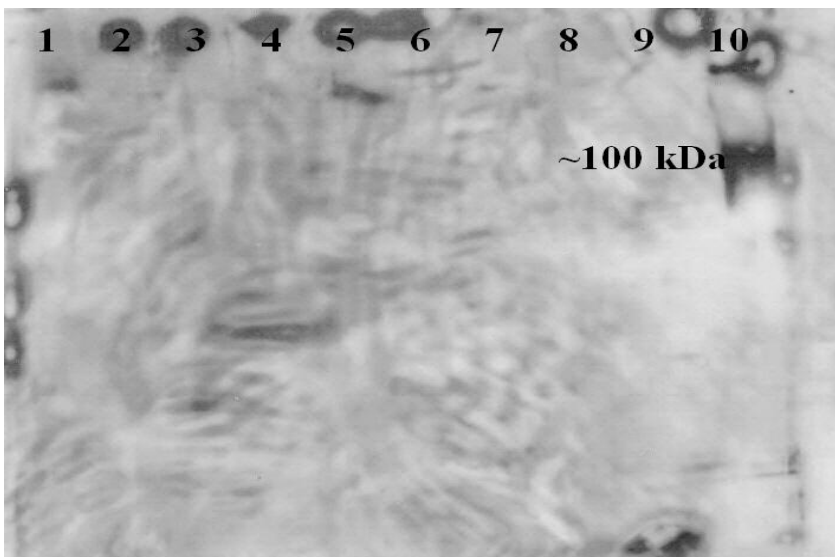


Figure 2. Western blot results of normal avian organs stained with CA125. Lane 2 represents liver. Lane 3 represents kidney. Lane 4 represents small intestine. Lane 5 represents large intestine. Lane 6 represents ovary. Lane 7 represents oviduct. Lanes 8-9 were kept blank for separation. Lane 10 represents an avian ovarian cancer sample previously shown to be CA125 positive.

6. CA125 in Human Ovarian Cancer and the Role of E-cadherin in Human and Avian Ovarian Cancer

6.1 Abstract

6.1.1 Objective

CA125 is a high molecular weight glycoprotein with many repeats that can result in different protein sizes. The first objective is to analyze the difference between CA125 expression in human versus avian ovarian tumor samples. E-cadherin is a cell adhesion molecule that is controversial in ovarian cancer studies. Knowing whether E-cadherin is expressed in correlation with CA125 could shed light onto the etiology of ovarian cancer. The second objective is to determine if CA125 is expressed in conjunction with E-cadherin in both human and avian ovarian cancer samples

6.1.2 Methods

Western blotting was employed to determine CA125 and E-cadherin expression in human and avian ovarian cancer samples. Chemiluminescence was used to detect the protein present on a PVDF membrane.

6.1.3 Results

Analysis of a human ovarian tumor resulted in a ~200 kDa band that represents CA125. Following the same sample preparation resulted in a ~100 kDa band in an avian ovarian cancer sample. E-cadherin was found in the human ovarian cancer sample by a band of ~50 kDa. There was no E-cadherin detected in any avian ovarian cancer sample or avian oviductal tumor sample. E-cadherin was also not detected in any normal organ samples taken from a healthy chicken.

6.1.4 Conclusion

CA125 is sensitive to temperature and proteases. Differences in sample preparation and heat exposure can result in bands of different sizes on a western blot. E-cadherin expression was not observed in avian ovarian cancer samples as it was in the human sample tested. It is possible that there are species differences in E-cadherin that allow it to escape detection with the currently used antibody. It also cannot be ruled out that E-cadherin is not present in the chicken species. Further experimentation should be performed to determine E-cadherin status in the gallus gallus species.

6.2 INTRODUCTION

Despite years of research and advances in surgery and chemotherapy, ovarian cancer remains the most lethal of all gynecological malignancies. Ovarian cancer is the fourth leading cause of cancer death in women living in the United States (1). It is estimated that in the United States alone in the year 2007 there will be 22,430 new cases of ovarian cancer diagnosed and 15,280 deaths from the disease (2). The high mortality rate is due to the fact that ovarian cancer typically presents itself in late stage disease as there are few to no noticeable symptoms in early disease stages.

CA125, along with transvaginal sonography, remains the conventional way to diagnose ovarian cancer. However, the problems with CA125 specificity and sensitivity remain. CA125 is elevated in several other conditions, including benign ovarian conditions, menstruation, pregnancy, pancreatitis, pelvic inflammatory disease, etc. Also, other forms of cancer show elevated CA125 levels, such as lung, breast, and cancers of the gastrointestinal tract (3). Up to 20% of women with ovarian cancer never express elevated CA125 levels and only about 3 % of women with elevated CA125 have ovarian cancer. Therefore, while CA125 remains useful in following progression of disease and response to treatment, it is still not a sufficient marker alone to be used in diagnosing the disease. For this reason it is important to find another marker to use in conjunction with CA125 to aid in diagnosing ovarian cancer and eliminate the number of false positives.

E-Cadherin is a 120 kDa transmembrane, Ca dependent glycoprotein. A shorter 80 kDa tryptic fragment has also been reported to be released from the core protein (4, 5). This cell adhesion molecule is responsible for maintaining the dedifferentiated noninvasive state of an epithelial monolayer (5,6). A 30 amino acid segment near the C-terminus of the

cytoplasmic domain has been shown to bind to catenins, which are cytoplasmic proteins, and this junction forms zonula adherens which are involved in cell junction formation, tissue stabilization, morphogenesis and signal transduction (4, 7, 10). The extracellular portion of cadherins contains ~110 amino acids consisting of four tandem repeats (12). Within the first extracellular domain of E-cadherin there is a specific sequence that is important for the binding specificity of the cell-adhesion molecule and for the binding of monoclonal antibodies (10). Down-regulation of E-cadherin leads to loss of adhesive function and is correlated with malignant stage, highly invasive dedifferentiated tumors (4,5).

It is controversial whether E-cadherin is expressed in the human ovary. There are data both supporting and refuting the presence of the cell-adhesion molecule in the ovary and ovarian surface epithelial (OSE) cells. Sundfeldt et al (4) found that E-cadherin is expressed in ovarian cancer tissue, while being down-regulated in normal ovaries. The researchers suggest that the switch from down-regulation to E-cadherin expression supports a role in transition of normal OSE cells to a malignant phenotype, which may be caused by the surrounding stromal cells which secrete paracrine factors that activate transcription factors (4). While in most malignant phenotypes E-cadherin acts as a tumor suppressor, the opposite is thought to be true in ovarian cancer.

In the current study, CA125 expression in a solid tumor from a two year old laying hen was compared to a human ovarian tumor to see how the expression varies between the two species. E-Cadherin expression was also analyzed in both species to see how this marker correlates with CA125 expression.

6.3 Materials and Methods

6.3.1 Tissue Collection

Hens utilized in this study were commercial Single Comb White Leghorn [*Gallus domesticus*] that had been maintained for two years at the Piedmont Research Station in Salisbury, NC. Each hen had ovulated approximately 450 times prior to death, and had never been subjected to a synchronized molt. All procedures involving animals were approved by the North Carolina State University Institutional Animal Care and Use Committee. When an ovarian adenocarcinoma was identified it was photographed, removed, and placed into RNA later solution until processed for protein analysis.

6.3.2 Western Blot

Tumor tissue was taken from the ovaries or oviducts of birds who exhibited ovarian and/or oviductal tumors. Human ovarian cancer tissue was received from Northwestern University. Normal organs were taken from a bird with no observable signs of disease. The tissue was weighed and put into tubes with lysis buffer (20mM Tris-HCL pH 7.5, 10 mM NaCl, 10 mM KCl, and 3 mM MgCl₂). Additionally, 1.0 mm mini glass beads were added to each tube to dissociate the tissue. The tubes were then placed into a mini bead beater and homogenized at 30 second intervals for a total time of 4 min. The protein suspension was spun for 4 min. at 10000 x g. A Bradford assay was performed to quantitate the amount of protein per sample. Protein was mixed 1:1 with Laemmli sample buffer (0.0625 M Tris-HCl, 2% SDS, 10% Glycerol, 5% 2-Mercaptoethanol, and 0.001% bromophenol blue). The avian samples were run at a quantity of 30 micrograms and the human sample at a quantity of 0.5 micrograms. The protein was fractionated through an 8% SDS-PAGE. The resolving gel consisted of ultrapure dH₂O, 30% acrylamide mix, 1.5M Tris pH 8.8, 10% SDS, 5%

glycerol, 10% ammonium persulfate, and TEMED. The 5% stacking gel contained ultrapure dH₂O, 30% acrylamide mix, 1.0M Tris pH 6.8, 10% SDS, 5% glycerol, 10% ammonium persulfate, and TEMED to accelerate polymerization. The gel was run at a constant current of 35 mAmps using the Hoefer Mighty Small II (Hoefer Scientific Instruments, San Francisco CA, USA) with 1X running buffer (.25M Tris, 1.92M Glycine, and .5% SDS) until the dye front reached the bottom of the gel.

Once electrophoresis was completed the gels were laid on top of PVDF transfer membranes (Immobilon Millipore Bedford MA, USA). Protein transfer onto the membrane was accomplished by running the transfer at a constant current of 400 mAmps for 1 h using 1X transfer buffer (Tris, Glycine, ultrapure dH₂O, and methanol) in the Hoefer Mighty Small transfer apparatus (Amersham Pharmacia Biotech AB, San Francisco CA, USA).

Nonspecific protein binding was blocked by immersing the membranes in 5% non-fat dry milk plus Tris-buffered saline Tween-20 (TBST) for 2 h at room temperature with gentle agitation. The membranes were immunostained with mouse anti-CA125 (Zymed, San Francisco CA, USA) at a dilution of 1:300 or supernatant from 8C2 cells at a volume of 15 mL in 5 mL of blocking solution overnight at 4°C. Secondary antibody incubation followed with a 1:3000 dilution of goat anti-mouse IgG-HRP conjugate for 2 h at room temperature with gentle agitation. The protein was then detected using the Immuno-Star HRP chemiluminescence kit (Bio-Rad).

6.4 RESULTS

A CA 125 positive ovarian cancer sample was compared to a human ovarian tumor. Figure 1 shows a band representing the human tumor at ~200 kDa. This is obviously different from the ~100 kDa avian ovarian cancer sample.

It was also determined whether or not CA 125 expression correlates with E-Cadherin expression in ovarian cancer samples. Figure 2 shows a band present in the human ovarian cancer sample lane representing E-Cadherin at ~50 kDa, however, no protein expression was present in avian ovarian cancer samples. Figure 3 shows a blot that contains normal avian organs. There are no bands present corresponding to E-Cadherin expression. The same negative result is seen for Figures 4-6. Figure 4 shows a blot that contains samples with different stages of avian ovarian tumors. Figure 5 shows a blot that contains samples of avian ovarian tumors with different levels of metastasis. Lastly, Figure 6 shows a blot with avian oviductal tumor samples.

6.5 DISCUSSION

It has been well documented that CA125 in human ovarian cancer samples is represented by a band that is approximately 200 kDa. CA125 is a temperature and protease sensitive glycoprotein. It has been suggested that CA125 protein samples not be boiled or immersed in sample buffer containing 2-mercaptoethanol. The result is a band of approximately 200 kDa for the human ovarian cancer sample fractionated using SDS-PAGE. In all previous experiments avian ovarian cancer samples were boiled at 100°C for 3 min in sample buffer containing 2-mercaptoethanol prior to electrophoresis.

In an effort to keep conditions consistent between samples, the avian sample was treated in the same manner as the human sample with no heat or 2-mercaptoethanol exposure. The result is a band in the avian lane that is significantly higher in molecular weight than previously reported. In prior experimentation we have reported a CA125 band of sizes 25 and 50 kDa. CA125 is a conformationally dependent protein with heavy O- and N- linked glycosylations (8, 9). Exposure to heat can cause the conformation of the protein to change resulting in a smaller protein size than would otherwise be observed. Figure 1 shows western blot results with a human CA125 size of ~200 kDa and an avian CA125 size of ~100 kDa. To ensure that harsh sample treatment was not the reason for other negative CA125 results, all samples previously tested were repeated with the new preparation conditions. The results consistently came back with no bands present. It is therefore reasonable to speculate that heat exposure does not eliminate the presence of CA125, but instead it results in a smaller CA125 protein size in samples where CA125 is expressed.

While the etiology and function of CA125 is still in question, one of the plausible reasons for CA125 expression is for cellular adhesion. The cell adhesion hypothesis would

allow the CA125 expressed on the surface of one tumor cell to adhere to CA125 on an adjacent cell resulting in tumor formation. CA125 could also aid in metastasis by allowing tumor cells shed from the primary tumor site to adhere to other peritoneal sites. The possible role of CA125 in cell adhesion and metastasis leads one to be curious about the presence of other established cell adhesion molecules on the surface of ovarian tumor cells.

E-cadherin is the cell adhesion molecule in question in the current study. A correlation between CA125 and E-cadherin expression would lend further evidence to CA125 as a cell adhesion molecule. Figure 2 shows E-cadherin expression in a CA125 positive human ovarian cancer sample. Alternatively, there is no band present in the lanes representing a CA125 positive avian ovarian cancer sample (Fig. 2). It was also worthwhile to test E-cadherin expression in samples previously tested to observe whether E-cadherin is specific to different levels of metastasis or cancer stage (Fig. 3 and Fig. 4). Normal avian organ samples and also avian oviductal tumor samples were tested to determine whether E-cadherin is expressed in normal tissue or oviductal avian tumor tissue (Fig. 5 and Fig. 6). E-cadherin was not expressed in any avian sample regardless of CA125 or tumor status.

The explanation behind the results described could lie in species differences between the human and the chicken. While the human expresses E-cadherin, the chicken is known to express LCAM. It has often been thought that based upon sequence homologies the chicken LCAM is the chicken homologue of human E-cadherin (11). However, there is evidence that this may not be true. The interaction formed between LCAM and E-cadherin when the two are mixed has been shown to be relatively weak which implies that these two molecules are not functional homologues of one another. When fibroblastic L-cells which do not express E-cadherin were transfected to become cadherin-expressing cells they were shown to

associate with other cadherin expressing cells (13). If LCAM was a true homologue one would expect that it would associate highly with other E-cadherin molecules. The fact that LCAM formed weak interactions supports the hypothesis that the chicken homologue of E-cadherin has not yet been identified. This would explain the results seen in the current study where the human sample displayed E-cadherin expression while there was no protein observed in any avian sample tested.

It has been shown that there are species-specific cadherins in liver tissue. The presence of species-specific cadherins leads one to hypothesize that not every cadherin has a similar homologue in other vertebrate species (12). There are large sequence divergences between mouse E-cadherin and chicken L-CAM on the phylogenetic tree. This suggests that there is sequence heterogeneity between species and gives more evidence to the idea that perhaps L-CAM is not a functional homologue of E-cadherin. Further experimentation should be performed to determine the presence of an E-cadherin homologue in the avian species.

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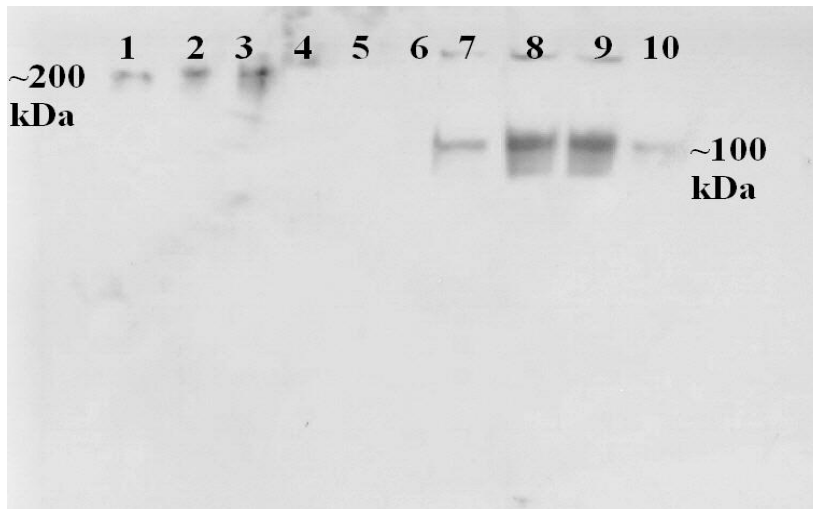


Figure 1. Western blot results of a human versus avian ovarian cancer sample stained with CA125. Lanes 1-4 represent a human ovarian tumor with a CA125 reactive band at ~ 200 kDa. Lanes 5-6 were kept blank for separation. Lanes 7-10 represent an avian ovarian cancer sample with a CA125 reactive band at ~100 kDa.

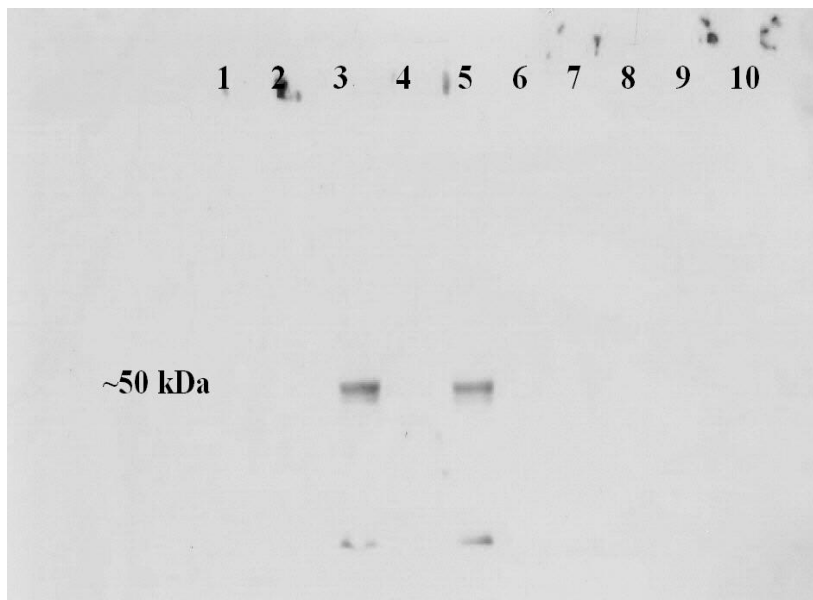


Figure 2. Western blot results of a human versus avian ovarian cancer sample stained with E-cadherin. Lanes 1-2 are blank. Lanes 3 and 5 represent a human ovarian cancer sample with an E-cadherin reactive band at ~50 kDa. Lanes 7 and 9 represent an avian ovarian cancer sample known to be CA125 positive that have no E-cadherin reactive protein present. Lanes 4, 6, 8, and 10 were kept blank for separation.

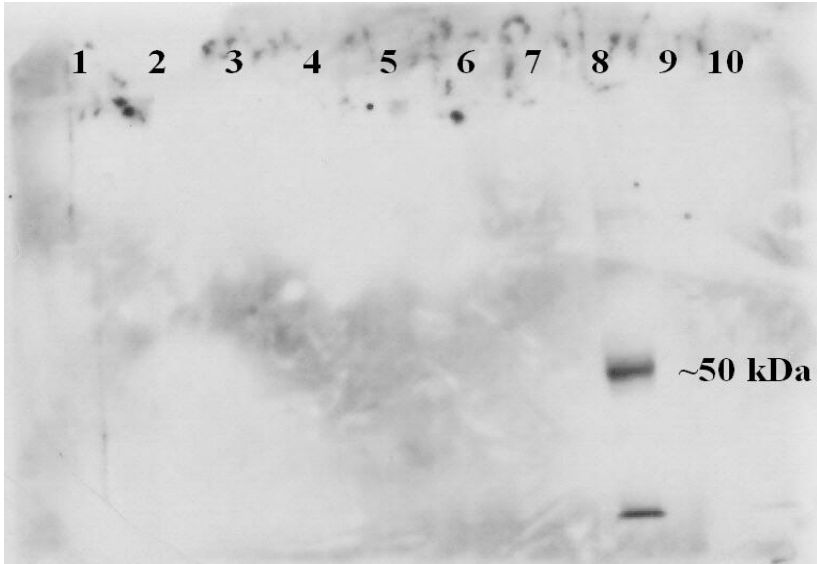


Figure 3. Western blot results of normal avian organs stained with E-cadherin. Lane 1 represents liver. Lane 2 represents kidney. Lane 3 represents small intestine. Lane 4 represents large intestine. Lane 5 represents oviduct. Lane 6 represents ovary. Lanes 7 and 9 are kept blank for separation. Lane 8 represents a human ovarian cancer sample with an E-cadherin reactive band of ~50 kDa. Lane 10 represents an avian ovarian cancer sample with no E-cadherin expression.



Figure 4. Western blot results of avian ovarian cancer samples at different stages stained with E-cadherin. Lane 10 represents an avian ovarian cancer sample known to stain positive with CA125.

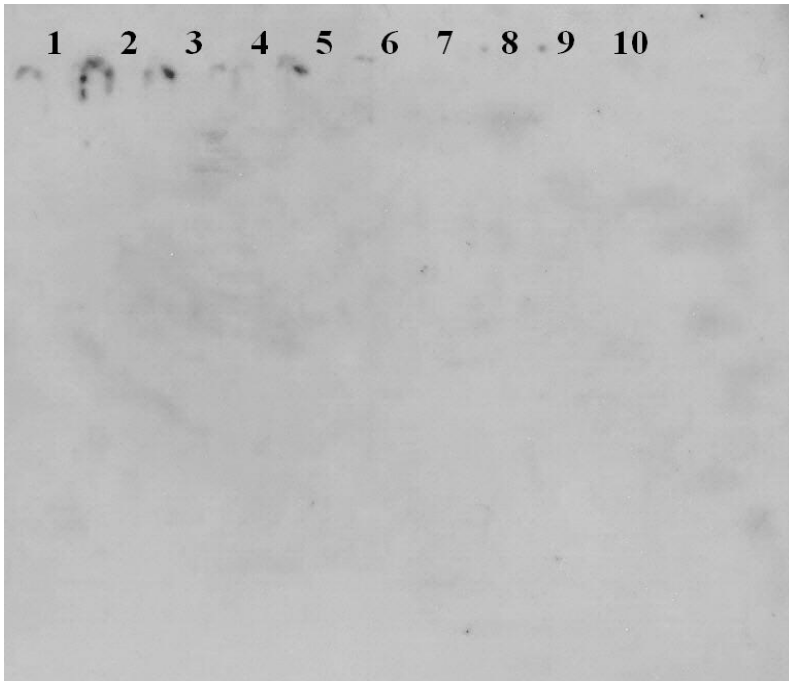


Figure 5. Western blot results of avian ovarian cancer samples at varying levels of metastasis stained with E-cadherin. Lane 8 represents an avian ovarian cancer sample known to stain positive for CA125. Lane 9 represents normal avian small intestine. Lane 10 represents normal avian large intestine.



Figure 6. Western blot results of avian oviductal tumor samples stained with E-cadherin. Lane 10 represents an avian ovarian cancer sample known to stain positive for CA125.

7. SUMMARY

Ovarian cancer has been mystifying doctors and researchers for years. It is relatively asymptomatic until late disease stages when the cancer has spread to other organs in the peritoneal cavity and successful treatment is rarely possible (1). Treatment is further complicated by the absence of an adequate tumor marker to screen for early stage disease.

Since its discovery in 1981, CA125 has been the most commonly used cancer antigen in studying ovarian cancer. The glycoprotein is found in many benign and malignant conditions which makes it an inadequate diagnostic tool due to problems in specificity and sensitivity (2). However, the tumor marker is very significant in following progression of the disease. A rise or fall in serum CA125 levels correlates well with remission or recurrence of disease during and after therapy is administered (3).

Until recently little was known about the biological significance of the cancer antigen CA125. However, with the cloning of CA125 in 2001 came insight into the structure and possible functions of CA125 in ovarian cancer. The N terminal of the glycoprotein is large and contains approximately 60 repeats, 9 of them being tandem repeats, of 156 amino acids. This region is heavily glycosylated with O- and N- linked glycans. There is a sequence of 25 hydrophobic amino acids representing a transmembrane spanning region. Following this is a short cytoplasmic tail of approximately 256 amino acids, 31 of which constitute a possible tyrosine phosphorylation site (4).

CA125 is sensitive to proteolytic cleavage and there are several sites along the molecule that could be potential sites for protease activity. One of these sites could be a sea-urchin sperm protein, enterokinase, and agrin domain (SEA) in the extracellular region. The cleavage causes release of CA125 from the cell surface into the peritoneal cavity and the

circulation. One of the hypothesized functions of CA125 is cell adhesion and metastatic potential. It is known that CA125 has the ability to bind to mesothelin (3). Mesothelin is a glycoprotein located on the peritoneal mesothelium. CA125 is shed from the primary tumor site and binds to mesothelin in the peritoneum resulting in cell adhesion and metastasis within the abdominal cavity.

CA125 is present in several species, one of which is the domestic laying hen. In contrast to the other species, the hen spontaneously develops ovarian tumors and is therefore the most biologically similar to the human. It was important to establish the chicken as a model for the human disease. Once the chicken was established as an animal model for human disease, it was important to study the different circumstances in which CA125 could be expressed in the avian model. It was not found that CA125 is expressed in a similar manner as it is in human disease. CA125 was not more prevalent in advanced stages of disease or with level of tumor burden as is seen in the human. CA125 was also not expressed in the oviduct of the hen. It has been reported that CA125 is present in cancers of the fallopian tube (5).

It was also significant to show CA125 as a cell adhesion molecule by observing a correlation between the tumor antigen and E-cadherin. It is controversial whether E-cadherin is present in ovarian cancer and also whether the LCAM chicken form of E-cadherin is truly a homologue of the human version of E-cadherin (6). E-cadherin was not observed in any of the avian samples tested, however, it was observed in the human ovarian cancer sample immunoblotted with E-cadherin.

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