Published in final edited form as:

Int J Gynecol Cancer. 2009 May ; 19(4): 500–507. doi:10.1111/IGC.0b013e3181a39db1.

Prevalence of anti-tumor antibodies in laying hen model of human ovarian cancer

Animesh Barua^{a,*}, Seby L Edassery^a, Pincas Bitterman^{b,c}, Jacques S. Abramowicz^c, Angela L Dirks^e, Janice M Bahr^e, Dale B. Hales^f, Michael J. Bradaric^a, and Judith L. Luborsky^{a,b,d}

^a Department of Pharmacology, Rush University Medical Center, Chicago

^b Department of Pathology, Rush University Medical Center, Chicago

^c Department of Obstetrics and Gynecology, Rush University Medical Center, Chicago

^d Department of Preventive Medicine, Rush University Medical Center, Chicago

^e Department of Animal Sciences, University of Illinois at Urbana-Champaign, Champaign

^f Department of Physiology and Biophysics, University of Illinois at Chicago, Chicago, IL

Abstract

Anti-tumor antibodies are associated with tumors in human cancers. There is relatively little information on the timing and progression of antibody response to tumors. The objective of the study was to determine if spontaneous ovarian cancer in the egg-laying hen is associated with anti-tumor antibodies. Antibodies were detected by immunoassay and immunoblotting using proteins from normal ovary and ovarian tumors. Candidate antigens were identified by mass spectrometry of immunoreactive spots cut from two-dimensional gels and Western Blot. Anti-tumor (serum reacting against tumor ovarian extract) and anti-ovarian (serum reacting against normal ovarian extract) antibodies were significantly associated with ovarian cancer (67%, $P \leq 0.001$) compared to normal control hens. Hens with abnormal histology but no gross tumor had anti-tumor antibodies (63%, $P \leq 0.025$) but not anti-ovarian antibodies. There were common as well as different immunoreactions against normal ovary, and homologous and heterologous tumor proteins in two-dimensional Western blots. The candidate antigens included those commonly associated with human cancers and other diseases such as vimentin, apolipoprotein A1, Annexin II, enolase, DJ-1 etc. The results suggest that anti-tumor antibodies are associated with ovarian cancer in hens, similar to human ovarian cancer. The egg-laying hen may be a model for understanding the anti-tumor humoral immune response, particularly at early tumor stages that are not readily accessible in human ovarian cancer.

Keywords

ovarian cancer; chicken; anti-tumor antibodies; ovarian antigens

Despite several decades of research, there is no reliable screening or diagnostic test for ovarian cancer (OvCa). Consequently, OvCa tends to be diagnosed at later stages and is the most fatal gynecological cancer(1). A pre-clinical animal model that recapitulates spontaneous OvCa, would supplement studies in humans, and improve understanding of disease progression and potentially facilitate the identification and validation of markers of early disease.

*Corresponding author: Animesh Barua, PhD, Department of Pharmacology, Rush University Medical Center, Cohn Research Building, 1735 W. Harrison St., Chicago, IL 60612, Tel: 312-942-6666; Fax: 312-942-6616, Animesh_Barua@rush.edu.

Cancers arise from the host's tissues and modifying the tissues as an altered "self" against which the immune system produces an immune response(2). There is clear evidence that there are both humoral and cellular immune responses to tumors(3,4). Anti-tumor antibodies are associated with most cancers(5,6) and antibodies to a number of immunoreactive intracellular and cell surface antigens have been reported for OvCa(7,8). Correlation between humoral and cellular immune responses against same tumor antigen has been reported(9). We(10) and others (11,12) have shown anti-tumor antibody responses to OvCa in humans. This suggests that immune responses against tumor antigens have potential as diagnostic markers for OvCa.

As shown in autoimmune diseases, antibodies are excellent disease markers because they are specific and biochemically stable(5). The advantage of anti-tumor antibodies as early cancer markers is that they can detect antigens at low levels that might not be detectable by protein measurement. Published reports on individual antigens support the concept that only cancer patients tend to have anti-tumor antibodies to normally occurring antigens that are expressed in tumors(12–15). Anti-tumor antibodies have been used to identify candidate cancer markers by screening tumor derived, cDNA expression libraries with patient sera(16,17). Analytic modeling showed the feasibility of using antigen specific antibodies to diagnose cancers in a cohort of patients with breast, lung, prostate, colorectal, gastric and hepatocellular cancers(6), although few studies have validated the early detection of cancers with anti-tumor antibodies (18,19).

Furthermore, there are few studies of antibodies associated with early stage OvCa(5). Our overall objective is to demonstrate the utility of anti-tumor antibodies to detect early stage OvCa. Since there is a paucity of early stage patients and since following the disease trajectory in humans is extremely difficult, we examined animal models of OvCa.

Historically, animal models have facilitated understanding of disease-related events that are difficult to study in humans. Models are selected as much as possible because of their similarity to the human disorder. For OvCa, most of the common models are rodents in which ovarian tumors are artificially induced by transplant of human tissue, chemical or genetic manipulation (20). Frequently, the tumor types and histopathology of ovarian carcinomas induced by chemical or genetic manipulation are not similar to spontaneous human OvCa(20,21). In contrast, the egg-laying hen (*Gallus domesticus*) is the only available animal known to develop OvCa spontaneously(22). Ovarian carcinoma in hens has remarkable similarities with human OvCa based on incidence, histopathology and expression of molecular markers. Similar to humans(23), the incidence of OvCa in egg-laying hens increases with age and, depending on the flock, up to 40% of hens may have ovarian tumors by 6 years of age(22). Although there is no comprehensive summary of the histopathology of malignant ovarian tumors in hens, the available published reports show that histological types of ovarian tumors in hens are similar to human tumors(24–26). Furthermore, common human tumor associated molecular markers including cytokeratin, several growth factors and their receptors, carcinoembryonic antigen, proto-oncogene, onco-fetal tumor markers and PCNA (proliferation cell nuclear antigen)(27) are also expressed in hen ovarian tumor tissues. CA125, the only marker in routine diagnostic use to gauge tumor recurrence, was recently shown to be expressed in hen ovarian tumors by immunohistochemistry(28). Furthermore, COX-1 is elevated in hen ovarian tumors(29) consistent with the specific effect of COX-1 inhibitors on reduction of human ovarian tumors. We recently showed that ovarian tumors could be detected by ultrasound using the similar equipment used to evaluate human ovaries, thus making it possible to monitor the morphological and blood vessel changes associated with onset and progression of ovarian cancer in egg-laying hens. Therefore, given the similarities in hen and human ovarian cancer, we investigated the anti-tumor antibody response in hens. This represents the first investigation of an immune response to ovarian tumors in the hen. The specific goal of this study was to determine if ovarian tumors in hens were associated with circulating anti-tumor antibodies.

The results would provide the basis for subsequent studies of the onset and progression of immune responses to ovarian tumors.

Materials and Methods

Animals

Commercial strains of Single Comb White Leghorn egg-laying hens (*Gallus domesticus*) 2.5–3 years old were kept in individual cages under a light regimen of 14h light:10h dark with *ad libitum* access to feed and water. Approximately 10–20% of laying hens at this age with low egg production (≤ 3 eggs/clutch/hen) will have spontaneous ovarian tumors(24). A commercial White Leghorn hen with normal ovarian function lays 5 or more eggs in a clutch. The ovary of young normally laying hens (11 months old, serum of these hens were used as negative control in immunoassay) contained 5 large yolk-filled preovulatory follicles, numerous developing small follicles and 2–3 degrading postovulatory follicular tissues indicating their frequent laying status. Hens lay an egg/day and a clutch is defined as the number of days of continuous egg laying by a hen in a sequence. After laying eggs in a sequence, a hen pauses for one or two days and then resumes laying. A smaller clutch size indicates partially reduced ovarian function. All procedures including the care and management, handling as well as subsequent euthanasia of animals were performed according to the Institutional Animal Care and Use Committee approved guidelines. A work flow diagram showing tissue collection, processing and subsequent steps are summarized in Figure 1.

Serum and tissue

Blood was collected into a 10ml red top tube from wing veins, centrifuged ($1,000 \times g$, 20 min) and sera stored at -80°C . Hens were euthanized by carbon dioxide inhalation just after oviposition approximately 30 minutes before the expected time of ovulation[30]. Hens with apparent non-ovarian pathologies upon post-mortem gross examination were excluded from the study. In the chicken, only the left ovary is functional while the right is rudimentary. The gross morphology of ovaries was recorded and included number of viable or atretic large preovulatory follicles and cystic and solid masses. A portion of the ovary was frozen immediately and stored at -80°C for later biochemical analysis. Another portion was processed for histological examination. In some cases, the ovary may be a secondary tumor site as tumors may metastasize to the ovary from the oviducts. Therefore, the oviducts of all hens with or without ovarian tumors were excised to confirm that the ovary was the primary site of malignancy. Hens with tumor seeding in the inner wall of the oviduct (oviductal mucosa) were excluded from the study. Ovaries were classified into normal ($n=39$, age-matched hens without any ovarian abnormality), ovaries with tumor ($n=18$) and ovaries with non-tumor pathology ($n=8$). Non-tumor pathology included atrophied ovaries with no preovulatory follicles, ovaries with atretic or fluid filled cystic preovulatory follicles without any solid tissue masses. Although atresia of small developing follicles is the normal physiological phenomena, the developed larger preovulatory follicles usually do not undergo atresia in hens and such conditions are considered abnormal.

Histology

Ovarian tissues were fixed in neutral-buffered formalin and embedded in paraffin. Sections ($6\mu\text{m}$ thick) were cut from each block, stained with hematoxylin and eosin, examined by light microscopy and classified as normal, ovaries with tumor or ovaries with non-tumor pathology. Microscopically, ovarian malignancies were confirmed by the presence of focal lesions, glandular structures, cells with pleomorphic nucleus with mitotic bodies and hyperplastic surface or stromal hyperplasia. Non-tumor pathologies included increased atresia of developing stromal follicles, cystic structures, hyperplasia without any focal lesion or malignant cells with a pleomorphic nucleus or glandular structures. Tumors were classified

based on histological types with reference to the FIGO classification for human. Staging of tumors were performed based on the gross morphology(24).

Preparation of ovarian homogenates for biochemical analysis

Frozen tissues were pulverized in a dry ice-acetone bath and homogenized with a Polytron (Brinkman Instruments, Westbury, NY) in ice cold Tris-sucrose buffer (40 mM Tris, 4mM MgCl₂, 0.25 M sucrose, 1 µl/ml protease inhibitor cocktail (Sigma, St. Louis, MO), pH 7.4). The homogenate was centrifuged (1,000×g, 10 min) and the supernatant was collected. The protein content of the extract was measured (Bradford protein assay kit, BioRad, Hercules, CA) with bovine serum albumin (BSA) as a standard (Sigma, St. Louis, MO). Extracts were stored at -80°C.

Immunoassay

Autoantibodies were detected by minor modification of a previously described tissue-based immunoassay(31). Immunoassay plates (MaxiSorp, Nunc) were coated with the extracts of normal ovaries or malignant ovarian tumors diluted in a phosphate based buffer (0.2 µg protein/well). Sera were diluted (1:100) in Wash buffer (10 mM phosphate buffer, 30 mM NaCl, 1mM MgCl₂, pH 7.4; 0.05% Triton X-100) containing 1% BSA (Sigma, St. Louis, MO) and tested in duplicate. Sera were tested using 100, 200, 500, 1000, 1500 and 2000 dilutions. The 1:1000 dilution was found to be the minimum threshold for positive reactivity against ovarian antigens. The bound autoantibodies were detected with alkaline phosphatase conjugated affinity purified rabbit anti-chicken IgY (IgG) (H+L) (Jackson Immuno Research Laboratories, Inc., West Grove, PA) and washed to remove unbound conjugate. The bound alkaline phosphatase was reacted with p-nitrophenylphosphate substrate (Sigma, St. Louis, MO) and plates were read at 405 nm in a plate reader (Thermomax; Molecular Devices, Sunnyvale, CA).

Two-dimensional electrophoresis and Western Blots

Two-dimensional gel electrophoresis is a process of protein separation from a complex proteome homogenate based on their isoelectric point (is the pH at which a particular molecule or surface carries no net electric charge and will not migrate in an electric field) and mass. In the first dimension, proteins are separated by their isoelectric point and in the second dimension by their mass (MW) perpendicularly to the first dimension. Two dimensional gel electrophoresis was performed as reported previously[32] with minor modification. Extracts (300µg protein/strip) were diluted in rehydration buffer (Bio-Rad ReadyPrep 2-D rehydration buffer) containing 1% (v/v) TBP reducing agent (Bio-Rad), 0.5% (v/v) carrier ampholyte (Bio-Lyte 3–10, Bio-Rad, Hercules, CA) and 1.2% (v/v) destreaking agent (DeStreak Reagent, Amersham Biosciences, Upsala, Sweden). Samples were incubated with immobilized pH gradient (IPG) strips (Bio-Rad, Hercules, CA) according to the manufacturer's instructions (16 h, 20°C). Following rehydration of the strip, proteins were separated by isoelectric focusing in a PROTEAN IEF apparatus (Bio-Rad, Hercules, CA), in three steps according to the manufacturer's instructions (step 1: 250V, 15 min; step 2: 8,000V, 2.5 h; step 3: 8000V, 35,000 V-hr). After focusing, strips were equilibrated in two steps (20 min each, 20°C) using equilibration buffer 1 (6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol and 2.5% (w/v) DTT) and buffer 2 (6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol and 2.5% (w/v) iodoacetamide). Ovarian proteins in IPG strips were resolved in discontinuous 10% Tris-HCl SDS-PAGE preparative well precast gel (BioRad, Hercules, CA) in the second dimension. Molecular size was determined by comparison to a chemiluminescent protein standard (MagicMarker Mix, broad range, Invitrogen, Carlsbad, CA). Proteins were transferred to a nitrocellulose membrane (0.45 µm; BioRad, Hercules, CA)(33). and reactions of four representative sera (positive for antibodies in immunoassay) against normal and tumor ovarian proteins were assessed in immunoblotting as reported previously(10). Blots were blocked

overnight in Starting Blocker (Pierce, Rockford, IL) containing 0.05% Tween-20, and then incubated with sera (1:100, 2 h) and washed 3X with Tris-buffered saline containing 0.05% Tween 20. Blots were then incubated with peroxidase-conjugated F(ab)₂ fragment affinity purified rabbit anti-chicken IgY (IgG) (H+L) (Jackson Immuno Research Laboratories Inc., West Grove, PA) (1:10,000, 1 h, 20°C). Immunoreactions were visualized as a chemiluminescence product (Super Dura West substrate, Pierce, Rockford, IL) and the image was captured using a Chemidoc XRS (BioRad, Hercules, CA).

Mass Spectrometry (MS) and protein identification

Sypro Ruby stained protein spots matched with two-dimensional Western blots were excised manually from gels and proteins were identified by LC-MS/MS. Proteins were digested with trypsin according to the manufacturer's protocol (Pierce, In-Gel Trypsin Digestion kit). Cystine disulfides were reduced to free cysteine and alkylated with iodoacetamide. Peptide digests were desalted on a Michrom (Auburn, CA) peptide CapTrap. The CapTrap was placed in-line as a pre-column, to a New Objective (www.newobjective.com) 10cm × 75 Qm i.d. C18 ProteoPrep nano-HPLC column attached to a NewObjective nano-ESI source interfaced to ThermoFinnigan (San Jose, CA) LTQ ion trap mass spectrometer. Peptides were separated and eluted into the nano-ESI interface with a reverse-phase LC gradient of solvent A (0.1% formic acid, Fluka)/water (Riedel-deHaen) versus solvent B (0.1% formic acid/70% acetonitrile (Riedel-deHaen). A linear gradient of 5% to 100% solvent B, at a flow rate of 200 nanoL/min for 70 min was delivered by a Dionex (www.dionex.com) Ultimate 3000 nanoLC. Full scan MS spectra, m/z 440–2000, high resolution zoom scan, and MS/MS spectra were obtained by a data dependent data acquisition “Triple Play” experiment. Instrument parameters include: ESI voltage 2.1kV, heated capillary 180°C; for MS/MS, isolation width 1.5 m/z, activation Q 0.25, activation time 30 msec, collision energy 35%, 2 × 100 msec microscans. The resultant MS/MS spectra were assessed for peptide sequence with SEQUEST. A peptide mass tolerance of 1.0 amu and fragment ion tolerance of 1.0 amu, strict trypsin cleavage at R & K, dynamic differential modifications of 16 amu for M and 1 amu for N. Spectra were then searched against a complete set of Avian proteins in GenBank v.156.

Analysis

In immunoassay, samples were assessed against the same negative control sera from young laying hens (11 months old, laying rate of 5 or more eggs/clutch, $n=5$) in every assay. Values considered positive for antibody had an optical density greater than three standard deviations ($P<0.01$) above the mean optical density value for negative control sera (31). The proportion of sera positive for ovarian or tumor antibodies was determined for each group and results were compared using a Chi-square test with $P<0.01$ considered significant. In addition, the mean optical density values were compared using a two tailed Student's t test for unequal variance with $P<0.05$ considered significant. The correlation of optical density values for normal ovarian antigens and tumor antigens was compared using Spearman's rho two-tailed test, means were compared by ANOVA using Games-Howell GH method for unequal variances and unequal sample sizes with $p<0.05$ considered significant. Statistical analysis was performed with SPSS (Chicago, IL)

Results

Gross morphology and histology

Upon necropsy, 2 or 3 large yolk-filled preovulatory follicles, few developing small follicles and 1 or 2 degrading postovulatory follicles were observed consistent with reduced ovarian function (Fig. 1A). In hens with abnormal ovaries, the 2–3 large preovulatory follicles were either atretic or cystic (Fig. 1B). In hens with OvCa, ovaries contained solid nodular masses or firm white cauliflower-like structures, with no yolky preovulatory follicles (Fig. 1C). In

some hens, tumors were metastasized to peritoneal organs accompanied by profuse reddish brown ascites (Fig. 1D).

More developing follicles were seen in ovarian sections of hens with normal ovaries than in hens with tumors or abnormal ovaries. Frequent atresia of follicles containing fat globules with ruptured theca and granulosa layers and cysts were seen in the ovary of hens with non-tumor abnormalities. Although occasional stromal hyperplasia was seen, neither focal lesions nor cells with pleomorphic nuclei were seen in abnormal ovaries. Serous OvCa (n=5, 2 early and 3 late stages) was characterized by malignant cells with pleomorphic nuclei and mitotic figures. Endometrioid carcinoma (n=5, 2 early and 3 late stages) was identified by glandular structures resembling endometrial glands with a lining of columnar, cuboidal or oval epithelial cells. In mucinous carcinoma (n=4, 3 early and 1 late stages), glandular ciliated goblet cells with luminal secretion were present and clear cell carcinoma (n = 1, early stage) was identified by cells with clear cytoplasm (Fig. 2a–d). In addition, poorly differentiated or mixed ovarian carcinomas (n = 3, 2 early and 1 late stage) were observed occasionally in some hens.

Anti-tumor antibodies detected by immunoassay

The mean optical density values for both anti-tumor and anti-ovarian antibodies in the serum of hens with OvCa was significantly higher than in hens with a normal ovary or hens with abnormal ovarian pathologies ($P < 0.05$) (Fig 3). Moreover, the optical density values for anti-tumor and anti-ovarian antibodies were significantly correlated except for hens with ovarian abnormalities [experimental controls (young hens), correlation co-efficient = 0.92, $P = 0.05$; normal (age matched old hens without any ovarian abnormality), correlation co-efficient = 0.90, $P = 0.01$; ovarian cancer, correlation co-efficient = 0.88, $P = 0.01$).

The proportion of sera positive for anti-tumor antibodies (67%, $P < 0.001$) or anti-ovarian antibodies (56%, $P < 0.01$) in hens with OvCa was significantly higher than hens with a normal ovary (15% and 21%, respectively). Anti-tumor antibodies (63%) but not anti-ovarian antibodies (38%) were more prevalent in hens with abnormal ovaries than in hens with normal ovary ($P < 0.03$). The proportion of serum antibodies in hens with OvCa using tumor or normal ovarian antigens did not differ significantly. Differences in the prevalence of anti-tumor antibodies by tumor type were not observed.

Anti-tumor antibody reactions with normal and tumor ovarian antigens in western blot

Two-dimensional electrophoresis and Western blots were performed using selected OvCa sera ($n = 4$, 1 each of serous, endometrioid, mucinous and clear cell carcinoma) with representative immunoreactions based on immunoassay and one-dimensional Western blot (data not shown). Owing to the heterogeneity in protein expression, serum from individual hens with OvCa was examined against homologous as well as heterologous tumor antigens (Fig. 4). Common immunoreactive spots of approximately 50 kDa were seen against normal or tumor ovarian antigens (Fig. 4b–d). Some spots at approximately 30–40 kDa range were different between normal and tumor ovarian antigens. In addition, among the tumors of different sources, spots of similar (dotted lines and circles) as well as different (triangular and square) molecular weights (20–40 kDa, Fig. 4) were seen. Two dimensional electrophoresis and western blot separates the individual immunoreactive antigens from the whole ovarian proteome but it does not identify the ovarian antigenic proteins. Therefore, mass spectrometry was used to identify these immunoreactive ovarian tumor antigens.

Identification of antigens

Proteins identified are summarized in Table 1 and an example of a representative 2-DE gel with hen ovarian tumor ovarian proteins indicating their approximate location are shown in Figure 5. Among identified proteins, number of low molecular wt (20–50 kDa) proteins was

more than high molecular wt (higher than 50kDa) proteins. Heat shock cognate and chaperonin GroEL were the two identified proteins from spots at 60–80 kDa. Proteins identified on the 50–60 kDa range include vimentin and ATP synthase subunit β . Enolase and protein disulfide isomerase A6 precursor were the two proteins identified at the range of 40–50 kDa. Proteins identified in the low molecular wt range (20–40 kDa) include apolipoprotein A1, Annexin A2/Annexin II, UDP-glucose-4-epimerase, ester hydrolase, peroxiredoxin (PRX), Rho GDP-dissociation inhibitor-1, D4-GDP-dissociation inhibitor, Protein DJ-1 (Fig. 5).

Discussion

The results of this study, for the first time, demonstrated an anti-tumor antibody response in the egg-laying hen, a spontaneous animal model of OvCa. The prevalence of antibodies associated with OvCa in hens is similar for ovarian antigens from both normal and tumor ovary. This suggests the reactive antigens are not unique to the tumors, similar to anti-tumor antibodies in human OvCa(10). The ovarian tumors have the same epithelial cell derived histology as human tumors. Antibodies were associated with all histological types including endometrioid, mucinous, clear cell and mixed types of OvCa. Furthermore, anti-tumor antibodies were detected in hens irrespective of OvCa stage suggesting the possibility that the antibody response occurs early in tumor progression.

The antibody response to tumors is a characteristic feature of cancer including OvCa(3,10, 34). The frequency of anti-tumor antibodies to individual antigens in patients with OvCa ranges from 5–67%[35]. Previously, we reported that 69% of patients with OvCa had anti-tumor antibodies that reacted to antigens in ovarian tumor homogenates(10). In the present study, a similar prevalence of anti-tumor antibodies was observed in hens with OvCa. Immunoreactive antigens detected in 2D-WB showed some commonality in their expression in both normal ovaries and ovarian tumors with a predominant reaction at 50 kDa. However, there were obvious differences in immunoreactions between antigens from normal and tumor ovaries as well as between homologous and heterologous (different) sources. Therefore, as reported in humans with OvCa, immunoreactive ovarian antigens are also heterologous in hens OvCa(10). Thus, in both hen and human OvCa, there is a humoral immune response against ovarian tumor antigens detected by serum anti-tumor antibodies.

This is the first report on the immunoreactive ovarian antigens detected by serum anti-tumor antibodies. Fourteen ovarian proteins were identified as the best match in this study as possible antigens for anti-tumor antibodies. Some of the identified proteins are structural or regulatory proteins where as others are members of enzyme families. None of these proteins is known to be unique to OvCa either in hens or humans. No published information on the ovarian antigens associated with OvCa in hens is available. Among these proteins, increased expression of vimentin was reported in the pulmonary sarcoma in hens(36). Interestingly, some of the identified proteins were suggested to be expressed in the several cancers and autoimmune diseases in humans and other animals. Apolipoprotein A1 is a by-product of host's response to tumor and down regulated in OvCa in humans(37). Annexin II and DJ-1 were reported to be involved in the tumor invasion and metastasis of breast, prostate GI and lung cancers(38–40). Vimentin and enolase are the two antigens reported in several autoimmune diseases including animal model of lupus, inflammatory diseases, endometriosis and tumors of several organs(41–44).

One of the limitations of the present study is that the immunoassay using ovarian antigens could not differentiate hens with non-tumor ovarian abnormalities and those with ovarian tumors with regard to the presence of ovarian autoantibodies. Abnormal ovaries may be an early event in the onset of malignancy. Hens with abnormal ovaries including, those neither with developed preovulatory follicles, follicular atrophy nor with gross tumors also showed

the presence of anti-ovarian antibodies as compared to their old normal counterpart. It is possible that these hens might have developed ovarian autoimmunity. Previous study reported that circulatory ovarian autoantibodies were associated with reduced ovarian function and decreased egg production in older hens(45). Therefore, it might be possible that ovarian autoimmunity may precede ovarian cancer. In contrast, it might also be possible that some of these hens were at the initial stage of molecular transformation of ovary that could not be perceived by light microscope. Another drawback of the present study is the differences in immunoreactions between hens that may limit the utility of normal ovarian and tumor antigen detection in routine and/or screening tests. However, the screening test could be based on more than one antigenic protein. In addition to the usefulness as detection tools, these antigens might be useful as markers for monitoring patient's response to treatment modalities.

The principle aim of our study is to understand the etiology and the early events in the progression of OvCa so as to develop an early detection tool for the disease. Over the years chickens have been used extensively as an established or emerging model in biomedical research and contributed immensely to the understanding of immune system as well as many other human diseases including genetic disorders, drug testing, immune suppression, multiple sclerosis and autoimmune diseases. Moreover, chickens are the only animal model in which a vaccine has been developed against cancer (Marek's disease)(46). Demonstration of anti-ovarian tumor immune response in hens similar to humans will be of immense significance to the understanding of OvCa in humans including diagnostic and therapeutic interventions. Numerous studies have attempted to evaluate the clinical value of circulating anti-tumor antibodies in the context of neoplastic process and the results were confusing. Following the detection of anti-tumor antibodies in circulation, hen can be followed for ovarian changes leading to OvCa by *in vivo* imaging. Recently, we have developed, for the first time, the modalities of *in vivo* imaging of ovarian tumors by transvaginal Doppler ultrasonography with using probes used for humans(47). The results of this study will reinforce the suitability of laying hen as a preclinical animal model of OvCa.

In conclusion, the result of the present study showed that majority of the serum from hens with OvCa contained anti-ovarian and anti-tumor antibodies. Thus, antibodies are a potential diagnostic marker for OvCa and laying hens can be used to understand the etiology of OvCa in humans. The current study confirms the existence of spontaneous OvCa in laying hens with similar metastasis to abdominal tissues and profuse ascites as in humans. All major types of epithelial ovarian tumors including serous, endometrioid, mucinous, clear cell and mixed types were observed in hens and are similar to humans in histological features.

Acknowledgments

This study was supported by NIH R01AI055060 (JL), the Daniel F. and Ada L. Rice Foundation (JL), the Ovarian Cancer Survivor Network (JL), and DOD OC050091 (DBH).

References

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics. CA Cancer J Clin 2007;57(1):43–66. [PubMed: 17237035]
2. Turk MJ, Wolchok JD, Guevara-Patino JA, et al. Multiple pathways to tumor immunity and concomitant autoimmunity. Immunol Rev 2002;188:122–35. [PubMed: 12445286]
3. Preuss KD, Zwick C, Bormann C, et al. Analysis of the B-cell repertoire against antigens expressed by human neoplasms. Immunol Rev 2002;188:43–50. [PubMed: 12445280]
4. Guevara-Patino JA, Turk MJ, Wolchok JD, Houghton AN. Immunity to cancer through immune recognition of altered self: studies with melanoma. Adv Cancer Res 2003;90:157–77. [PubMed: 14710950]

5. Luborsky JL, Barua A, Shatavi SV, et al. Anti-tumor antibodies in ovarian cancer. *Am J Reprod Immunol* 2005;54(2):55–62. [PubMed: 16105096]
6. Koziol JA, Zhang JY, Casiano CA, et al. Recursive partitioning as an approach to selection of immune markers for tumor diagnosis. *Clin Cancer Res* 2003;9(14):5120–6. [PubMed: 14613989]
7. Hanash S. Harnessing immunity for cancer marker discovery. *Nat Biotechnol* 2003;21(1):37–8. [PubMed: 12511908]
8. Imafuku Y, Omenn GS, Hanash S. Proteomics approaches to identify tumor antigen directed autoantibodies as cancer biomarkers. *Dis Markers* 2004;20(3):149–53. [PubMed: 15502247]
9. Gnjjatic S, Atanackovic D, Jager E, et al. Survey of naturally occurring CD4+ T cell responses against NY-ESO-1 in cancer patients: correlation with antibody responses. *Proc Natl Acad Sci U S A* 2003;100(15):8862–7. [PubMed: 12853579]
10. Barua A, Bradaric MJ, Kebede T, et al. Anti-tumor and anti-ovarian autoantibodies in women with ovarian cancer. *Am J Reprod Immunol* 2007;57(4):243–9. [PubMed: 17362385]
11. Taylor DD, Gercel-Taylor C. Tumor-reactive immunoglobulins in ovarian cancer: diagnostic and therapeutic significance? (review). *Oncol Rep* 1998;5(6):1519–24. [PubMed: 9769398]
12. Stockert E, Jager E, Chen YT, et al. A survey of the humoral immune response of cancer patients to a panel of human tumor antigens. *J Exp Med* 1998;187(8):1349–54. [PubMed: 9547346]
13. Sugita Y, Wada H, Fujita S, et al. NY-ESO-1 expression and immunogenicity in malignant and benign breast tumors. *Cancer Res* 2004;64(6):2199–204. [PubMed: 15026363]
14. Jager E, Stockert E, Zidianakis Z, et al. Humoral immune responses of cancer patients against “Cancer-Testis” antigen NY-ESO-1: correlation with clinical events. *Int J Cancer* 1999;84(5):506–10. [PubMed: 10502728]
15. Scanlan MJ, Gout I, Gordon CM, et al. Humoral immunity to human breast cancer: antigen definition and quantitative analysis of mRNA expression. *Cancer Immun* 2001;1:4. [PubMed: 12747765]
16. Chen YT, Scanlan MJ, Sahin U, et al. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc Natl Acad Sci U S A* 1997;94(5):1914–8. [PubMed: 9050879]
17. Li G, Miles A, Line A, Rees RC. Identification of tumour antigens by serological analysis of cDNA expression cloning. *Cancer Immunol Immunother* 2004;53(3):139–43. [PubMed: 14722670]
18. Li Y, Karjalainen A, Koskinen H, et al. p53 autoantibodies predict subsequent development of cancer. *Int J Cancer* 2005;114(1):157–60. [PubMed: 15523685]
19. Suzuki H, Graziano DF, McKolanis J, Finn OJ. T cell-dependent antibody responses against aberrantly expressed cyclin B1 protein in patients with cancer and premalignant disease. *Clin Cancer Res* 2005;11(4):1521–6. [PubMed: 15746055]
20. Vanderhyden BC, Shaw TJ, Ethier JF. Animal models of ovarian cancer. *Reprod Biol Endocrinol* 2003;1:67. [PubMed: 14613552]
21. Stakleff KD, Von Gruenigen VE. Rodent models for ovarian cancer research. *Int J Gynecol Cancer* 2003;13(4):405–12. [PubMed: 12911715]
22. Johnson PA, Giles JR. Use of genetic strains of chickens in studies of ovarian cancer. *Poult Sci* 2006;85(2):246–50. [PubMed: 16523622]
23. Goodman MT, Howe HL, Tung KH, et al. Incidence of ovarian cancer by race and ethnicity in the United States, 1992–1997. *Cancer* 2003;97(10 Suppl):2676–85. [PubMed: 12733132]
24. Fredrickson TN. Ovarian tumors of the hen. *Environ Health Perspect* 1987;73:35–51. [PubMed: 3665870]
25. Giles JR, Shivaprasad HL, Johnson PA. Ovarian tumor expression of an oviductal protein in the hen: a model for human serous ovarian adenocarcinoma. *Gynecol Oncol* 2004;95(3):530–3. [PubMed: 15581958]
26. Damjanov I. Ovarian tumours in laboratory and domestic animals. *Curr Top Pathol* 1989;78:1–10. [PubMed: 2651020]
27. Rodriguez-Burford C, Barnes MN, Berry W, et al. Immunohistochemical expression of molecular markers in an avian model: a potential model for preclinical evaluation of agents for ovarian cancer chemoprevention. *Gynecol Oncol* 2001;81(3):373–9. [PubMed: 11371125]

28. Jackson E, Anderson K, Ashwell C, et al. CA125 expression in spontaneous ovarian adenocarcinomas from laying hens. *Gynecol Oncol* 2007;104(1):192–8. [PubMed: 16942793]
29. Urlick ME, Johnson PA. Cyclooxygenase 1 and 2 mRNA and protein expression in the *Gallus domesticus* model of ovarian cancer. *Gynecol Oncol* 2006;103(2):673–8. [PubMed: 16797680]
30. Warren D, Scott HM. Time factor in poultry production. *Poultry Science* 1935;14:195–207.
31. Luborsky JL, Visintin I, Boyers S, et al. Ovarian antibodies detected by immobilized antigen immunoassay in patients with premature ovarian failure. *J Clin Endocrinol Metab* 1990;70(1):69–75. [PubMed: 2104631]
32. Le Naour F, Brichory F, Misek DE, et al. A distinct repertoire of autoantibodies in hepatocellular carcinoma identified by proteomic analysis. *Mol Cell Proteomics* 2002;1(3):197–203. [PubMed: 12096119]
33. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 1979;76(9):4350–4. [PubMed: 388439]
34. Dong H, Strome SE, Salomao DR, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* 2002;8(8):793–800. [PubMed: 12091876]
35. Naora H, Montz FJ, Chai CY, Roden RB. Aberrant expression of homeobox gene HOXA7 is associated with mullerian-like differentiation of epithelial ovarian tumors and the generation of a specific autologous antibody response. *Proc Natl Acad Sci U S A* 2001;98(26):15209–14. [PubMed: 11742062]
36. Hafner S, Goodwin MA, Smith EJ. Pulmonary sarcomas in a young chicken. *Avian Dis* 1998;42(4):824–8. [PubMed: 9876857]
37. Zhang Z, Bast RC Jr, Yu Y, et al. Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer. *Cancer Res* 2004;64(16):5882–90. [PubMed: 15313933]
38. Sharma MR, Koltowski L, Ownbey RT, et al. Angiogenesis-associated protein annexin II in breast cancer: selective expression in invasive breast cancer and contribution to tumor invasion and progression. *Exp Mol Pathol* 2006;81(2):146–56. [PubMed: 16643892]
39. Singh P. Role of Annexin-II in GI cancers: interaction with gastrins/progastrins. *Cancer Lett* 2007;252(1):19–35. [PubMed: 17188424]
40. Clements CM, McNally RS, Conti BJ, et al. DJ-1, a cancer- and Parkinson's disease-associated protein, stabilizes the antioxidant transcriptional master regulator Nrf2. *Proc Natl Acad Sci U S A* 2006;103(41):15091–6. [PubMed: 17015834]
41. Vossenaar ER, Despres N, Lapointe E, et al. Rheumatoid arthritis specific anti-Sa antibodies target citrullinated vimentin. *Arthritis Res Ther* 2004;6(2):R142–50. [PubMed: 15059278]
42. Thebault S, Gilbert D, Hubert M, et al. Orderly pattern of development of the autoantibody response in (New Zealand White × BXSb)F1 lupus mice: characterization of target antigens and antigen spreading by two-dimensional gel electrophoresis and mass spectrometry. *J Immunol* 2002;169(7):4046–53. [PubMed: 12244208]
43. Gitlits VM, Toh BH, SENTRY JW. Disease association, origin, and clinical relevance of autoantibodies to the glycolytic enzyme enolase. *J Investig Med* 2001;49(2):138–45.
44. He P, Naka T, Serada S, et al. Proteomics-based identification of alpha-enolase as a tumor antigen in non-small lung cancer. *Cancer Sci* 2007;98(8):1234–40. [PubMed: 17506794]
45. Barua A, Yoshimura Y. Ovarian autoimmunity in relation to egg production in laying hens. *Reproduction* 2001;121(1):117–22. [PubMed: 11226034]
46. Millers SH. Increased Marek's Condemns....New Strains or Old Problems? *Broiler Industry* 1996;(May):26–31.
47. Barua A, Abramowicz JS, Bahr JM, et al. Detection of ovarian tumors in chicken by sonography: a step toward early diagnosis in humans? *J Ultrasound Med* 2007;26(7):909–19. [PubMed: 17592054]

Figure 1.

Workflow schematic diagram showing the methods used for the separation and identification of immunoreactive ovarian antigens from laying hens with or without ovarian tumors. Please refer to the materials and methods for detailed description.

Figure 2.

Gross morphology of ovaries of White Leghorn egg-laying hens (2.5–3.0 years old) with or without tumor. A) normal ovary showing 3 large preovulatory follicles (F₃-F₁). Following ovulation of the largest one (F₁), F₂ becomes F₁; F₃ becomes F₂ and one from the stock of growing follicles will be recruited to the set of largest preovulatory follicles. In between the preovulatory follicles, is a degrading postovulatory follicle, B) abnormal ovary. All the large preovulatory follicles are atretic and shrunken with yellow or black brown color (arrows). However, small growing follicles are present, C) early stage ovarian cancer. The tumor is solid with a cauliflower-like appearance (circle), well formed nodules and is limited to the ovary without ascites or preovulatory follicles, D) late stage ovarian cancer with extensive metastasis to the abdominal cavity and extra-ovarian solid masses accompanied by profuse ascites (*).

Figure 3.

Histology of ovarian carcinoma in egg-laying hens. A) Clear cell carcinoma showing solid sheets of polyhedral cells with abundant clear cytoplasm with dense hyalinized fibrotic stroma. B) Well developed endometrioid carcinoma with confluent pattern of back to back glands. C) Mucinous carcinoma showing glandular epithelium containing ciliated goblet cells and mucin-like secretory products in the lumen of the gland. D) Serous carcinoma. Solid pattern of sheets beneath the surface epithelium containing moderate to large-sized cells with scanty cytoplasm and pleomorphic nuclei separated by septal tissue. Hematoxylin and eosin stain, 40X.

Figure 4.

Anti-tumor antibodies in sera of hens with or without ovarian cancer determined by immunoassay. Optical density values are displayed as a box-and-whiskers plot with median, range (whiskers), 25th to 75th percentile (box) and outliers (circles). Antibodies were detected in two assays using antigens either from normal ovary or from ovarian tumors. Sera ($n = 26$; age range 2.5 to 3.0 years) from hens with ovarian cancer ($n = 18$), abnormal (non-tumor ovarian pathology, $n = 8$) were compared with normal hens (healthy hens of the same age, $n = 39$) and experimental controls (young laying hens). The mean optical density of ovarian cancer sera was significantly higher than those of controls.

Figure 5.

An example of the immunoreactivity of a serum from a hen with ovarian cancer against normal ovarian antigens and autologous or heterologous tumor antigens in two-dimensional Western blot (2D-WB). A) control 2D-WB with ovarian tumor antigen but omitting serum shows the lack of background reaction except for interaction of the second antibody with IgY. Ovarian cancer sera reacted against B) normal ovarian antigens, C) homologous ovarian tumor antigens and D) heterologous ovarian tumor antigens. Anti-tumor antibodies reacted with both normal and ovarian tumor antigens, consistent with immunoassay results (see Fig. 3). Although variations in the immunoreactivities among different sources of antigens are seen, common immunoreactions at approximately 50 kDa are also observed. Sera reacted more with homologous tumor antigens than heterologous tumor antigens. Spots of similar (dotted lines and circles) as well as different (triangular and square) molecular weights (20–40 kDa) were seen in heterologus tumors.

Figure 6.

Representative two-dimensional gel of hen ovarian tumor protein (corresponding to Western blot immunostained with serum from same hen) stained with Sypro Ruby. The tryptic peptides from proteins in spots corresponding to Western blot reactions with ovarian cancer sera were analyzed by LC-MS/MS (see the methods for detail).

Table 1

Immunoreactive ovarian antigens identified by mass spectrometry.

Acc number	Title	Mass (kDa)	pI	sequence score	fllicka hit	coverage
NP_990856.1	apolipoprotein AI (put.); putative	30.7	5.6	19	15	51
XP_429266.1	heat shock cognate 70	71	5.6	6.2	5	15
CAG31521	Chaperonin GroEL (HSP60 family)	60	5.7	94	26	54
NP_990451.1	enolase	47.3	5.9	60.6	20	38.5
NP_990682.1	annexin A2; annexin II	38.6	6.9	2.6	5	20
XP_417833.1	UDP-glucose 4-epimerase	38.25	5.58	5.72	3	13.5
XP_417195.1	Predicted similar to Ester hydrolase	34.6	5.9	22.7	6	26.6
CAG32139.1	Peroxioredoxin (PRX) family, 1-cys PRX subfamily	24.5	5.8	41.5	11	44
BAB79527.1	Protein DJ-1.	19.9	6.8	12.5	7	51.3
hm11604	~human Rho GDP- dissociation inhibitor 1 (Rho GDI 1)	20		18.7	11	44
XP_416182.1	PREDICTED: ~ D4-GDP-dissociation inhibitor	22.8	4.9	9.6	5	49
CAG31468.1	ATP synthase subunit beta.	56.6	5.6	56.2	24	65.9
XP_418622.1	~ vimentin	53.1	5	30	26	57.6
XP_419952.1	PREDICTED: ~ Protein disulfide isomerase A6 precursor	48.8	5.1	12.5	10	29