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Ovarian Adenocarcinomas in the Laying Hen and Women Share Similar Alterations in p53, ras, and HER-2/neu

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Abstract We examined alterations in the *p53* tumor suppressor gene and the *ras* and *HER-2/neu* oncogenes in chicken ovarian cancers to determine if these tumors have genetic alterations similar to those in human ovarian adenocarcinomas. Mutations in the *p53* tumor suppressor gene and the H-*ras* and K-*ras* oncogenes were assessed by direct sequencing in 172 ovarian cancers obtained from 4-year-old birds enrolled at age 2 in two separate 2-year chemoprevention trials. Birds in trial B had approximately twice as many lifetime ovulations as those in trial A. Immunohistochemical staining for the *HER-2/neu* oncogene was done on a subset of avian ovarian and oviductal adenocarcinomas. Alterations in *p53* were detected in 48% of chicken ovarian cancers. Incidence of *p53* alterations varied according to the number of lifetime ovulations, ranging from 14% in trial A to 96% in trial B ($P < 0.01$). No mutations were seen in H-*ras*, and only 2 of 172 (1.2%) tumors had K-*ras* mutations. Significant HER-2/neu staining was noted in 10 of 19 ovarian adenocarcinomas but in only 1 of 17 oviductal adenocarcinomas. Similar to human ovarian cancers, *p53* alterations are common in chicken ovarian adenocarcinomas and correlate with the number of lifetime ovulations. *Ras* mutations are rare, similar to high-grade human ovarian cancers. *HER-2/neu* overexpression is common and may represent a marker to exclude an oviductal origin in cancers involving both the ovary and oviduct.

Epithelial ovarian cancer remains a highly lethal malignancy. It is the fifth leading cause of cancer deaths among women in the United States and causes more deaths than all other gynecologic malignancies combined (1). Major advances in our understanding and treatment of ovarian cancer have occurred over the past decade, yet the long-term cure rate of women with this disease has only improved modestly. The feasibility of early detection is uncertain, and highly effective curative therapy for women who present with metastatic disease is lacking.

The pathogenesis of ovarian cancer is not completely understood, but it is believed that the process of ovulation leads to genetic damage in the ovarian epithelium. Ovarian cancer risk has been shown to correlate with the number of ovulatory cycles in a woman's life time, whereas factors associated with decreased ovulation such as increased parity, breast-feeding, and oral contraceptive use have been shown to have a protective effect (2). These observations have led to the "incessant ovulation" hypothesis, which purports that repeated cycles of epithelial disruption and repair may facilitate neoplastic transformation of the ovarian epithelium in susceptible individuals and that the risk of ovarian cancer may be proportional to the number of ovulatory cycles in a woman's lifetime (3). Repeated cycles of rupture and repair of the ovarian epithelium associated with ovulation may predispose the ovarian epithelium to DNA damage, inclusion cyst formation, and dysplastic changes that can lead to neoplastic transformation. In addition, repetitive ovulation may expose the ovarian epithelium to high levels of estradiol or gonadotropins, which may increase ovarian cancer risk (4).

Until recently, the molecular events involved in the development of human cancers were largely unknown. It is now thought that most cancers arise due to inherited or acquired alterations in oncogenes and tumor suppressor

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genes that encode molecules normally involved in regulating cellular growth and differentiation. The specific molecular alterations vary considerably between different types of cancers. Among the known genes, the *HER-2/neu* oncogene and the *p53* tumor suppressor gene are among the most frequently altered in ovarian cancers (5–9). In contrast, although mutations in members of the *ras* family of oncogenes are among the most common genetic events described in human cancers, these mutations occur less frequently in ovarian cancers and are confined primarily to specific ovarian cancer histologic subtypes, including borderline, mucinous, or endometrioid tumors (10–14).

The lack of a valid ovarian cancer animal model has been a major obstacle to ovarian cancer prevention research. To develop effective chemopreventive strategies for ovarian cancer in a timely fashion, animal models that closely mimic human ovarian cancer are desperately needed. Human prevention trials are costly, requiring large numbers of subjects and many years to complete. Development of an animal model for ovarian cancer prevention research would represent a significant breakthrough, allowing the expedited evaluation of numerous agents. Ideally, this would lead to the rapid identification of a select number of agents with the greatest potential for ovarian cancer prevention, which could then be evaluated in human trials.

Among the candidate ovarian cancer animal models, the egg-laying hen may have great potential, with characteristics that make it especially attractive for chemoprevention research. The most relevant feature of the domestic hen is its high incidence of spontaneous ovarian cancer, which ranges from 11% to 35% between 4 and 6 years of life (15, 16). This makes the laying chicken unique relative to other animals that require either experimental induction or genetic engineering to induce the development of ovarian tumors (17–39). In addition, the egg-laying hen has a high ovulatory rate (almost daily), raising the possibility that chicken and human ovarian cancers have a common pathogenesis related to ovulation-induced genetic damage to ovarian epithelial cells. The chicken ovarian cancer model, however, requires validation. To gather evidence to critically evaluate the chicken model, we examined genetic alterations in the *p53* tumor suppressor gene and the *ras* and *HER-2/neu* oncogenes in chicken ovarian cancers to determine whether chicken ovarian cancers have alterations similar to those in women.

Materials and Methods

Chicken ovarian adenocarcinomas

Chicken ovarian adenocarcinomas (n = 172) were collected at trial termination from two flocks (flocks A and B) of 4 y-old domestic egg-laying hens (*Gallus domesticus*; Single Comb White Leghorn laying hens). Details about the reproductive performance of the flocks before the current study was initiated have been previously described for both flocks (40, 41).

The flocks had completed 2-y prospective studies evaluating candidate ovarian cancer preventives including the retinoid *N*-(4-hydroxyphenyl)retinamide, vitamin D (cholecalciferol), and a progestin. These preventives had been administered in low human-equivalent dosages, adjusted for the size and metabolic rate in the hen, including (*a*) twice the recommended daily allowance for vitamin D, (*b*) a dose of progestin comparable to that contained in either standard oral contraceptives or low continuous combined hormone replacement therapy, or (*c*) a dose of *N*-(4-hydroxyphenyl)retinamide equivalent to 200 mg/d in humans. The chemopreventive outcomes of the trials are being submitted in a separate article. The prevention trials had begun in both flocks with birds at ~2 y of age. Flock A (n = 102) had decreased lighting and a calorie-restricted diet from age 2 to 4 y to minimize ovulation during the prevention trial, thus ovulating almost daily for only their first 2 y of life, but not during the prevention trial. Caloric restriction in flock A was limited to ~100 kcal/d and was aimed at maintaining hen weight. This strategy did not starve the birds. Rather, it maintained healthy birds that were not receiving sufficient additional calories required to support daily egg production. As a consequence of caloric restriction, ovulation ceases. No hormonal manipulation was used to achieve inhibition of ovulation, and ovulation did not differ among groups receiving different chemopreventive interventions. Flock B (n = 70) received normal caloric intake, which supported almost daily ovulation. Thus, this flock ovulated regularly throughout its 4-y life span. For each study, the complete flock was kept in the same research house, with uniform environmental conditions including the light cycle. In flock A, light was reduced to 10 h of light and 14 h of dark. In flock B, lighting was held constant at 12 h of light and 12 h of dark.

In both studies, the hens were housed at the North Carolina Department of Agriculture and Consumer Services, Piedmont Research Facility in Salisbury, North Carolina. The flocks were managed in accordance with the Institute for Laboratory Animal Research Guide with all of the husbandry practices being approved by and under the oversight of North Carolina State University Institutional Animal Care and Use Committee. The hens were randomly assigned to the treatment replicates in each study. The diets were provided to the research station in a mash form and the prescribed medications were incorporated on site to ensure the best drug activity. The diets were prepared as needed and temporary storage was in sealed containers

Table 1. PCR and sequencing primers

Gene	Forward primer	Reverse primer	Size (bp)
Chicken H-*ras*	TCAGCTGGAAGATGACCGAGT	TTGTGGGGTCGTACTCATCAA	114
Turkey K-*ras*	CGCCGGGCAGGTCTGCTAAAA	AGAGACAGGTTTCCCCATCAA	174
P53-1	GCGGAGGAGATGGAACCATTG	GGGGAGTAAGTGCAGGTGACC	332
P53-2	CCCATCCACGGAGGATTATGG	GGTCTCGTCGTCGTGGTAACG	342
P53-3	GCCGTGGCCGTCTATAAGAAA	CGGAAGTTCTCCTCCTCGATC	396
P53-4	CCTCACCATCCTTACACTGGA	GGTCCCTCCACCTTCACACGG	319
P53-5	GCTGAACCCCGACAATGAGAT	GCGTGGCTAAAGGAAAAAGGG	222

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Table 2. *p53* mutations in chicken ovarian tumors

Mutation	AA	Flock	Frequency
G 368 C	G 102 A	A	1/102
393 del 57	110	A	1/102
451 del 147	130	A	2/102
649 del 3	217 T	A	1/102
750 del 51	229	A	1/102
795 del 107	244	A	1/102
800 del 122	246	A	1/102
803 del 122	247	A	4/102
803 del 202	247	A	1/102
1073 del 15	337	A	1/102
C 178 G	Q 39 E	B	1/70
178 del 3	39 Q	B	3/70
183 del 3	40 E	B	1/70
CT 184 AG	L 41 K	B	1/70
AG 187 GA	S 42 D	B	1/70
TG 194 GT	L 44 R	B	1/70
G 203 A	S 47 N	B	1/70
GC 203 AA	S 47 K	B	1/70
T 236 C	L 58 P	B	14/70
T 242 C	L 60 S	B	2/70
T 242 G	L 60 W	B	1/70
G 247 C	A 62 P	B	1/70
C 251 T	A 63 V	B	2/70
263 ins C	67	B	1/70
A 277 C	T 72 P	B	44/70
C 283 T	P 73 S	B	1/70

labeled with the treatment codes. All diets were provided *ad libitum* and the drug dosages were controlled based on the feed intake of the hens. The house temperature was maintained at 80 ± 5°F.

The hens were monitored daily. Birds displaying signs of illness during study were euthanized via cervical dislocation. Dead hens were necropsied for gross examination and sample collection. At study completion, the remaining birds were examined, bled, euthanized, and necropsied. Samples of ovary and oviduct were collected from each chicken, fixed in 10% buffered neutral formalin, transferred to 70% ethanol after 72 h, trimmed, and processed by paraffin embedding for preparation of H&E-stained slides. Tissues from selected hens were snap frozen in optimum cutting temperature compound on dry ice and stored at −80°C until processed. Ovaries and oviducts showing gross lesions of ovarian or oviductal cancer were photographed, and additional samples (both frozen and fixed) were collected for immunohistochemistry and molecular analyses. Ovarian and oviductal tumors underwent gross and microscopic examinations by a board-certified avian pathologist (H.J.B.) to confirm the origin of the lesion.

Overall, the 172 avian ovarian cancer specimens were selected from more than 400 specimens that were collected from necropsies at the end of each trial from the two flocks. All 172 cases that were included for sequencing had been carefully characterized and annotated by an avian pathologist (H.J.B.) as being ovarian. Tumors were excluded from analysis if there was insufficient material or if the site of origin was not clear. In total, the study was composed of 105 tumors selected from 154 collected from flock A and 70 tumors selected from 276 collected from flock B. The specimens were equally distributed across chemopreventive interventions. All of the ovarian and oviductal adenocarcinomas that were analyzed for HER-2/neu were from flock A, and these too had been carefully annotated by an avian pathologist (H.J.B.).

Table 3. Type and location of *p53* mutations by flock

	Flock A	Flock B
Type of mutation		
Deletion	13	4
Insertion	0	1
Missense	1	71
Total	14	76
Location of mutation		
Transactivation domain (aa 1-45)	0	8
Proline-rich region (aa 46-90)	0	68
DNA binding domain (aa 101-336)	14	0
Total	14	76

NOTE: Seventy-six mutations identified in flock B came from 67 birds.

p53 DNA sequencing

Frozen tissues were ground to a fine powder in liquid nitrogen with a mortar and pestle. Total RNA was extracted from ~50 mg of powdered tissue using a commercial, nonphenol RNA extraction kit (Purescript). cDNA was synthesized from total RNA using Moloney murine leukemia virus reverse transcriptase and random primers following the manufacturer's protocol (Pharmacia Biotech). The entire *p53* coding region was amplified from cDNA in five PCR amplification reactions using overlapping primer pairs based on the chicken cDNA sequence (accession no. X13057; ref. 42). The sequences of PCR primers used are shown in Table 1. Five microliters of each PCR product were analyzed by electrophoresis in 1.5% agarose gels to check for amplification fidelity. Sequencing was done using an ABI 377 Automated Fluorescent Sequencer with Prism Dye Terminator Cycle Sequencing Kit (Applied Biosystem). Sequence electropherograms were compared with the wild-type chicken sequences to identify mutations.

Ras mutation screening

The H-*ras* and K-*ras* genes were evaluated in the chicken ovarian cancers by direct sequencing around and including codons 12 and 13 to examine them for mutations similar to those described in human ovarian cancers (10, 12–14). The *ras* oncogene has been highly conserved throughout evolution, and its sequence in the chicken as H-*ras* has been reported (accession no. X03578), whereas the sequence of chicken K-*ras* is not known. Comparing the sequences of chicken H-*ras*, turkey K-*ras* (accession no. X85754), and human K-*ras* (accession no. M54968) using the BLAST suite of programs[8] shows that both turkey and human K-*ras* genes are 83% identical at the nucleic acid sequence level around codon 12. Comparison of turkey K-*ras* and chicken H-*ras* shows that they are 86% identical at the nucleic acid sequence level around codon 12. Based on these homologies, primers for amplification of the chicken K-*ras* and H-*ras* genes around codons 12 and 13 were designed (Table 1). Codons 12 and 13 of H-*ras* and K-*ras* chicken tumor genes were evaluated for mutations by direct sequencing.

HER-2/neu immunostaining

The v-erbB oncoprotein is an oncogenic form of the chicken epidermal growth factor receptor and the homologue of HER-2/neu. Expression of the v-erbB oncoprotein via immunohistochemistry has been

[8] http://www.ncbi.nlm.nih.gov/BLAST/

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Table 4. Distribution of HER-2/neu staining scores for adenocarcinomas from laying hens

	n	Score negative	Score positive
Ovarian cancer, type 1	4	4	*
Ovarian cancer, type 2	1	1	*
Ovarian cancer, type 3	14	4	10
Oviductal cancer	17	16	1

NOTE: Type 1 tumors represent lesions occupying <10% of the ovarian total volume; type 2, lesions occupying 11% to 50% of total ovarian volume; and type 3, lesions occupying >50% of ovarian volume.
*No hens in this category.

described previously in the chicken, and immunostaining was done using this technique in paraffin sections of ovarian (n = 19) and oviductal adenocarcinomas (n = 17; ref. 43) with modifications as indicated. All of these specimens were from birds in flock A. Specimens were heated at 58°C for 1 h, deparaffinized, and transferred to Tris-buffered bath (0.05 mol/L Tris base, 0.15 mol/L NaCl, 0.0002% Triton X-100, pH 7.6). They were then processed for antigen retrieval by boiling in 10 mmol/L citrate buffer (pH 6.0) for 15 min and cooling to room temperature. Sections were then rinsed with deionized water and immersed in 3% hydrogen peroxide in 100% ethanol for 5 min to block endogenous peroxidase activity. Deionized water was gradually added and the slides were incubated in blocking solution (3% normal horse serum in PBS) for 20 min to block unspecific binding sites. Immunostaining was done with either negative control IgG1 (1.0 μg/mL in 3% horse serum) or the erb-B2 antihuman monoclonal antibody (synthetic peptide of the COOH terminus of human c-erbB2 protein; c-erbB2/HER-2/neu AB-15 mouse monoclonal antibody, clone 3B5, Lab Vision Corporation) at a concentration of 1 μg/mL in PBS with 3% horse serum for 40 min. The sections were then treated with biotinylated horse antimouse immunoglobulin (1:200; Vector Laboratories, Inc.) and avidin-biotin peroxidase complex (Vectastain ABComplex, Vector Laboratories). The peroxidase staining was visualized with 3,3′-diaminobenzidine (DAB Substrate Kit, Vector Laboratories) and the sections were counterstained with Mayer's hematoxylin. To confirm the specificity of the staining, human mammary carcinoma was used as a positive control; human tonsilar tissue was run as a negative control. In addition, a histologic slide stained with H&E was prepared for each case. Sections were examined in a blinded fashion by three investigators, including an avian pathologist (H.J.B.), and a subjective scoring system was created for individual tumors based on the intensity and number of positive immunostaining cells, whereby moderate to marked staining in as many as 50% or more of cells was defined as high expression.

Results

Mutations in *p53*

Overall, mutations in *p53* were identified in 81 of 172 (48%) chicken ovarian adenocarcinomas, and several tumors had more than one mutation (Tables 2 and 3). In flock A, 14 of 102 (14%) hens had a total of 14 *p53* mutations, compared with 67 of 70 (96%) hens having a total of 76 mutations in flock B (P < 0.01). In flock A, 13 of the 14 mutations were deleterious, with 10 of these being sizeable deletion mutations (>50 nucleotides). The remaining mutation was a missense mutation resulting in an amino acid substitution.

In flock B, the vast majority of *p53* mutations were missense mutations, not deletion mutations as seen in flock A. Four tumors had mutations at nucleotide 178; three had deleted nucleotides 178 to 180, thus eliminating glutamine (amino acid 39) from the protein product, whereas the fourth tumor had a missense mutation that changed glutamine to glutamic acid. Fourteen tumors had a missense mutation at nucleotide 236, changing amino acid 57 from leucine to proline within the proline-rich region of the gene. The most frequent mutation (found in 44 of 70 flock B tumors) was a missense mutation that changed amino acid 277 from threonine to proline, also in the proline-rich region of *p53*. Thus, 76% of the flock B mutations added a proline residue to the final *p53* protein within the proline-rich region of the gene. Only one insertion mutation was found in this study, in flock B, creating a frameshift that would terminate at 118 amino acids after incorrectly encoding 50 amino acid residues.

The locations and types of *p53* mutations differed between the two flocks (Table 3). Unlike flock A, which had all 14 mutations in the DNA binding domain (amino acids 101-336), none of the mutations in flock B were located in this region. Instead, 8 of 76 (11%) mutations occurred in the transactivation domain (amino acids 1-45), whereas the other 68 (89%) mutations were in the proline-rich domain (amino acids 46-90). Of note, there was no difference in the prevalence or type of mutations identified according to chemopreventive intervention. In other words, hormonal or vitamin interventions did not influence the incidence or types of *p53* alterations that were observed.

Mutations in the *ras* genes

Evaluation of the H-*ras* and K-*ras* genes in the chicken ovarian cancers revealed infrequent alterations. In flock A, no mutations were seen in either gene. However, a silent single nucleotide polymorphism was common in codon 39 of K-*ras* (36% of tumors), and in H-*ras*, one tumor also had a silent single nucleotide polymorphism in codon 12. In flock B, two missense mutations were detected in codon 28 of the K-*ras* gene (2 of 70; 3%). Both had a G residue at nucleotide 176 instead of a T, resulting in an amino acid change from phenylalanine to cysteine. No mutations were found in the H-*ras* gene in any of the flock B tumors.

HER-2/neu immunohistochemistry

Overall, 10 of 19 ovarian adenocarcinomas but only 1 of 17 oviductal adenocarcinomas showed significant HER-2/neu expression (Table 4). In addition, significant HER-2/neu staining was more likely in the large as compared with the small ovarian lesions (Table 4). The pattern of HER-2/neu staining in chicken tumors was distinctly different from that of control human mammary adenocarcinoma. Staining of both human and chicken tumor cells was cytoplasmic; the nucleus was unstained. However, in the human tumor, staining of the cytoplasm was diffuse and the cell membrane was intensely stained. In the chicken, there was multiple punctuate staining in the cytoplasm, especially in the basal and apical parts of the cell when they lined tubules, and the cell membrane was not stained (Fig. 1A-C). Tissue and tumor cells that stained positive were not stained in the negative control sections, indicating that the staining for HER-2/neu was specific even though the pattern of staining was different (Fig. 1D). Tumor emboli

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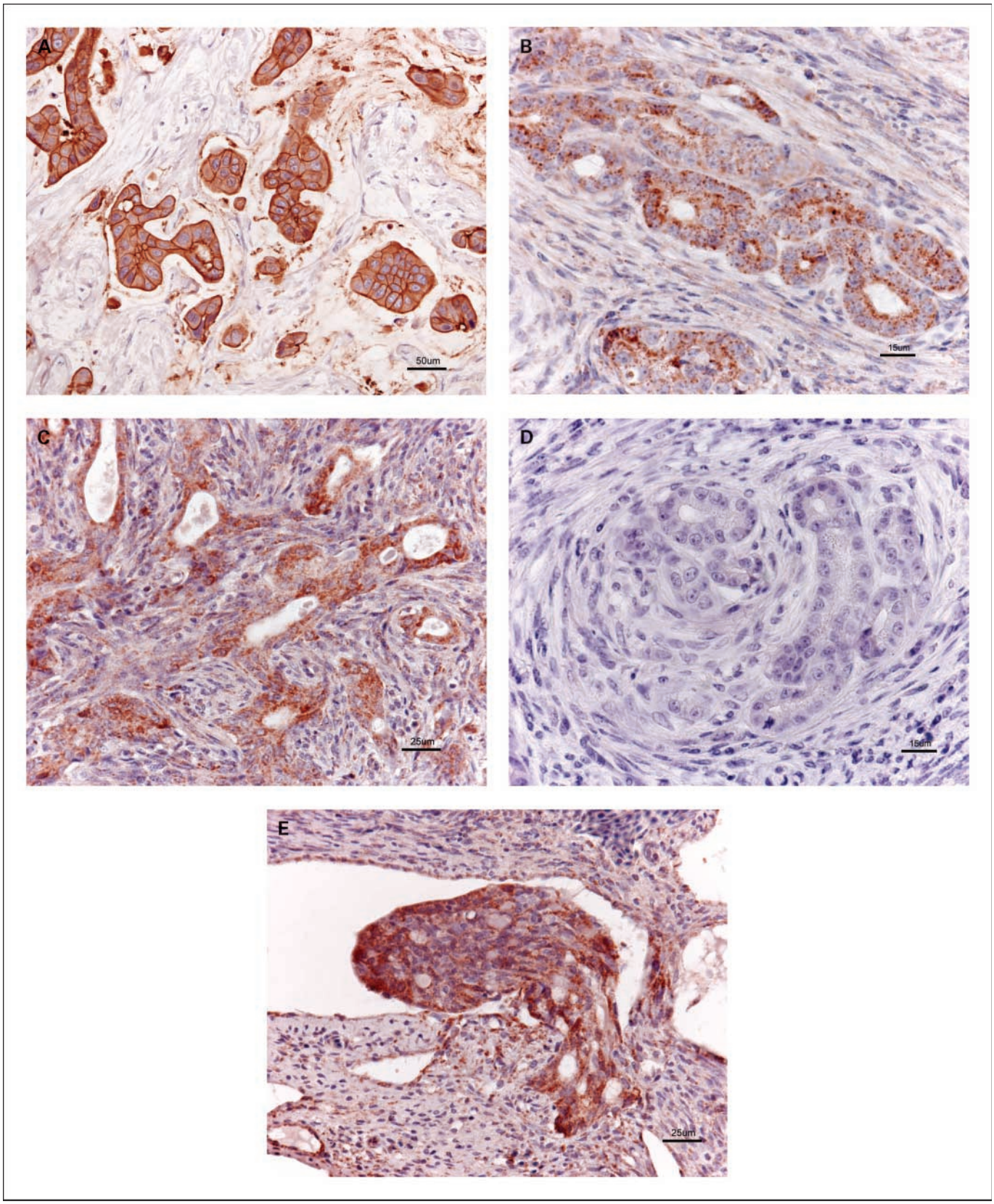

Fig. 1. *A,* adenocarcinoma, human, breast, HER-2/neu, positive control. Diffuse cytoplasmic and intense cell membrane staining. *B,* adenocarcinoma, ovary, chicken, HER-2/neu. Punctate staining in cytoplasm especially apically; nucleus and cell membrane were not stained. *C,* adenocarcinoma, ovary, chicken, HER-2/neu. Moderate staining of tumor cells and concomitant stromal hyperplasia. *D,* same as *C*, negative stain control. *E,* adenocarcinoma, ovary, chicken, HER-2/neu. Intense staining of cells in a tumor embolus in a perifollicular vessel consistent with a lymphatic.

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in thin-walled perifollicular vessels, interpreted as lymphatics, were usually intensely stained (Fig. 1E). Leiomyomas and leiomyocarcinomas did not stain for HER-2/neu (data not shown).

Discussion

Great progress has been made over the past two decades in the development of animal models for ovarian cancer. Each generation of animal model has had its advantages and limitations. The earliest models used a xenograft approach in which human ovarian tumor cells or tissues were grown in immunodeficient mice (17, 27, 32, 33, 36). The xenograft model preserved the complex interactions that occur between cancer cells and their microenvironment, including stromal-epithelial cell interactions, as well as influences of matrix proteins, growth factors, and angiogenesis. Thus, this model was a great advance over cell culture model systems and advanced the study of therapeutic interventions. However, an important weakness in the xenograft model was the lack of host immunity, which severely limited the ability to reliably predict the effect of noncancer immune-host influences on outcomes. In addition, tumors were introduced in the xenograft model rather than arising as primary lesions in the ovary, thus allowing the investigation of therapeutic, but not chemopreventive, interventions.

Very recently, the advent of several genetically engineered mouse models has facilitated the investigation of ovarian cancer pathogenesis and pharmacologic interventions in the context of both an intact tumor microenvironment and a host immune system (18, 19, 21, 22, 25). These genetically engineered models feature both the development of ovarian cancers *in situ* in the ovary and a metastatic pattern similar to human ovarian cancer. In addition, some of these models have one or more oncogene or tumor suppressor gene alterations similar to human ovarian cancer (19, 22). However, even these models have features that significantly limit their usefulness for studying human ovarian carcinogenesis. Importantly, genomic alterations occur in the germ line and, thus, may confer abnormal influences during embryonic development, in contrast to human ovarian cancer, which occurs spontaneously in the mature ovary. An additional limitation is the induction of ovarian tumors by genomic alterations that occur infrequently in human ovarian cancer (18, 21). Thus, some of these models may have utility for studying interventions directed against specific ovarian cancer genotypes, but may not be useful for studying the natural evolution of the disease as it occurs in humans.

For an ovarian cancer animal model to yield insights that are likely to have a meaningful effect on the prevention or treatment of women with the disease, it is important that the model recapitulates as many aspects of human ovarian cancer as possible. Ideally, ovarian cancers in the model should be adenocarcinomas that arise in the ovarian surface epithelium and have an i.p. spread pattern similar to human ovarian cancer. Preferably, the neoplastic process should arise in the mature ovary and not be subject to abnormal influences present during embryologic development. To account for the effect of the host on tumor growth and response to therapy, the animal should have intact immunity. In addition, the tumors that develop should have a genetic profile similar to human ovarian cancer. Finally, for the purpose of chemoprevention research, it is ideal that the tumors have a long latent phase.

The chicken ovarian cancer animal model poses an attractive alternative to genetically engineered mouse models and has features that make it ideal for ovarian chemoprevention research. Ovarian tumors occur with high frequency spontaneously in the absence of any manipulation. Thus, the developing ovary in the chicken is not subject to the abnormal influences associated with tissue-specific promoter-driven oncogenes. In addition, the chicken model shares many of the characteristics of human ovarian cancer. Ovarian cancers arise in the adult, mature ovarian surface epithelium in animals that have intact immunity. Tumors develop after a long latent phase, making the model well suited for investigation of chemopreventive strategies. Similar to human ovarian cancer, tumor incidence is affected by the number of lifetime ovulatory events, and progestins confer chemopreventive effects (44). Finally, as we report here, a number of genetic features parallel those in human ovarian cancer, including frequent alterations in *p53* and *HER-2/neu* and infrequent alterations in *ras*.

Alterations in *p53* have been described in more than 50% of human ovarian cancers (5, 7–9). In addition, the proportion of human ovarian cancers with *p53* mutations has been shown to increase commensurate with the number of lifetime ovulatory events in women (9). Similarly, in the chicken we found alterations in *p53* in a significant proportion of cases. Overall, we measured alterations in *p53* in 48% of cases (81 tumors from 172 hens had 90 mutations). Similar to human ovarian cancers, a significantly greater proportion of chicken ovarian cancers had *p53* alterations in birds with a higher number of lifetime ovulations [tumor rate of 14% in flock A, versus 96% in flock B ($P < 0.01$); birds in flock B had approximately twice the number of lifetime ovulatory events as birds in flock A]. Thus, in chickens as in women, the process of ovulation with repeated cycles of rupture and then repair of the ovarian epithelium may increase the number of proliferative events and genetic errors in the ovarian surface epithelium, leading to more *p53* mutations.

The relative significance of the various *p53* alterations that we observed in chicken ovarian cancers is unclear, but there are similarities to the types and locations of alterations in *p53* in human ovarian cancer. Most of the *p53* mutations found here in chicken tumors were in the proline-rich and DNA binding domains of the *p53* protein (82 of 90; 91%), similar to where mutations are most frequently mapped in human ovarian cancers. For instance, Havrilesky et al. (5) examined *p53* mutations and overexpression in primary tumors from 125 human patients with advanced epithelial ovarian cancer. The majority of the mutations detected were missense mutations that were in the DNA binding domain (89%) versus the proline-rich region. Whereas all of the alterations that we measured in chickens from flock A were clustered within the DNA-binding domain of the *p53* gene, only one mutation was a missense mutation (1 of 14; 0.7%). In contrast, 71 of 76 (93%) mutations in ovarian cancers from hens in flock B were missense mutations, and all of them were before the DNA binding domain. The most common mutation resulted in a change of threonine to proline at amino acid 72 (A 214 C) and occurred at the rate of 63% (44 of 70) in the flock B tumors.

It is interesting to note that most of the flock B mutations (58 of 76; 76%) result in changing an aliphatic amino acid at position 62 or 72 (alanine and threonine, respectively) to proline

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within the proline-rich region of *p53*. Ueda et al. (45) have studied the biological significance of a single *p53* germ-line polymorphism in the development of gynecologic cancer. They conducted genotype analysis of *p53* codon 72 in a total of 354 blood samples from normal healthy women and gynecologic cancer patients, including 95 normal controls and 83 cervical, 108 endometrial, and 68 ovarian cancer patients with invasive disease. They classified *p53* codon 72 genotype into two subgroups: Arg/Arg and Arg/Pro + Pro/Pro. The Arg/Arg genotype was associated with an increased risk for the development of endometrial cancer, but there was no significant difference in genotype or allele prevalence between control subjects and cervical or ovarian cancer patients. In chickens, we found a high frequency of missense mutation at amino acid 277, but the significance of this alteration and whether it may actually represent a polymorphism similar to that described by Ueda et al. are unclear. Given that the chicken and human *p53* protein sequences are not identical, direct comparisons of particular missense mutations are not possible between the species.

H-*ras* and K-*ras* oncogenes belong to the *ras* family of oncogenes and encode for a 21-kDa protein known as p21. *Ras* genes are implicated in a wide range of human tumors, including colon, lung, breast, uterus, kidney, and stomach, and mutations in these genes are most often point mutations, which cause p21 GTPase activity to stop, and thus constitutively activate the *ras* genes (14). In human ovarian cancer, mutations in codons 12 and 13 of the H-*ras* and K-*ras* genes are hallmarks of low malignant potential tumors but are rare in invasive ovarian carcinomas (10, 12–14). Overall, in the chicken, no mutations were seen in H-*ras*, and only 2 of 172 tumors (1.1%) had K-*ras* mutations. Thus, mutations in the *ras* oncogene are rare in chicken ovarian cancers, similar to human ovarian cancers.

Finally, *HER-2/neu* overexpression has been reported in as high as 50% of human ovarian cancers and has been associated with tumors that are more aggressive (6, 46). Similarly, in the chicken, 10 of 19 (52.6%) ovarian adenocarcinomas showed significant HER-2/neu staining. In addition, significant expression of HER-2/neu was associated with the larger ovarian tumors, suggesting that it may be a marker of cancer aggressiveness in the chicken. The pattern of staining in chicken tumors was distinctly different from the human controls in that it was confined to the cytoplasm and often concentrated in the apical parts of the cell when they lined tubules. These results are similar to those found by Rodriguez-Burford et al. (43), who studied two hundred 2-year-old hens and found eight animals with gross ovarian adenocarcinomas. They also found strong staining for HER-2/neu in the cytoplasm (and in the membrane) in their adenocarcinomas. Staining in human tumors overexpressing HER-2/neu is typically membranous. The significance of this difference in staining pattern is unclear. However, it seems to be specific in that ovarian tumors are frequently positive whereas oviductal tumors are rarely positive, and the degree of signal intensity correlates with the degree of histologic anaplasia of the ovarian tumors; signal was greatest in metastasizing tumor emboli (Fig. 1E). Clearly, this deserves further study.

An unexpected finding was that only 1 of 17 oviductal adenocarcinomas had significant staining for HER-2/neu, suggesting that HER-2/neu staining may provide a means to help differentiate ovarian from oviductal cancer. Both types of tumors are common in the chicken and can, in some cases, share similar histologic features. In the past, the presence of ovalbumin in chicken ovarian carcinomas was thought to be a marker of an oviductal origin of the cancers. However, Giles et al. (47) recently reported expression of ovalbumin in chicken ovarian tumors in the absence of any oviductal involvement, indicating that ovalbumin cannot be used to distinguish between ovarian and oviductal adenocarcinomas in the chicken. Differential HER-2/neu staining of our ovarian and oviductal tumors, if validated, could be a significant tool for distinguishing between these two common cancers in the chicken.

In summary, we have shown that ovarian adenocarinomas in the chicken share alterations in *p53, ras*, and *HER-2/neu* similar to those described in ovarian cancer in women. P53 alterations are common and are associated with the number of lifetime ovulatory events. *Ras* mutations are infrequent, and increased expression of *HER-2/neu* is both common and associated with more advanced disease. Clearly, the chicken, like any other animal model, is far removed from humans, and thus suffers from this great limitation. Nonetheless, the findings from our study suggest a similar evolution of chicken and human ovarian adenocarcinomas, thereby providing support for the chicken ovarian cancer animal model. More work is needed to further validate the model and, importantly, to test whether chemopreventive interventions in the chicken can be reliably translated to humans.

Although the best use of the chicken model remains to be determined, we believe that the model is ideally suited for chemoprevention research. By 2 years of age, young birds will have had more ovulatory events than occur in one to two lifetimes in women. These young birds, which rarely have malignancy but which presumably have incurred initiating events in the ovary, can be randomized to chemopreventive interventions with the goal of determining the effect of these interventions on ovarian cancer incidence 12 to 24 months later.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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