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CYP1B1 expression in ovarian cancer in the laying hen *Gallus domesticus*

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Abstract

Objectives—Ovarian carcinoma is the most lethal gynecological malignancy. The genetic and molecular mechanisms that cause it still remain largely unknown. CYP1B1 is a cytochrome P450 enzyme that catalyzes the conversion of estrogens to genotoxic catechol estrogens which may cause DNA mutations and initiate ovarian epithelial cancer. Our objectives were to evaluate CYP1B1 expression, distribution and localization in the hen ovary and to determine if there is an increased CYP1B1 expression associated with, and possibly involved in the initiation of ovarian cancer.

Methods—Two groups of hens were used: 1. young (50 weeks of age; devoid of cancer) and 2. old (165 weeks of age; divided into two groups: age-matched normal and ovarian cancer). CYP1B1 mRNA and protein expression were analyzed in cancerous ovaries, ovaries of age-matched normal and/or young hens by quantitative real-time PCR (qRT-PCR), *in situ* hybridization (ISH) and immunohistochemistry (IHC). RNA was extracted from tissue preserved in RNAlater for qRT-PCR. Tissue frozen in liquid nitrogen was used for ISH. Tissue fixed in neutral buffered formalin was subjected to IHC.

Results—Higher expression of CYP1B1 mRNA was observed in cancerous ovaries as compared to ovaries of young and age-matched normal hens by qRT-PCR. ISH and IHC confirmed that the expression of CYP1B1 was much higher in ovarian tumors compared to ovaries of age-matched normal hens. CYP1B1 mRNA and protein were distributed extensively throughout the carcinoma, while primarily localized to the granulosa layer surrounding the follicle in age-matched normal hens. IHC also showed nuclear localization of CYP1B1. Highly expressed CYP1B1 was found in POF-3 from young and age-matched normal hens as compared to POF-1 and POF-2 by qRT-PCR. No significant difference was found in the expression of CYP1B1 between the distal (site of rupture) and the proximal (site of attachment to the ovary) of POF-1 from young and age-matched normal hens.

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Conflict of interest statement

The authors have no conflicts of interest to declare.

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Conclusions—High expression of CYP1B1 in the hen ovary is associated with ovarian cancer and our data suggest that CYP1B1 may play an important role in the initiation of ovarian cancer and may prove to be a target for intervention. Moreover, the results of this study further confirm that the laying hen is a good model to study human ovarian cancer.

Keywords

Ovarian epithelial cancer; Estrogen metabolism; Chicken model; Postovulatory follicle; Ovarian surface epithelium; Cytochrome P450

Introduction

Ovarian carcinoma is the fifth leading cause of cancer death in women and the most lethal of gynecological malignancies [1]. The high mortality rate is due to the late stage of detection when approximately 75% of ovarian cancers are diagnosed. More than 90% of human ovarian cancers are epithelial in origin and are thought to arise from invaginations of the specialized layer of tissue that covers the ovary [2]. The genetic and molecular mechanisms that cause ovarian cancer still remain largely unknown. The lack of a reliable and specific method for early detection results in clinical presentation at later stages when treatment is less effective [3]. Research into the cause of ovarian cancer has been hampered by lack of suitable animal models. Ovarian cancer is rarely observed in most species with the notable exception of the domestic hen, which, like humans, spontaneously develops ovarian cancer.

CYP1B1 (Cytochrome P450, family 1, subfamily B, polypeptide 1) encodes a monooxygenase capable of metabolizing both xenobiotics, such as polycyclic aromatic hydrocarbons [4] and endogenous compounds, such as estrogen and testosterone [5]. CYP1B1 is a unique member of the cytochrome P450 family because of its dual modes of regulation. It can be regulated by polycyclic aromatic hydrocarbons via the aryl hydrocarbon receptor (AhR) and AhR nuclear translocator (ARNT) typical of other xenobiotic metabolizing isoforms; and CYP1B1 is also regulated by hormones acting via membrane receptors coupled to cAMP production or by steroid hormones acting via their nuclear receptors. Mutations in this gene have been associated with primary congenital glaucoma [6]. CYP1B1 is mostly expressed in estrogen target tissues including the mammary, ovary and uterus and specifically catalyzes the hydroxylation of estradiol to 4-hydroxy estradiol (4-OHE₂) [7]. 4-OHE₂ is a highly reactive catechol estrogen which is further oxidized to estrogen-3, 4-semiquinone and quinone that can react with DNA to form depurinating adducts. These adducts are released from DNA to generate apurinic sites. Error-prone base excision repair of this damage may lead to DNA mutations and may possibly result in carcinogenesis [5,8–10]. Higher expression of CYP1B1 has been observed in several types of cancer, including ovary, breast, colon and lung [11–17]. CYP1B1 expression in various cancers and its role in the metabolic activation of endogenous pro-carcinogens implicate it as one possible cause of these cancers.

There are several proposed hypotheses about the etiology of ovarian cancer. The “incessant ovulation hypothesis” was first proposed by Fathalla in 1971 [18]. In support of this hypothesis, multiparity, duration of lactation and use of birth control pills all decrease the risk of epithelial ovarian cancer (EOC). Other hypotheses concerning the origin of ovarian cancer have been proposed, as reviewed by Landen et al. [19]. The hen model strongly supports the incessant ovulation hypothesis because domestic hens ovulate almost daily and frequently develop ovarian cancer. By the time a hen has completed two years of egg laying, she has ovulated about as many times as a woman approaching menopause. The incidence of ovarian cancer in hens rises dramatically with age, with a 4% incidence at 2 years of age, increasing to as high as 40% by 6 years [20]. After each ovulation, the rapid repair of the ovarian surface epithelium (OSE) may cause the formation of inclusion cysts where the surface cells become trapped.

Estrogen concentrations are high in the ovary which may provide stimuli for proliferation of the OSE via estrogen receptor mediated actions. In addition, estradiol may also be a substrate for CYP1B1 which could convert it to form genotoxic intermediates. These intermediates may cause mutations of trapped OSE cells and possibly lead to the initiation of cancer.

CYP1B1 expression in the hen ovary has not been examined. The objectives of this investigation were to characterize the expression, distribution and localization of CYP1B1 in the ovaries of cancerous, age-matched normal and young hens, and to determine if CYP1B1 expression and distribution is correlated with, and possibly involved in the initiation of ovarian cancer.

Materials and methods

Animals and tissue collection

Single-comb White Leghorn hens, 50 weeks (young) and 165 weeks (old-cancer or age-matched normal), were used for sample collection. Hens were maintained three per cage, provided with feed and water ad libitum and exposed to a photoperiod of 17 h light:7 h dark, with lights on at 05:00 h and lights off at 22:00 h. Oviposition was monitored daily. Animal management and procedures were reviewed and approved by the Institutional Animal Care and Use Committees at the University of Illinois at Urbana-Champaign and University of Illinois at Chicago.

Hens were euthanized by CO₂ asphyxiation. Ovaries were immediately removed and small yellow follicles (6–8 mm) and preovulatory follicles (F1–F5, 9–35 mm) were separated from ovaries of the young and age-matched normal hens (see Fig. 1). Chickens with ovarian cancer rarely have small yellow follicles and preovulatory follicles. The ovarian tissue consisting of the stroma, cortical (<1 mm), small and large white follicles (1–5 mm), was cut into smaller portions and divided for three analyses: 1) snap frozen and stored at –80 °C for biochemical analysis; 2) placed into RNAlater (Applied Biosystems, CA) and stored at 4 °C for RNA isolation; and 3) fixed in neutral-buffered formalin (NBF) (Sigma-Aldrich, MO) for routine histological and immunohistochemical analysis. The three postovulatory follicles (POF-1, POF-2 and POF-3) were removed from ovaries of young and age-matched normal hens. The cancerous ovary lacks POFs. To compare the difference between the site of follicular rupture (distal) and the point of follicular attachment (proximal), the POF-1 was cut in half into distal and proximal pieces. All POFs were placed into RNAlater for RNA isolation.

Histology and immunohistochemistry

Ovarian tissues fixed in NBF solution were processed and paraffin embedded. 5 µm sections were cut and mounted on SuperFrost Plus microscope slides (Fisher Scientific, IL). Slides were deparaffinized and rehydrated through xylene and graded ethanol solutions (Fisher Scientific). Hematoxylin and eosin (H&E) staining was done as described [21].

Anti-human CYP1B1 polyclonal antibody used in IHC was a generous gift from Dr. Craig Marcus (Oregon State University). CYP1B1 between human and chicken shows about 70% identity at both the nucleotide and protein levels. A 15-mer peptide of 14 amino acids spanning positions 301–314 of the deduced amino acid sequence of human CYP1B1 plus an additional carboxy terminal cysteine for conjugation was synthesized and purified by HPLC for preparation of rabbit antiserum [22]. Immunohistochemistry was performed by using the Vectastain Elite ABC kit (Vector Laboratories, CA). Antigen retrieval was achieved using Antigen Unmasking Solution (Vector Laboratories) and heated under pressure at 20 psi for 5 min in a Decloaking Chamber electric pressure cooker (Biocare Medical, CA). Slides were cooled and quenched in 0.3% H₂O₂ (Sigma-Aldrich) in methanol for 15 min. Slides were

blocked with normal serum, and incubated in anti-human CYP1B1 polyclonal antibody at a dilution of 1:1000 overnight at 4 °C. After rinsing in Tris-buffered saline, sections were incubated with anti-rabbit secondary antibody and avidin–biotin complex (Vector Laboratories). Specific binding was visualized using diaminobenzidine (DAB) (Sigma-Aldrich) in the presence of H₂O₂ and sections were counter-stained with Gills hematoxylin (Sigma-Aldrich), mounted with Histomount (Fisher Scientific), examined on a Nikon ECLIPSE E400 microscope and were documented using SPOT Advanced version 4.0.1 software (Diagnostic Instruments, MI).

IHC staining intensity was analyzed using the computer assisted image analysis method (CAIA) with Adobe Photoshop 7.0 and with NIH ImageJ [23].

RNA extraction and analysis

Total RNA was extracted from POF1–3 and ovaries using Trizol reagent (Invitrogen, CA) and was quantified by determination of absorbance at A₂₆₀. RNA samples were then treated with RQ1 RNase-free DNase (Promega, WI) prior to reverse transcription reaction. Synthesis of single-stranded cDNA was performed using the high capacity cDNA Archive Kit (Applied Biosystems) and cDNA was quantified by QuantiT fluorescent reagent (Invitrogen). Equal amounts of cDNA from all samples were subjected to quantitative real-time PCR.

In situ hybridization

In situ hybridization followed the protocol as previously described [24]. In brief, frozen sections (10 µm) were mounted onto poly-L-lysine-coated slides (Sigma-Aldrich) and fixed in cold 4% paraformaldehyde (Polysciences, INC) in PBS. Sections were prehybridized and then hybridized at 45 °C for 4 h in 50% formamide hybridization buffer containing ³⁵S-labeled antisense or sense cRNA probes. RNase A-resistant hybrids were detected by autoradiography. Sections were post-stained with H&E. Sections hybridized with sense probes did not exhibit positive signals and served as negative controls.

Hybridization probes

The cDNA clone for CYP1B1 (pgn1c.pk007.j13) was obtained from Delaware Biotechnology Institute (Newark, DE) and verified by sequencing. The plasmid was linearized by using NotI (Invitrogen) for the sense and KpnI (Invitrogen) for the antisense probe. For *in situ* hybridization, sense and antisense ³⁵S-labeled cRNA probes were generated using Sp6 and T7 polymerases (New England Biolabs), respectively. Probes had specific activities of 2×10⁹ dpm/µg.

Quantitative real-time PCR (qRT-PCR)

Chicken specific primers were designed to recognize target genes using Primer Express software (Applied Biosystems) and obtained from Sigma-Genosys (Sigma-Aldrich). The qRT-PCR primer pairs were designed to span an intron. CYP1B1 (XM_419515) forward primer: 5' GCATCCCAAAGCAAAATCCC 3'; reverse primer: 5' CGGCAGGTTCCAGTAGAATGA 3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (NM_204305) was used as internal control gene. GAPDH forward primer: 5' GATGGGTGTCAACCATGAGAAA 3'; reverse primer: 5' CAATGCCAAAGTTGTCATGGA 3'. Plasmid standards for CYP1B1 and GAPDH were used for absolute quantification. To clone the GAPDH plasmid, total RNA was extracted from hen ovarian tissue, pooled, and reversed transcribed into cDNA with the Reverse Transcription System Kit (Promega). The GAPDH cDNA fragments were amplified by Taq DNA Polymerase (Qiagen, CA) and primer sequences used were: forward: 5' GCAGATGCAGGTGCTGAGTATG 3'; reverse: 5' GGCAGGTCAGGTCAACAACAGA 3'. The GAPDH fragments were then cloned by using a TOPO TA cloning Kit (Invitrogen) and

verified by sequencing. The corresponding copy numbers of plasmids were calculated based on the formula that 1 µg of 1000 bp of DNA=9.1×10¹¹ molecules. qRT-PCR was conducted by amplifying cDNA with SYBR Green Master Mix (Applied Biosystems) on ABI 7900HT using a 384 well plate and analyzed with ABI Prism software. Control reactions lacking the template were run for each gene. Reactions were 10 µl in total volume and 200 nM of each primer was applied. The plasmid standards and cDNA were simultaneously assayed in duplicate reactions. The amplification conditions were as follows: 50 °C 2 min, 95 °C 10 min, then 40 cycles of: 95 °C 15 s, 60 °C 1 min.

Statistical analysis

Statistical analysis was performed with GraphPad InStat by using one-way ANOVA with Student–Newman–Keuls comparison. A value of $p<0.05$ was considered significant whereas a value of $p<0.01$ was considered as highly significant.

Results

Immunohistochemistry

H&E staining shows an ovary from an age-matched normal hen with a cortical follicle containing an oocyte surrounded by prominent granulosa and theca cell layers (Fig. 2A). Fig. 2B shows H&E staining of a poorly differentiated ovarian tumor with endometrioid-like glands from an age-matched hen with ovarian cancer. Expression of CYP11B1 protein in cancerous ovaries and in ovaries of age-matched normal hens was examined by IHC. Higher expression of CYP11B1 protein was observed in cancerous ovaries (Fig. 2D) as compared to ovaries from age-matched normal hens (Fig. 2C). Computer assisted image analysis method (CAIA) provided semi-quantitative data for IHC. Much higher pixel area was obtained (286.31±13.08) in cancer as compared to normal ovaries (162.86±9.16) ($p<0.0001$), in good agreement with subjective analysis. In the age-matched normal hen ovaries, CYP11B1 was localized primarily to the granulosa cell layer surrounding the follicle, with some staining also in the theca layer and cortical stroma. Extensive CYP11B1 protein expression was found throughout the ovarian carcinoma. IHC also showed nuclear localization of CYP11B1 (Figs. 2E, F).

In situ hybridization

Expression of CYP11B1 mRNA in ovaries of cancerous and age-matched normal hens was analyzed by ISH. Figs. 3A, B showed the H&E staining of the hybridized sections. The expression of CYP11B1 was considerably higher in cancerous ovarian tissue (Fig. 3D) as compared to the ovary of age-matched normal hens (Fig. 3C). CYP11B1 was localized to the granulosa cell layer surrounding the follicle in the ovary of age-matched normal hens (Fig. 3C), whereas extensive CYP11B1 mRNA expression was observed throughout the entire carcinoma tissue (Fig. 3D). No signal above background was observed when sections were hybridized to sense probe (Figs. 3E, F).

qRT-PCR

CYP11B1 mRNA expression in cancerous ovaries, ovaries of age-matched normal and young hens was compared by qRT-PCR. There was a significant ($p<0.05$) increase of CYP11B1 mRNA in cancerous ovaries as compared to ovaries of age-matched normal and young hens after normalization to GAPDH expression. No significant difference of CYP11B1 mRNA expression was found between ovaries from age-matched normal and young hens (Fig. 4).

CYP11B1 mRNA in POFs from age-matched normal and young hens was analyzed by qRT-PCR. There was significantly higher expression of CYP11B1 mRNA in POF-3 as compared to POF-1 and POF-2 from both young hens (Fig. 5A) and age-matched normal hens (Fig. 5B).

No significant difference was detected in CYP1B1 expression between POF-1 proximal and distal parts from both young and age-matched normal hens. There were also no significant differences in CYP1B1 expression in the POFs of young hens as compared to their counterparts in the age-matched normal hens.

Discussion

We are reporting that CYP1B1 mRNA and protein are highly expressed in cancerous ovaries compared to ovaries of age-matched normal and/or young hens. qRT-PCR analysis indicated that CYP1B1 mRNA was elevated in the hen ovarian tumor. This observation is in agreement with the ISH and IHC. CYP1B1 mRNA and protein were localized primarily to the granulosa cell layer in normal hen ovaries, whereas CYP1B1 mRNA and protein were distributed throughout the entire cancerous ovarian tissue. CYP1B1 protein appeared to be nuclear, as was reported in the human ovary [25].

We have chosen to measure the expression of CYP1B1 in normal and cancerous ovarian tissue because of its possible role in cancer. This enzyme converts 17 β -estradiol (E2) to 4-OHE2 which is further oxidized to the catechol estrogen-3, 4-quinones (CE-3, 4-Q) which can react with DNA to form depurinating adducts that may cause mutations and initiate cancer. The observation that CYP1B1 was highly expressed in cancerous ovaries agrees with previous studies [11–17]. The hen's ovarian stromal tissue with its cortical and small follicles is a rich estrogenic environment thus providing sufficient substrate for CYP1B1. The majority of estradiol produced by the hen's ovary is produced by these small follicles and not the larger preovulatory follicles [26].

We also observed that POF-3 had significantly greater expression of CYP1B1 mRNA compared to POF-1 and POF-2, the more recent postovulatory follicles. The measurement of CYP1B1 in the POFs is particularly meaningful and may provide support for the incessant ovulation theory. POFs are the sites where ovulation had occurred 1 day (POF-1), 2 days (POF-2) or 3 days (POF-3) earlier. If the incessant ovulation theory is correct, one would expect to find oxidative damage in the POF and more significant damage in the older POFs. These POFs become apoptotic and are incorporated into the ovarian stroma which could eventually be the site where ovarian cancer is initiated. Interestingly, we found that CYP1B1 mRNA was highly expressed in the POF-3 from young and age-matched normal hens which could be an important phenomenon that may help to understand the origin of ovarian cancer.

We compared the expression of CYP1B1 mRNA between the distal (site of ovulation) and proximal parts of the POF-1 from both young and age-matched normal hens. Murdoch et al. have reported more oxidative damage in the distal surface of POF-1 and POF-2 as compared to the proximal part [27,28]. We, however, found no difference in the expression of CYP1B1 between the distal and proximal parts of the POFs-1 from both young and age-matched normal hens. The hen ovary differs morphologically from the human ovary but has the same basic cellular components, steroidogenic pathways, and gonadotropic support as present in the human ovary. The hen ovary has three POFs and four to six preovulatory follicles which are arranged in a distinct hierarchy based on size [29] (see Fig. 1). The pre- and postovulatory follicles consist of a granulosa layer and a theca layer (interna and externa) surrounded by OSE. The POFs are important endocrine tissues in the hen ovary. They produce prostaglandins for shell egg formation and oviposition [29,30]. The hen POFs, unlike their counterpart in mammals, do not form a corpus luteum. However, they are similar to the human corpus luteum, regressing and assimilated finally into the ovary stroma. POF-3 is the last remnant of the POFs and is resorbed into the ovarian stroma. The process of ovarian surface remodeling after follicular rupture is similar in both humans and hens [20]. Remodeling of the surface epithelium after ovulation is a dynamic process and we speculate that POF-3 might become trapped during

resorption and result in the formation of the inclusion cysts. In these inclusion cysts, the high levels of CYP1B1 from POF-3, in the presence of the high concentrations of estradiol from the ovarian stroma, could provide substrate for the production of genotoxic metabolites. These metabolites could potentially cause the malignant transformation of the trapped ovarian surface epithelial cells and result in the initiation of ovarian cancer. Thus the molecular stage may be set for the development of cancer. Our data support CYP1B1's role in the initiation of ovarian cancer. Further studies are required to confirm CYP1B1's contribution to the initial events and its regulation in the hen.

The laying hen model shows marked similarities to human ovarian cancer based on histology and in the pattern of protein expression, for example, cytokeratin, PCNA [31], COX-1 and COX-2 [32,33], SELENBP1 [34] and anti-tumor antibodies [35]. The results of this study have further demonstrated that ovarian cancer in the hen has elevated CYP1B1 expression, similar to human ovarian carcinoma, thus further supporting the laying hen as an important model for the human disease. Moreover, our results show for the first time that CYP1B1 mRNA is highly expressed in POF-3 which is being incorporated into the ovarian stroma. During the dynamic process of ovarian surface remodeling, the POF-3 may invaginate to form inclusion cysts rendering the cells susceptible to subsequent malignant transformation in the presence of both high concentrations of estrogens and CYP1B1. Our findings suggest that CYP1B1 may play an important role in the initiation of ovarian cancer and may prove to be a target for intervention.

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Fig. 1. Anatomy of the hen ovary. (A) Normal ovary showing follicular hierarchy (F1–F5) and postovulatory follicles (POF1–3). (B) Normal ovary showing cortical follicles, <1 mm diameter, embedded in the ovarian stroma; hundreds of small and large white follicles, 1–5 mm diameter; 5–6 small yellow follicles, 6–8 mm diameter. The largest preovulatory follicle (F1) is the follicle that will ovulate next, followed by F2, F3 and so on.

Fig. 2. CYP11B1 H&E staining and immunohistochemistry. (A) Age-matched normal ovary stained by H&E; (B) ovarian tumor stained by H&E; (C) CYP11B1 staining in age-matched normal ovary; (D) CYP11B1 staining in ovarian tumor (insert; non-immune IgG, 20X); (E) nuclear-located CYP11B1 in age-matched old normal ovary; (F) nuclear-located CYP11B1 in ovarian tumor. Bar for A, B, C and D: 100 μ m; bar for E and F: 50 μ m.

Fig. 3.

In situ hybridization of CYP1B1 in ovaries of age-matched normal hens and ovaries from hens with ovarian cancer. A–B: Stained with H&E; C–D: sections of ovaries of age-matched normal hens and ovaries from hens with ovarian cancer hybridized with antisense CYP1B1 probe; E–F: sections of ovaries of age-matched normal hens and ovaries from hens with ovarian cancer hybridized with sense CYP1B1 probe. Bar–400 μ m.

Fig. 4. CYP1B1 mRNA expression in ovarian cancer (165 weeks, $n=17$), age-matched normal hens (165 months, $n=10$) and young hens (50 weeks, $n=6$). Asterisks indicate a significant increase of CYP1B1 in ovaries with tumors as compared to ovaries from age-matched normal and young ($p < 0.05$). Bars indicate standard error.

Fig. 5.

CYP11B1 mRNA expression in POF1–3 from age-matched normal and young hens. (A) POFs from young hens: POF-1 distal ($n=7$), POF-1 proximal ($n=6$), POF-2 ($n=6$), POF-3 ($n=6$); (B) POFs from age-matched normal hen: POF-1 distal ($n=7$), POF-1 proximal ($n=6$), POF-2 ($n=6$) and POF-3 ($n=6$). a vs. b, $p<0.05$; a vs. c, $p<0.01$. Bars indicate standard error.