Published in final edited form as:

Gynecol Oncol. 2008 April ; 109(1): 115–121.

Selenium-Binding Protein 1 expression in ovaries and ovarian tumors of in the laying hen, a spontaneous model of human ovarian cancer

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Abstract

Objective—Reduced Selenium-Binding Protein 1 (SELENBP1) expression was recently shown in multiple cancers. There is little information on the expression and function of SELENBP1 in cancer progression. In order to develop a better understanding of the role of SELENBP1 in ovarian cancer, our objective was to determine if SELENBP1 is expressed in the normal ovaries and ovarian tumors in the egg-laying hen, a spontaneous model of human ovarian cancer.

Methods—SPB1 mRNA expression in normal ovary (n=20) and ovarian tumors (n=23) was evaluated by RT-PCR. Relative levels of mRNA were compared by quantitative RT-PCR (qRT-PCR) in selected samples. SELENBP1 protein expression was evaluated by 1D Western Blot and immunohistochemistry with a commercial anti-human SELENBP1 antibody.

Results—SELENBP1 mRNA and protein was expressed in 100% of normal and ovarian tumors and qRT-PCR confirmed decreased mRNA expression in 80% of ovarian tumors. SELENBP1 was primarily localized in surface epithelial cells of normal ovaries. In ovaries containing early tumor lesions, SELENBP1 expression was reduced in the surface epithelium near the tumor and was expressed in tumor cells, while more distant regions with normal histology retained SELENBP1 expression in the surface epithelium.

Conclusions—We have shown for the first time that SELENBP1 is expressed in both normal ovaries and ovarian tumors in the hen and that SELENBP1 expression is altered in the vicinity of the tumor. Furthermore, SELENBP1 expression in normal ovarian surface epithelium and in ovarian tumors parallels that previously reported for ovarian cancer in women.

Keywords

Selenium-Binding Protein 1; Ovarian Cancer; Chickens; Animal Model

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Introduction

Selenium is an essential micronutrient known for its role in the alleviation or prevention of inflammatory and autoimmune diseases, infertility, immunodeficiency diseases (HIV), thyroid function, cardiovascular disease and neurological diseases [1–5]. Because selenium inhibits cellular proliferation and promotes apoptosis of prostate, breast, endometrial, and lung cancer cells directly, it also plays a significant role in the reduction or prevention of multiple cancers [6–8]. The importance of the anti-cancer action of selenium has been well documented in epidemiological studies which have shown a correlation between high cancer rates and low dietary selenium intake [9]. Clinical trial results for the preventative role of selenium in prostate cancer are particularly strong [10–13].

Selenium uptake and distribution in mammals involves a variety of mechanisms and biochemical pathways [14]. In cancer progression, attention has primarily focused on glutathione peroxidase, a selenoprotein known to mediate oxidative stress in cancer cells [3, 6]. There is relatively little information on Selenium-Binding Protein 1 (SELENBP1) which was originally identified from mouse liver [15] and subsequently cloned from human liver [16]. Interestingly, SELENBP1 may have an important role in cancer because significantly decreased SELENBP1 mRNA expression was observed in ovarian cancer [17] as well as colorectal [18], prostate [19], lung [20], gastrointestinal [21] and papillary thyroid cancer [22] by proteomic and differential array analysis. Decreased expression of SELENBP1 was associated with poorer survival of patients diagnosed with poorly differentiated lung tumors [20]. In addition, a paired comparison of colorectal adenoma and carcinoma tissues revealed decreased SELENBP1 levels in 87.5% of carcinomas versus 12.5% of adenomas, providing additional support for the potential role of SELENBP1 in malignant transformation and cancer progression [18]. This suggests that loss of SELENBP1 has a significant impact on the ability of selenium to control tumor cell growth. However, there is little information on expression and regulation of SELENBP1 during early tumor stages.

In ovarian cancer, decreased SELENBP1 expression was observed in 87% of borderline and invasive tumors [17]. A greater decrease in SELENBP1 expression was associated with better survival unlike other cancers in which a greater decrease was associated with poorer survival. Thus, the role of decreased SELENBP1 in ovarian cancer prognosis is not clear. Ovarian cancer remains the most lethal gynecologic malignancy for women and it represents 2.5% total cancer deaths in the United States [23–26]. Identifying altered SELENBP1 expression may be significant for the treatment of ovarian cancer since selenium has been shown to have an important role in the reduction and prevention of other cancers.

Currently, diagnosis of ovarian cancer primarily occurs at advanced stages, making it difficult to study progressive events involved in ovarian cancer in women. Animal models have historically complemented the discovery of disease etiology and progression by making it possible to examine events that are difficult to study in humans [27–29]. The egg-laying hen is the only spontaneous model of ovarian cancer. Ovarian cancer in the hen has significant similarities to human ovarian cancer. The incidence of ovarian cancer in hens is strain and flock dependent and in general the incidence of ovarian cancer is high (up to 40% by age 6) which contributes to its utility as a model [30]. Also, the incidence of ovarian cancer in hens is age dependent [30], similar to ovarian cancer in women [25]. Hormone cycles, hormone regulation, and ovulation are also similar in women and hens. Hens ovulate about 250 eggs a year [31], which is the equivalent of 20 years of ovulation for a woman. An accepted epidemiologic risk factors for human ovarian cancer is the number of life-time ovulations, because reduced lifetime ovulation -- due to pregnancy and birth control pills (elevated progesterone) -- reduces ovarian cancer risk [32–35]. Likewise, inhibition of ovulation with progesterone partly reduces ovarian cancer incidence in hens [36].

The morphologic, histological and molecular features of hen ovarian tumors are remarkably similar to human ovarian cancer. Epithelial tumors represent 60% of human ovarian tumors, 90% of which are malignant [33,37]. In the hen, most tumors are malignant and have epithelial cell histology [30,38–40]. In addition, the common histological subtypes of tumors that are seen in humans, such as, serous, endometrioid, clear cell and mucinous, are represented in hen tumors [41]. Hen ovarian tumors express molecular markers that are expressed in human ovarian tumors [42] such as cytokeratin AE1/AE3, pan cytokeratin, TGF- α (growth factor), EGF-R (growth factor receptor) erbB-2 (proto-oncogene), Lewis Y, CEA and Tag 72 (oncofetal tumor markers), PCNA (proliferation marker), p27 (cell cycle inhibitor) [42] and the well known CA-125 [43]. In addition, we showed for the first time that hens with ovarian cancer have anti-tumor antibodies [44,45], similar to humans.

Ovarian adenocarcinomas that bear a striking histological resemblance to human ovarian cancer develop spontaneously in the egg-laying hen [29,30]. In addition, proteins commonly expressed in human ovarian cancer have been shown to occur in the laying hen using antibodies to human proteins [42]. In order to study the changes in SELENBP1 expression during tumor development, our objective was to determine if SELENBP1 is expressed in the normal ovary and ovarian tumors of the laying hen. This would permit subsequent studies of the role of SELENBP1 in ovarian tumor progression and its regulation by selenium.

Materials and Methods

Animals

Commercial strains of White Leghorn laying hens (n=43, 2.5 to 3 years old) were housed at the Poultry Research Farm of the University of Illinois at Urbana-Champaign and kept under a controlled light regimen (14h light:10h dark) with food and water provided *ad libitum*. Egg production and mortality records were maintained on a daily basis. Hens with normal ovarian morphology and histology had ≥ 5 eggs per clutch, while those with ovarian tumors had ≤ 2 eggs per clutch. Hens were euthanized according to an approved Institutional Animal Care and Use Committee (IACUC) protocol and the presence of tumors was detected by gross morphology and histology.

Tissue

Normal ovaries (n=23) and ovarian tumors (n=20) were collected at the time of euthanasia and portions snap frozen and stored at -80°C for biochemical analysis or fixed for histological 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 HCl, 5mM MgSO_4 , 0.25 M sucrose) containing 1 $\mu\text{l/ml}$ protease inhibitor cocktail (Sigma, St. Louis, MO), pH 7.4. The homogenate was centrifuged (1,000xg, 10 minutes) and the supernatant was collected. The protein content of the extract was measured with a Bradford protein assay kit (BioRad, Hercules, CA) with bovine serum albumin as a standard.

For RNA isolation, tissues were homogenized in Trizol reagent (1ml Trizol/100mg tissue, Invitrogen) and phase separation by adding 0.2 ml chloroform/ml of trizol used. The homogenate was centrifuged (10,000xg, 15 minutes, 4°C) and the aqueous, RNA-containing phase was collected. RNA was precipitated from the aqueous phase with isopropanol (0.5ml/1ml Trizol; 10 minutes, 22°C). Samples were centrifuged (10,000xg, 10 minutes, 4°C) and the supernatant was removed. The remaining RNA pellet was washed with 75% ethanol (1ml/1ml Trizol), centrifuged (7,500xg, 5 minutes, 4°C) and allowed to air dry. The RNA pellet was dissolved in 250 μL of diethylpyrocarbonate (DEPC) treated water and stored at -80°C . The RNA-containing solution was diluted (1:100) in HPLC grade water and concentrations as well as RNA quality were measured.

Western Blot

Thirty micrograms of total protein lysate was loaded per lane onto a 10% Tris-HCL gradient gel (Bio-Rad Laboratories, Hercules, CA). Proteins were resolved by one-dimensional SDS PAGE and transferred to a nitrocellulose membrane. Membranes were blocked (16 hours, 4°C) in Starting Block in Tris-Buffered Saline (TBS), Pierce Biotechnology, Rockford, IL) containing 0.05% Tween. Blots were washed with TBST (TBS containing 0.05% Tween-20). The membranes were incubated (1 hour, 22°C) in mouse anti-human SELENBP1 antibody (1:1000, purified IgG clone 4D4, MBL, Nagoya, Japan) and washed in TBST prior to incubation (1 hour, 22°C) with horseradish peroxidase-conjugated secondary antibody (1:1,000, goat anti-mouse IgG HRP, Pierce Biotechnology, Rockford, IL). Antibody reactions were visualized with an enhanced chemiluminescence reagent (Pierce Biotechnology, Rockford, IL) and images were acquired using a Chemidoc XRS (Bio-Rad Laboratories, Hercules, CA).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

First strand synthesis was completed according to the manufacturer's recommendations using the Superscript III first strand synthesis (Invitrogen, Carlsbad, CA). Oligoperfect Designer software (Invitrogen, www.invitrogen.com) was used to design SELENBP1 (BX935001.2) and actin (endogenous control, NM_205518.1) *Gallus gallus* primer sequences as follows: SELENBP1 Forward (5' – TGC TGC AGA AGG ATT TGT TG – 3') and Reverse (5' – CAC CAC AGT CAC AGG TCC AC – 3'); Actin Forward (5' – TGC GTG ACA TCA AGG AGA AG – 3') and Reverse (5' – ATG CCA GGG TAC ATT GTG GT – 3').

The PCR reaction was performed according to the suggested protocol for taq DNA polymerase (Invitrogen, Carlsbad, CA). The PCR reaction included one denaturation step (94°C, 3 minutes) and 35 cycles of amplification (94°C, 30 seconds; 53°C, 30 seconds; 72°C, 60 seconds; 72°C, 5 minutes). PCR products were visualized using agarose (2%) gel electrophoresis and ethidium bromide staining. SELENBP1 amplicon was observed at 312 base pairs.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Quantitative RT-PCR primers for SELENBP1 and β -actin were designed using Primer Express software (Applied BioSystems, Foster City, CA). SELENBP1 sequence (BX935001.2) was used to design the qRT-PCR primer as follows: SELENBP1 Forward (5' - GGA TGG CTC CTC CCT GAC A - 3') and Reverse (5' – TCG TCC AGC GAG ATG AGG AT - 3'). β -actin (endogenous control) sequence (NM_205518.1) was used to design the qRT-PCR primer as follows: Forward (5' – GCC CTC TTC CAG CCA TCT TT) and Reverse (5' – TGG AGT TGA AGG TAG TTT CAT GGA T - 3').

Total RNA was isolated by methods described previously. 1.0 μ g of total RNA was treated with DNaseI according to the manufacture's recommended protocol (DNaseI, catalog number EN0521, Fermentas, Hanover, MD). cDNA synthesis and first strand synthesis were completed according to the manufacturer's recommendations using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA).

To perform a PCR assay, SYBR Green PCR Master Mix (Applied BioSystems, Foster City, CA), primers, template, and nuclease-free water (Applied Biosystems, Foster City, CA) were added to a reaction mixture (50 μ l final volume) according to the manufacturer's instructions. Primers were added to a final concentration of 200nM. From each 50 μ l reaction mixture two 25 μ l aliquots were drawn, each containing the cDNA template from 25ng of total RNA, and placed into wells in a 96-well PCR reaction plate. For negative control reactions (minus template), nuclease-free water was substituted for solutions of template DNA. PCR assays were performed on a ABI 7500 System (Applied BioSystems, Foster City, CA)

Relative levels of SELENBP1 mRNA were calculated using the Relative Quantitation (RQ $\Delta\Delta C_t$) method. In order to use the RQ $\Delta\Delta C_t$ method, both the target gene and endogenous control should have similar amplification efficiency and that was evaluated in a preliminary qRT-PCR run that produced similar slopes for SELENBP1 and β -actin (-3.07 and -3.08). The average C_t value for each SELENBP1 and β -actin sample was determined and the ΔC_t for SELENBP1 in each sample was calculated by subtracting the average C_t value of β -actin from average C_t value of SELENBP1. The average ΔC_t value was also calculated for normal ovary and used as a calibrator. $\Delta\Delta C_t$ was determined by subtracting ΔC_t for β -actin (calibrator) from the ΔC_t for SELENBP1 (normalized target). The fold change for each tumor sample was calculated by converting the $\Delta\Delta C_t$ exponential values to linear values using the formula $2^{-\Delta\Delta C_t}$.

Histology and Immunohistochemistry

Tissues were fixed in a 10% buffered formalin, paraffin-embedded and 6 μ m tissue sections were cut, mounted on microscopic slides and incubated (2 hours, 35°C). Normal histology and ovarian tumor pathology were verified by H&E staining. In order to determine the location of SELENBP1 expression, selected normal (n=2) and tumor (n=8) tissues were further processed for immunohistochemistry. De-paraffinized sections were boiled (10 minutes) in an antigen unmasking solution (1:100, Vector Laboratories, Burlingame, CA) and incubated in 0.3% hydrogen peroxide-methanol (20 minutes, 22°C) to block endogenous peroxidase activity. Sections were washed in phosphate buffered saline (PBS) and blocked with normal horse serum (30 minutes, 22°C). Anti-human SELENBP1 monoclonal antibody (purified IgG Clone CD4, MBL, Nagoya, Japan) was diluted in PBS plus 1% BSA (1:250) and incubated with sections (1 hour, 22°C then 12 hours, 4°C). Control staining was performed by (1) omitting the primary antibody (replaced with PBS), or (2) by omitting the secondary antibody (replaced with PBS). Sections were washed in PBS and incubated (1 hour, 22°C) with a universal secondary antibody (pan-specific biotinylated anti-immunoglobulin, Vector Laboratories, Burlingame, CA) followed by incubation (1 hour, 22°C) with Avidin-Biotin Complex reagent (Vector Laboratories, Burlingame, CA). Reaction product was produced with diaminobenzidine substrate (R.T.U. Vectastain Kit, Vector Laboratories, Burlingame, CA). Sections were counterstained lightly in Hematoxylin.

Results

Expression of SELENBP1 in Normal Ovary and Ovarian Tumors

SELENBP1 PCR amplicon was detected at the expected size of 312bp in all normal ovaries (n=23) examined in the egg-laying hen (Figure 1). Immunoreactive SELENBP1 protein was detected at 52kD in all normal ovaries by Western blot (Figure 1). SELENBP1 amplicon and immunoreactive protein was expressed in all ovarian tumors (n=20) (Figure 2), including endometrioid, serous, mucinous and clear cell ovarian tumors.

Because mRNA density varied and because previous findings suggested that SELENBP1 expression in ovarian tumors in women is altered, we examined SELENBP1 mRNA levels by quantitative RT-PCR. Compared to normal hen ovaries, SELENBP1 mRNA was down regulated in 80% (12/15) of hen ovarian tumors (Figure 3).

Localization of SELENBP1 in Normal Ovary and Ovarian Tumors

In order to assess the location of SELENBP1 expression, SELENBP1 expression was detected by immunohistochemistry in selected normal (n=2) and tumor (n=8) ovaries. In normal ovaries, SELENBP1 was primarily expressed in ovarian surface epithelium (Figure 4). In cells of the normal surface epithelium, SELENBP1 was frequently localized in the cytoplasm adjacent to the plasma membrane region and to a lesser extent throughout the cytoplasm and nuclei.

Hen ovarian tumor histology was strikingly similar to the histology observed in ovarian tumors in women (Figure 5) and represented endometrioid, serous, clear cell, and mucinous tumor cell types. Within the tumors studied, (n=20), 6 (30%) had endometrioid, 4 (25%) had mucinous, 3 (15%) had serous, and the remaining had a mixed histology with 1 (5%) serous/mucinous, 2 (10%) serous/endometrioid and 2 were undetermined (late stage with extensive metastasis). SELENBP1 was expressed in all ovarian tumors and was down regulated in the surface epithelium of ovaries containing tumors. Moreover, in the vicinity of small focal lesions, decreased SELENBP1 expression at the nearby surface epithelium appeared to coincide with the presence of a tumor, while SELENBP1 was expressed in surface epithelium in more distant areas of the ovary (Figure 6). In contrast to cells of the normal surface epithelium, SELENBP1 in tumor cells was frequently located throughout the cytoplasm and in nuclei.

Discussion

We demonstrated for the first time that SELENBP1 mRNA and protein is expressed in both normal ovary and ovarian tumors in the hen. In addition, SELENBP1 mRNA and protein was similar in size to human SELENBP1 mRNA and protein, consistent with the nucleic acid homology of 73% and the protein homology of 76%. Furthermore, the tissue distribution of SELENBP1 in hens parallels its expression in the human ovary and ovarian tumors [17]. Likewise, hen SELENBP1 mRNA was decreased in 80% of ovarian tumors compared to the normal ovary. This is also consistent with the 87% decrease in SELENBP1 expression reported for human ovarian cancer [17].

The histology of ovarian tumors in the hen resembles the histology of human ovarian tumors. We observed expression of SELENBP1 in endometrioid, mucinous, serous, and clear cell tumors by immunohistochemistry, similar to that reported for human ovarian cancer [17]. However, the observations of human ovarian cancer by Huang *et al* [17] were based on late stage ovarian cancer in humans. The advantage of studies in the hen is that we routinely observed early stage focal lesions. At these early stages, large areas of the ovary retain normal morphology. Interestingly, reduced expression of SELENBP1 in the surface epithelium of the ovary was seen in the immediate vicinity of the focal lesions, while other more distant areas retained expression in the surface epithelium. The disappearance of SELENBP1 in the epithelial layer appeared to coincide with SELENBP1 expression the focal lesion. This is a novel result, which supports the concept that epithelial ovarian cancer arises from the surface epithelium. Furthermore, the intracellular distribution of SELENBP1 differed in tumor cells; in normal surface epithelium SELENBP1 is located in the sub-plasma membrane region of the cell while in tumor cells it appeared to be more diffusely distributed throughout the cytoplasm and the nucleus. One interpretation of this result is that the functional relationships of SELENBP1 are altered in transformed cells.

Because of its similarity to human ovarian cancer, researchers have sought to utilize the egg-laying hen to examine the expression of proteins thought to play a role in human ovarian cancer. For example, hen ovarian tumors express molecular markers that are expressed in human ovarian tumors [42] such as cytokeratin AE1/AE3, pan cytokeratin, TGF- α (growth factor), EGF-R (growth factor receptor) erbB-2 (proto-oncogene), Lewis Y, CEA and Tag 72 (oncofetal tumor markers), PCNA (proliferation marker), p27 (cell cycle inhibitor) [42], CA-125 [43] and COX-1 and COX-2 [46]. In turn, these findings have helped to validate the use of the laying hen as an emerging model of ovarian cancer. Likewise, the findings of this study of SELENBP1 support the use of the hen as a model of human ovarian cancer.

While there are significant changes in the expression of SELENBP1 in ovarian cancer in both humans and hens, the role of SELENBP1 in selenium utilization and ovarian cancer progression remains to be determined. The demonstration that changes in SELENBP1 expression in the

hen are similar to those in humans will permit studies to examine the role of selenium and SELENBP1 in ovarian cancer progression.

Acknowledgements

This work was supported in part by NIH R01 A1055060, the Daniel F. and Ada L. Rice Foundation and the Ovarian Cancer Survivor network to JL and USAMRMC OC050091 to DBH

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Figure 1. Examples of SELENBP1 mRNA and protein expression in normal hen ovaries
 A) SELENBP1 mRNA expression was identified in 100% of normal ovaries (n=23) by RT-PCR. B) SELENBP1 protein was identified in 100% of normal ovaries (n=23) by one-dimensional Western Blot. β -Actin was used as an internal control for RT-PCR and one-dimensional Western Blot. In the examples shown, lanes 1–7 represent the same tissues for SELENBP1 mRNA and protein. For mRNA, lane 8 is a positive control and lane 9 is a negative control. For protein detection, no stain occurred in the absence of primary antiserum (not shown).

Figure 2. Examples of SELENBP1 mRNA and protein expression in hen ovaries containing tumors
 A) SELENBP1 mRNA expression was identified in 100% of ovaries containing tumors (n=20) by RT-PCR. B) SELENBP1 protein expression was identified in 100% of ovaries containing tumors (n=20) by one-dimensional Western Blot. β -Actin was used as an internal control for RT-PCR and one-dimensional Western Blot. In the examples shown, lanes 1–7 represent the same tissues for SELENBP1 mRNA and protein. The tumor types are, Lane 1: endometrioid, Lane 2: endometrioid, Lane 3: endometrioid, Lane 4: serous, Lane 5: mucinous, Lane 6: clear cell histology. For mRNA, lane 7 is a positive control and lane 8 is a negative control. For protein detection, no stain occurred in the absence of primary antiserum (not shown).

Figure 3. Relative expression of SELENBP1 mRNA in ovarian tumors

The graph shows that SELENBP1 mRNA expression, evaluated by qRT-PCR, is decreased in 80% (12/15) of ovarian tumors compared to normal ovaries. Fold changes were calculated by converting the $\Delta\Delta C_t$ exponential values to linear values using the formula $2^{\Delta\Delta C_t}$ as described in the Methods.

Figure 4. Immunohistochemical localization of SELENBP1 in normal hen ovaries

Each row contains an H&E stained section (A and D) and a corresponding example of the SELENBP1 expression (B, C, E and F) at two magnifications (20X and 120X). SELENBP1 is localized primarily in surface epithelium (arrows) of normal ovaries. In cells (C) of the normal surface epithelium, SELENBP1 is frequently located adjacent to the plasma membrane and characteristically exhibits a ring-like staining pattern. Cells (F) with stained nuclei and cytoplasm are observed less often.

Figure 5. Immunohistochemical localization of SELENBP1 in hen ovarian tumors

Each row contains an H&E stained section and an example of the same tumor type at two magnifications (20X and 120X). SELENBP1 expression is reduced in ovarian surface epithelium (SE) (middle column) and is localized in tumor cells (right column) in endometrioid (top row), clear cell (second row), serous (third row) and mucinous (bottom row) tumors. SELENBP1 expression is typically observed in the cytoplasm and nuclei of tumor cells (arrows).

Figure 6. Reduced SELENBP1 expression in the surface epithelium is associated with SELENBP1 expression in tumor cells of focal lesions

(A) Reduced expression of SELENBP1 in the surface epithelium (SE) of the ovary was seen primarily in the vicinity of focal lesions (FL) while other more distant areas retained SELENBP1 expression in the normal surface epithelium (NSE). (B) The area in (A) is enlarged to show the disrupted expression of SELENBP1 in the surface epithelium (arrow) near tumor cells. (C) The area in (A) is enlarged to show tumor cell staining in cytoplasm and nuclei (arrow). The intracellular distribution of SELENBP1 in tumor cells differs from cells (see Figure 4) in normal surface epithelium.