Original Research

Paradoxical expression of *AHCYL1* affecting ovarian carcinogenesis between chickens and women

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Abstract

We investigated *S*-adenosylhomocysteine hydrolase-like protein 1 (*AHCYL1*) gene expression in human epithelial ovarian cancer (EOC) using the chicken, which is the most relevant animal model. Ovarian cancer was detected in 10 of 136 laying hens (7.4%). Results of the present study indicated that AHCYL1 mRNA and protein are most abundant in the glandular epithelium of adenocarcinoma of cancerous, but not normal, ovaries of hens. In addition, bisulfite sequencing to examine methylation patterns in the promoter region of the *AHCYL1* gene revealed that 30–38% of the three CpG sites were demethylated in ovarian cancer cells as compared with normal ovarian cells. Furthermore, in human ovarian cancer cells such as OVCAR-3, AHCYL1 protein was predominantly in the nucleus and had a similar expression pattern to that in chicken ovarian cancer cells. Thereafter, we examined the prognostic value of AHCYL1 expression in patients with EOC using multivariate linear logistic regression and Cox's proportional hazard analyses. In 109 human patients with EOC, 14 (12.8%), 41 (37.6%) and 54 (49.6%) patients showed weak, moderate and strong expression of AHCYL1 protein, respectively. However, intermediate or high expression of AHCYL1 protein was a favorable factor for overall responses (adjusted odds ratio, 7.23; 95% confidence interval [CI], 1.36–38.39), and for progression-free survival (adjusted hazard ratio, 0.20; 95% CI, 0.07–0.55). From these results, we conclude that AHCYL1 expression is associated with ovarian carcinogenesis as an oncogene in chickens, whereas it plays the role of tumor suppressor in human EOC, suggesting a paradoxical function of AHCYL1 in ovarian carcinogenesis.

Keywords: AHCYL1, ovary, cancer, prognostic factor

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Introduction

Although maximal cytoreduction and various types of chemotherapeutic agents have contributed to improve clinical outcomes in patients with epithelial ovarian cancer (EOC), advanced-stage disease, poor differentiation and chemoresistance still lead to a poor prognosis.[1] In particular, more than two-thirds of patients with EOC are diagnosed with advanced-stage disease due to lack of specific symptoms, no effective screening methods and treatment methods that fail to prolong their survival, with only 25% of patients having a five-year survival rate.[2,3] Thus, recent strategies are being investigated to overcome the limitation in terms of both targeted molecular therapy,[4] and novel biomarkers for early diagnosis and prediction of prognosis.[5]

For developing biomarkers for EOC, the laying hen is the only animal that spontaneously develops tumors from ovarian surface epithelium in contrast to the artificial nature of the induced tumors in rodent models.[6–8] Recently, we have shown that serpin peptidase inhibitor, clade B, member 3 (SERPINB3) detected during ovarian carcinogenesis in the chicken model may be a prognostic factor for patients with EOC (unpublished data). In the current study, we investigated whether *S*-adenosylhomocysteine

hydrolase-like protein 1 (AHCYL1), another protein detected in ovarian carcinogenesis of the chicken model, could serve as a novel biomarker for human EOC.

AHCYL1 consists of an N-terminal hydrophilic domain (106 amino acids) and a C-terminal domain (424 amino acids), which is homologous with 51% protein identity to the methylation pathway enzyme, AHCY.[9] It is the evolutionarily conserved and ubiquitously expressed enzyme that catalyzes the reversible hydrolysis of *S*-adenosyl-L-homocysteine, a byproduct of the *S*-adenosyl-L-homomethionine-dependent methyltransferase reaction, into adenosine and homocysteine.[10] Furthermore, AHYCL1 acts like an inositol 1,4,5-triphosphate receptor (IP_3R)-binding protein (termed IP_3R-binding protein released with inositol 1,4,5-triphosphate, or IRBIT) by competitively inhibiting the interaction of IP_3 with IP_3R.[11,12] As a result, AHCYL1 regulates IP_3-induced Ca^{2+} signaling, which plays crucial roles in numerous cellular processes including gene expression and cell death.[13]

Recently, we have shown regulation of AHCYL1 mRNA and protein expression by estrogen in a tissue and cell-specific manner in chickens (unpublished data). However, there is little known about expression or function of AHCYL1 in ovarian carcinogenesis. Thus, we compared the distribution and localization of AHCYL1 between normal and cancerous ovaries of chickens, and then AHCYL1 expression among normal and cancer cells of their ovaries, human EOC cell lines (OVCAR-3 and SKOV-3) and a human ovarian teratocarcinoma cell line (PA-1). Finally, we investigated the prognostic value of AHCYL1 expression in patients with EOC.

Materials and methods

Experimental animals and animal care

Approval for the experimental use of chickens was obtained from the Institute of Laboratory Animal Resources, Seoul National University (SNU-070823-5). White Leghorn chickens were managed according to approved standards for operation of the University Animal Farm, Seoul National University, Korea, for reproduction and embryo manipulation procedures, as well as standard operating protocols in our laboratory. All chickens had free access to feed and water for *ad libitum* consumption.

Tissue samples in the chicken model

A total of 136 mature hens (88 over 36 months and 48 over 24 months of age), which had stopped laying eggs, were euthanized for collection of cancerous ovarian tissues. We obtained cancerous ovarian tissues from 10 hens and normal ovarian tissues from 10 egg-laying hens. We evaluated the tumor stage of 10 hens with cancerous ovaries according to characteristic features of chicken ovarian cancer.[6,14] Three hens had stage III disease as the ovarian tumor cells had metastasized to the gastrointestinal (GI) tract and liver surface with profuse ascites in the abdominal cavity. Five hens had tumor cells that had spread to distant organs, including the liver parenchyma, lung, GI tract and oviduct with profuse ascites, indicating stage IV disease. Two hens had stage I disease as the tumors were limited to their ovaries. Subsets of these samples were fixed in 4% paraformaldehyde for further analyses. After 24 h, fixed tissues were changed to 70% ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus (Leica Microsystems, Wetzlar, Germany). Paraffin-embedded tissues were sectioned at 5 μm and stained with hematoxylin and eosin (Supplementary Figure 1; please see http://ebm.rsmjournals.com/lookup/suppl/doi:10.1258/ebm.2012.011433/-/DC1). Epithelial ovarian cancers in chickens were classified based on the cellular subtypes and patterns of cellular differentiation with reference to ovarian malignant tumor types in humans.[6]

RNA isolation

Total cellular RNA was isolated from frozen tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. The quantity and quality of total RNA was determined by spectrometry and denaturing agarose gel electrophoresis, respectively.

Semi-quantitative reverse transcriptase polymerase chain reaction analysis

The expression of *AHCYL1* mRNA in normal and cancerous ovaries of chickens was investigated using semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) as previously described.[15] Briefly, complementary DNA was synthesized from 2 μg total cellular RNA using random hexamer (Invitrogen) and oligo (dT) primers and AccuPower® RT Premix (Bioneer, Daejeon, Korea). After PCR, equal amounts of reaction product were analyzed using a 1% agarose gel, and PCR products were visualized using ethidium bromide staining. The amount of DNA present was quantified by measuring the intensity of light emitted from correctly sized bands under ultraviolet light using a Gel Doc™ XR+ system with Image Lab™ software (Bio-Rad, Hercules, CA, USA).

Quantitative RT-PCR analysis

Gene expression levels were measured using SYBR® Green (Sigma, St Louis, MO, USA) and a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The *GAPDH* gene was analyzed simultaneously as a control and used for normalization to account for variation in loading. Each target gene and *GAPDH* were analyzed in triplicate. ROX dye (Invitrogen) was used as a negative control for the fluorescence measurements. Sequence-specific products were identified by generating a melting curve in which the C_T value represented the cycle number at which a fluorescent signal was statistically greater than background, and relative gene expression was quantified using the $2^{-\Delta\Delta C_T}$ method.[16] For the control, the relative quantification of gene expression was normalized to the C_T value for the normal ovaries.

In situ hybridization analysis

For *in situ* hybridization probes, PCR products were generated and gel-extracted, and then cloned into the pGEM-T vector (Promega, Madison, WI, USA) as described previously.[17] After verification of the sequences, plasmids containing the correct gene sequences were amplified with T7- and SP6-specific primers, and then digoxigenin (DIG)-labeled RNA probes were transcribed using a DIG RNA labeling kit (Roche Applied Science, Indianapolis, IN, USA). After hybridization and blocking, the sections were incubated overnight with sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche Applied Science). The signal was visualized by exposure to a solution containing 0.4 mmol/L 5-bromo-4-chloro-3-indolyl phosphate, 0.4 mmol/L nitroblue tetrazolium and 2 mmol/L levamisole (Sigma).

Cell culture

Three human ovarian epithelial cancer cell lines and two chicken primary ovarian cell lines were used in this study. Human ovarian cancer cell lines (OVCAR-3, SKOV-3 and PA-1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured according to the supplier's directions. In addition, three different primary ovarian surface epithelial cell lines from three normal and cancerous chickens, respectively, were isolated and cultured as previously described,[18,19] with some modifications. The purity of these cell cultures was confirmed based on their expression of cytokeratin (epithelial cells) or vimentin (mesenchymal cells) proteins.[19]

Western blot analysis

Ovarian cancer cells and normal ovarian cells obtained from hens were rinsed with cold phosphate-buffered saline and lysed by homogenization for five minutes in ice-cold lysis buffer. Proteins were denatured, separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose. Blots were developed using enhanced chemiluminescence detection (SuperSignal West Pico, Pierce, Rockford, IL, USA) and quantified by measuring the intensity of light emitted from correctly sized bands under ultraviolet light using the ChemiDoc EQ system and Quantity One software (Bio-Rad). Immunoreactive AHCYL1 protein was detected using an anti-human AHCYL1 monoclonal antibody (catalog number: ab56761; Abcam, Cambridge, UK) at a final dilution of 1:1000. As a loading control, Western blotting with mouse anti-beta actin IgG (catalog number: sc-47778; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was performed.

Immunofluorescence microscopy for detecting AHCYL1 activation

Each type of cell was seeded onto Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY, USA). After 24 h, cells were fixed with -20°C methanol and immunofluorescence staining was performed using an anti-human AHCYL1 monoclonal antibody (Abcam). Cells were then incubated with Alexa Fluor 488 rabbit anti-mouse IgG secondary antibody (A21204; Invitrogen). Slides were overlayed with 4′,6-diamidino-2-phenylindole before images were captured using a Zeiss confocal microscope LSM710 fitted with a digital microscope camera AxioCam and Zen 2009 software (Carl Zeiss, Jena, Germany).

Prediction of transcription factor-binding *cis*-elements

The presence of transcription factor-binding *cis*-elements within the AHCYL1 promoter region was predicted using a bioinformatics tool for orthologous sequences (Transcriptional Element Search System; http://www.cbil.upenn.edu/tess.html; Computational Biology and Informatics Laboratory, School of Medicine University of Pennsylvania, Philadelphia, PA, USA).

Bisulfite sequencing

DNA samples were prepared using an AccuPrep Genomic DNA Extraction Kit (Bioneer) and converted using an Epitect Bisulfite Kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions. For amplifying the converted DNA, PCRs were performed and the PCR products were cloned into the pGEM-T easy vector (Promega) and sequenced using an ABI Prism 3730 XL DNA Analyzer (Applied Biosystems).

Human study population

Clinicopathological data were extracted from a database of patients with EOC between June 2003 and March 2009. The current study was approved by the Institutional Review of Board of Seoul National University's Bundang Hospital. The inclusion criteria were as follows: patients diagnosed with EOC; those treated with maximum cytoreductive surgery followed by adjuvant taxane- and platinum-based chemotherapy if indicated; those showing Eastern Cooperative Oncology Group performance status of 0–2; and those without underlying disease affecting prognosis.

Optimal cytoreduction was defined as a residual tumor $\leq$1 cm while suboptimal cytoreduction was defined as a residual tumor $>$1 cm in maximal diameter. All patients except those with International Federation of Gynecology and Obstetrics (FIGO) stage IA or IB with grade 1 or 2 disease received adjuvant chemotherapy using paclitaxel (175 mg/m^2)/carboplatin (area under curve 5.0) or paclitaxel (175 mg/m^2)/cisplatin (75 mg/m^2) for one or two weeks after surgery, and chemotherapy was repeated every three weeks for six cycles.

Tumor response was evaluated by the Response Evaluation Criteria in Solid Tumors and serum CA-125 concentrations using a radioimmunoassay kit (Fujirebio Diagnostics, Malvern, PA, USA).[20,21] A complete response (CR) to treatment was defined as the disappearance of all measurable disease for at least four weeks and normalization of serum CA-125 concentrations at the end of therapy. A partial response (PR) was defined as a 30% reduction in the product of the transverse diameters of each measurable

lesion for at least four weeks or a CR without normalization of serum CA-125 concentrations. Overall response (OR) was defined as the disease state with CR or PR. Progression-free survival (PFS) was defined as the time elapsed from the date of completion of primary adjuvant chemotherapy to the date of clinically proven resumption of growth of the tumor. Overall survival (OS) was calculated from the date of staging laparotomy to the date of cancer-related death or the end of the study.

Immunohistochemistry

The localization of AHCYL1 protein in normal and cancerous ovaries of hens was evaluated by immunohistochemistry (IHC) using an anti-human AHCYL1 monoclonal antibody at a final dilution of 1:500 (1 μg/mL) (Abcam). Antigen retrieval was performed using the boiling citrate method as described previously.[22] A histological section of ovarian cancer tissue was used as a positive control, whereas negative controls included normal ovarian tissue or cancerous ovarian tissue where the primary antibody was substituted with purified non-immune mouse IgG at the same final concentration.

For IHC of human ovarian cancer tissues, representative core tissue sections (2 mm in diameter) were taken from paraffin blocks and arranged in new tissue microarray (TMA) blocks using trephine apparatus (Superbiochips Laboratories, Seoul, Korea). In cases with variable histological features, the most representative area was selected for TMA construction. The IHC staining of human TMA samples was performed using similar methods for the laying hen model. Human normal ovarian tissues were used as a positive control because they showed strong expression of AHCYL1. On the other hand, we used EOC tissues treated with purified non-immune mouse IgG at the same final concentration as a negative control due to no or weak expression of AHCYL1. The results of IHC were assessed semi-quantitatively by one pathologist, who was unaware of the clinicopathological status of the patient. Since nuclear staining was detected in both normal and cancerous ovarian tissues, expression of AHCYL1 was classified as weak (1+), moderate (2+) or strong (3+) based on staining intensity in tumor cells.

Statistical analysis

For investigating AHCYL1 expression associated with ovarian carcinogenesis in the laying hen model and evaluating its prognostic value in patients with EOC, we used analysis of variance, Chi-squared and Student's *t*-tests, Kaplan–Meier method with the log-rank test, logistic regression and Cox's proportional hazard analyses to determine odds ratio (OR), hazard ratio (HR) and 95% confidence interval (CI). Statistical analyses were performed using SPSS software (Version 19.0; SPSS Inc., Chicago, IL, USA). A probability value of $P < 0.05$ was considered statistically significant.

Results

Increased expression of AHCYL1 in normal and cancerous ovaries of chicken

In previous studies, morphological and IHC analyses compared normal and cancerous ovaries from hens.[6,14,23] The morphological characteristics of cancerous ovaries included more solid shapes with surface tumor lesions and numerous small regressed follicles as compared with normal ovaries that contained several preovulatory follicles that were hierarchically arranged for ovulation. Histological analysis between normal and cancerous ovaries revealed that cancerous ovaries had mostly gland-like structures in the compact stromal cell compartment with a few small follicles, whereas normal ovaries contained many small well-developed follicles surrounded by connective tissue. These results were consistent with those reported previously.[6,24–27] Based on the results and our recent finding that AHCYL1 is involved in growth and development of the chicken oviduct via an estrogen-mediated ERK1/2 MAPK signaling pathway (unpublished data), we hypothesized that the expression pattern of the *AHCYL1* gene may differ between normal and cancerous tissues from chicken ovaries. Expression of *AHCYL1* mRNA in normal and cancerous ovaries was performed using RT-PCR analysis. *AHCYL1* mRNA expression was predominantly found in

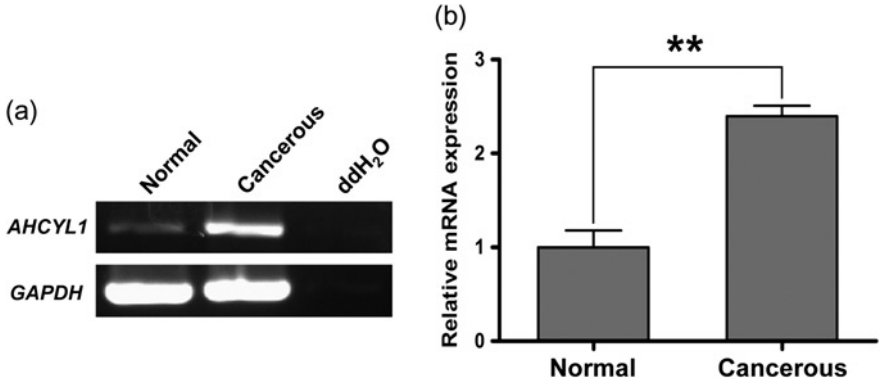

Figure 1 Expression and quantitation of *AHCYL1* mRNA in normal and cancerous ovaries from hens. (a) RT-PCR analyses were performed using mixed cDNA templates from normal ($n = 4$) and cancerous ($n = 10$) ovaries of chickens using chicken *AHCYL1* and *GAPDH*-specific primers. *AHCYL1* mRNA was predominantly found in cancerous ovaries with little or no expression in normal ovaries. (b) The quantitative PCR analysis indicated a 2.3-fold increase ($P < 0.01$) in *AHCYL1* mRNA in cancerous ovaries as compared with normal ovaries. Please see Materials and methods for a complete description of the methods. RT-PCR, reverse transcriptase polymerase chain reaction

cancerous ovaries with little or no expression in normal ovaries (Figure 1a). Quantitative RT-PCR analysis showed a 2.3-fold increase of *AHCYL1* mRNA in cancerous ovaries ($P < 0.01$), when compared with normal ovaries (Figure 1b). These results indicate that AHCYL1 may be a useful biomarker for chicken ovarian cancer as it is up-regulated during the development of ovarian cancer.

Localization of AHCYL1 mRNA and protein in normal and cancerous ovarian tissues

To examine cell-specific expression of *AHCYL1* mRNA and protein in normal and cancerous ovaries from hens, we performed *in situ* hybridization and IHC analyses. *AHCYL1* mRNA was predominantly and abundantly expressed in glandular epithelium of cancerous ovaries, but not in luminal epithelium, stroma or blood vessels (Figure 2a). Consistent with this result, IHC revealed that AHCYL1 protein was localized predominantly in glandular epithelium of cancerous ovaries, but not in any other cell types of the same tissues (Figure 2b). However, AHCYL1 protein was detectable at very low abundance around developing follicles and glandular epithelium of normal ovaries. In the negative control, mouse IgG failed to detect a signal. These results indicate that AHCYL1 is expressed specifically in the glandular epithelium of the adenocarcinoma of cancerous ovaries of hens.

Detection of AHCYL1 protein in cultured chicken ovarian carcinoma cells

To assess the quantity and intracellular localization of AHCYL1 protein in epithelia of cancerous ovaries, we performed Western blotting and immunofluorescence analyses to compare expression patterns of AHCYL1 protein between normal and cancerous ovaries from hens. As illustrated in Figure 3a, immunoreactive AHCYL1 was abundant only in cancerous ovarian cells with little or no detectable ACHYL1 in normal ovarian cells. In addition, immunofluorescence microscopy revealed that AHCYL1 protein was most abundant in nuclei of chicken ovarian cancer cells, but detectable only at a lower level in the cytoplasm of normal ovarian epithelial cells (Figure 3b). These findings and our preliminary results indicate that AHCYL1 may play an important role in an unknown signaling cascade(s) essential for ovarian carcinogenesis.

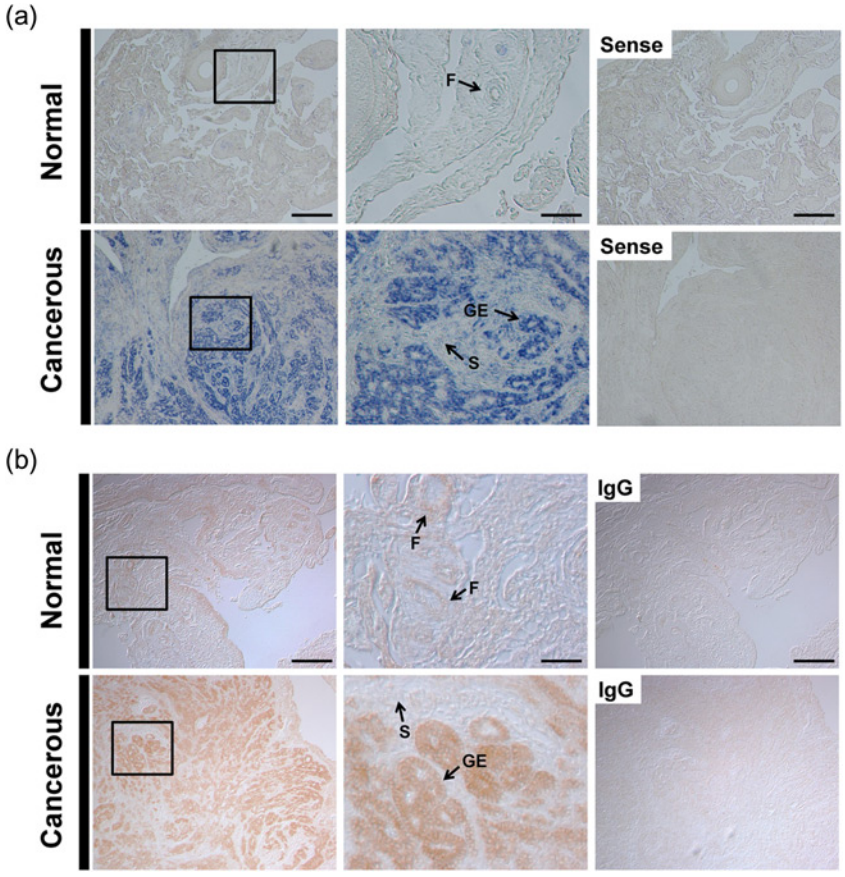

Figure 2 Expression of *AHCYL1* mRNA and protein is unique to glandular epithelium of endometrioid carcinoma (stage III) of cancerous ovaries from hens. (a) *In situ* hybridization analyses of *AHCYL1* mRNA. Cross-sections of normal and cancerous ovaries from chickens were hybridized with sense or antisense chicken *AHCYL1* cRNA probes. *AHCYL1* mRNA was predominantly and abundantly expressed in the glandular epithelium of cancerous ovaries, but not in luminal epithelium, stroma or blood vessels. (b) Immunohistochemical expression of AHCYL1 protein. Immunoreactive AHCYL1 protein was predominantly localized in glandular epithelium of cancerous ovaries, but not in any other cell types of the same tissues. However, AHCYL1 protein was detectable at very low abundance around developing follicles and glandular epithelium of normal ovaries. For a negative control, the primary antibody was substituted with purified non-immune mouse IgG. F, follicle; GE, glandular epithelium; S, stroma. Scale bar represents 200 μm (the first columnar panels and sense) or 50 μm (the second columnar panels). Please see Materials and methods for a complete description of the methods. (A color version of this figure is available in the online journal)

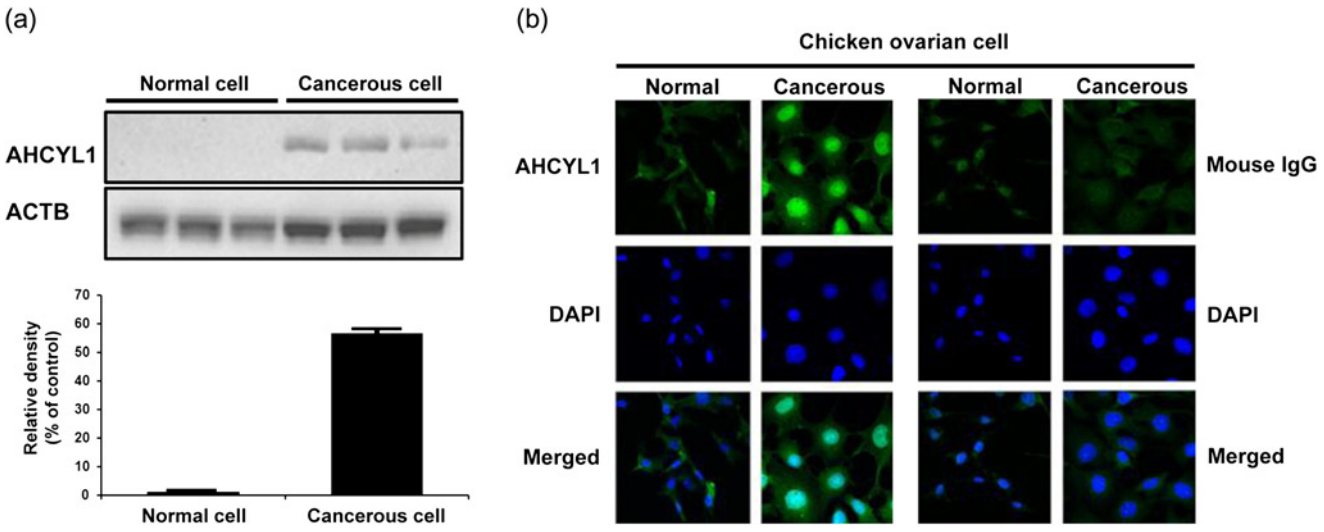

Figure 3 Differential expression of AHCYL1 protein in normal and cancerous ovarian cells from hens. a) Western blotting for AHCYL1 protein from normal and ovarian cancer cell lines. Three different primary ovarian surface epithelial cell lines from three normal and cancerous chickens, respectively, were isolated and cultured with some modifications. Western blotting analyses detected a 57-fold increase ($P < 0.001$) in AHCYL1 protein in ovarian cancer cells as compared with normal ovarian cells. (b) Immunofluorescence microscopy for AHCYL1 protein in normal and ovarian cancer cell lines. Immunoreactive AHCYL1 protein was abundant in nuclei of chicken ovarian cancer cells, but detectable at lower levels in the cytoplasm of normal ovarian epithelial cells. Please see Materials and methods for a complete description of the methods. DAPI, 4′,6-diamidino-2-phenylindole

Comparison of CpG methylation status in the 5′ promoter region upstream of the start site for the AHCYL1 gene between normal and cancerous ovarian cells in hens

The effect of CpG demethylation in the 5′ promoter region upstream of a gene is to increase transcriptional activity. Moreover, CpG methylation is closely associated with the development of cancers. Therefore, we investigated methylation patterns in the promoter region of the *AHCYL1* gene in normal and cancerous ovarian epithelia. Both normal and cancerous ovarian epithelial cells were extracted and cultured *in vitro* as previously reported.[14,28,29] Bisulfite sequencing results revealed CpG sites at −2721, −2799, −2835 and −2844 base pairs from the transcriptional start site that maintained a methylated and unmethylated status in both type of cells, respectively (Figure 4 and Table 1).

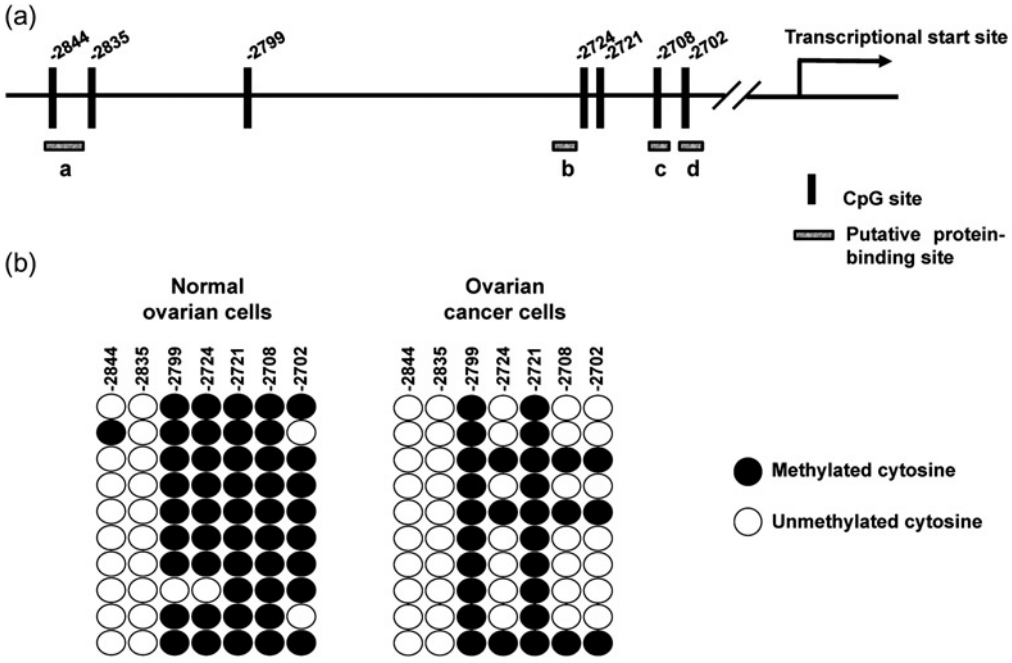

Figure 4 Bisulfite sequencing of CpG sites in 5′ promoter upstream of the transcription start site for the *AHCYL1* gene. (a) Schematic of the seven CpG sites in the promoter region of the *AHCYL1* gene are indicated by the heavy black vertical lines. The numbers on the line indicate positions relative to the transcription start site. (b) The CpG methylation status in the upstream region of the *AHCYL1* gene was analyzed in normal ovarian cells and ovarian cancer cells of hens by bisulfate sequencing. Each circle indicates a CpG site in the primary sequence, and each line of circles represents analysis of a single cloned allele. Closed and open circles are methylated and unmethylated CpGs, respectively. Please see Materials and methods for a complete description of the methods

Table 1 Putative protein-binding sites within the upstream 5′ promoter region of the *AHCYL1* gene

Mark	Sequences	Position	Binding TF	Species
a	GGGCTGGG	−2838, −2845	AP-2	Human, mouse, rat, chicken, clawed frog
			AP-2alpha	Human, mouse
			AP-2alphaA	Human
			AP-2alphaB	Human
b	GGGCA	−2725, −2729	ER-alpha	Human, rat, chicken, clawed frog
			LF-A1	Human
			Sp1	Human, mouse, rat, chicken, rabbit, pig
c	CCGA	−2706, −2709	RAF	Yeast
d	AGACGG	−2699, −2704	SIF	Human, mouse, rat

TF, transcription factor

However, about 37.5, 30 and 33.3 percent of the CpG sites at −2702, −2708 and −2724 were unmethylated in ovarian cancerous cells compared with normal ovarian cells. These results suggest that the decreased CpG methylation status in the upstream region of the *AHCYL1* gene may enhance transcription of the *AHCYL1* gene in chicken ovarian tumors.

Immunofluorescence detection of AHCYL1 protein in human immortalized ovarian carcinoma cells

To examine the existence and potential function of AHCYL1 in human ovarian cancer, we performed immunofluorescence analyses and compared expression patterns of AHCYL1 protein between chicken and human ovarian cancer cells. In this study, three human ovarian cancer cell lines (OVCAR-3, SKOV-3 and PA-1 cells) were prepared, cultured, fixed and then subjected to immunofluorescent microscopy. Similar to the localization of AHCYL1 in chicken ovarian cancerous cells, immunoreactive AHCYL1 protein was most abundant in the nucleus of human OVCAR-3 cells, whereas it was mainly detected in the cytoplasm of human SKOV-3 and PA-1 cells (Figure 5). These results suggest a functional role of AHCYL1 in the nucleus that may be involved in the activation of transcriptional cascades or signaling pathways associated with cell proliferation and development or inhibition of apoptosis during epithelial ovarian carcinogenesis in humans.

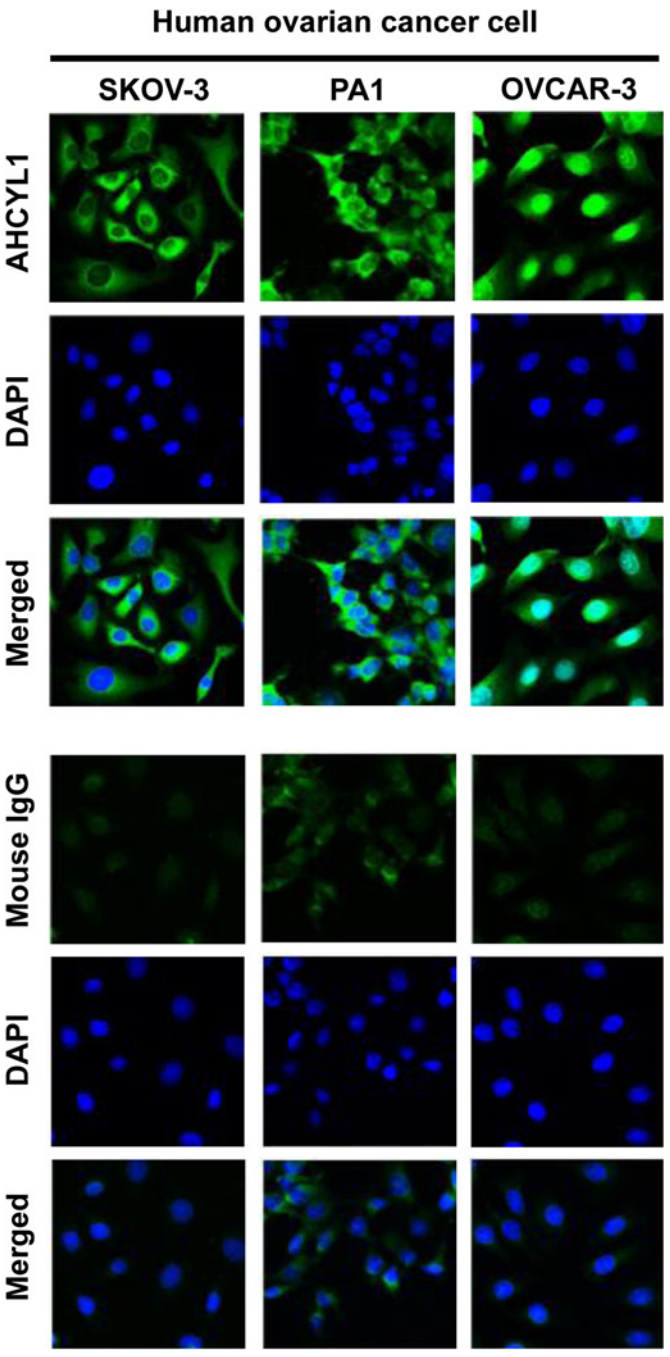

Figure 5 Differential localization of AHCYL1 protein in human immortalized ovarian carcinoma cells. Immunoreactive AHCYL1 protein was abundant in the nuclei of human OVCAR-3 cells, but the cytoplasm of human SKOV-3 and PA-1 cells. Cell nuclei were stained with DAPI (blue). All images were captured at 40× objective magnification. Please see Materials and methods for a complete description of the methods. DAPI, 4′,6-diamidino-2-phenylindole

Different expression of AHCYL1 affecting overall response and PFS in patients with ovarian epithelial cancer

Next, we examined the functional role(s) of AHCYL1 in human ovarian epithelial cancer. We enrolled 109 patients with a median age of 52 years (range, 23–82 years) in the study. Among them, 38 (34.9%) were in FIGO stage I, 20 (18.3%) in stage II and 51 (46.8%) in stage III of the disease. The tumor grade was G1 in 21 (19.3%), G2 in 41 (37.6%) and G3 in 47 patients (43.1%). Histologically, 62 tumors (57%) were diagnosed as serous carcinoma, 17 (15.6%) as mucinous carcinoma, 12 (11%) as clear cell carcinoma, nine (8.3%) as endometrioid carcinoma, four (3.6%) as undifferentiated carcinoma, three (2.7%) as endometrioid and clear cell carcinoma, and two (1.8%) as serous and clear cell carcinoma. The median time to follow up was 69.2 months (range, 2–88 months).

Optimal cytoreduction was performed in 65 patients (59.6%), whereas 44 (40.4%) underwent suboptimal

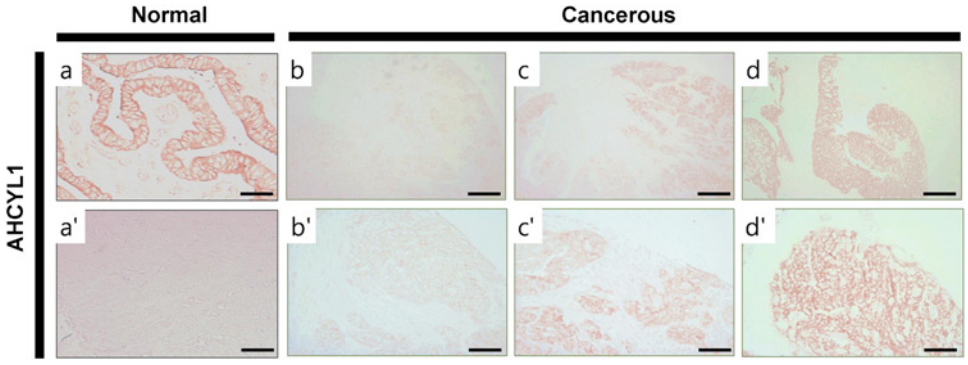

Figure 6 Immunohistochemical expression of AHCYL1 protein in normal and cancerous ovaries from women. Weak (b and b′), moderate (c and c′) and strong (d and d′) expression of AHCYL1 protein was detected in women with epithelial ovarian cancer. There was either no expression or very weak expression of AHCYL1 in cancerous ovaries, whereas AHCYL1 protein was readily detectable in normal ovaries (a and a′). The negative control was mouse IgG instead of primary antibody. Scale bar represents 200 μm (the first horizontal panels and IgG) or 50 μm (the second horizontal panels). (A color version of this figure is available in the online journal)

cytoreduction. Among all patients, 90 (82.6%) received adjuvant chemotherapy after surgery, of which 84 (93.3%) received paclitaxel/carboplatin while paclitaxel/cisplatin was administered to six (6.7%) patients. IHC analysis revealed that AHCYL1 protein was abundant in the nucleus of EOC cells, and 14 (12.8%), 41 (37.6%) and 54 patients (49.4%) had weak, moderate and strong expression of AHCYL1 protein, respectively (Figure 6).

After treatment, 75 (68.8%) and 18 patients (16.5%) showed CR and PR, suggesting an OR rate of 85.3%. Intermediate or high expression of AHCYL1 protein was more frequent in patients showing OR ($n = 85$, 91.4%) than in those showing non-OR ($n = 10$, 62.5%, $P < 0.01$). Furthermore, intermediate or high expression of AHCYL1 protein was a favorable prognostic factor for OR after adjustment using clinicopathological factors (adjusted OR, 7.23; 95% CI, 1.36–38.39) (Table 2). Although there was no difference in OS based on the expression of AHCYL1 protein (mean value, 46.9 versus 61.3 months, $P > 0.05$), intermediate or high expression of AHCYL1 protein was associated with longer PFS than weak expression (mean value, 33.8 versus 46.7 months, $P < 0.01$). Moreover, intermediate or high expression of AHCYL1 protein was also a favorable prognostic factor for PFS (adjusted HR, 0.20; 95% CI, 0.07–0.55) (Table 3).

Table 2 Multivariate linear logistic regression analysis for factors affecting overall response

Characteristics	Adjusted OR	95% CI	*P* value
Age ≥52 years	1.14	0.29–4.47	0.85
FIGO stage I or II disease	0.61	0.13–2.79	0.53
Grade 1 or 2 disease	3.98	1.02–15.43	0.04
Serous adenocarcinoma	5.17	1.12–24.02	0.04
Adjuvant chemotherapy	1.32	0.21–8.28	0.77
Optimal cytoreduction	11.87	2.35–60.02	<0.01
Intermediate or high expression of *AHCYL1*	7.23	1.36–38.39	0.02

OR, odds ratio; CI, confidence interval; FIGO, International Federation of Gynecology and Obstetrics

Table 3 Multivariate Cox's proportional hazard analysis for factors affecting progression-free survival

Characteristics	Adjusted HR	95% CI	*P* value
Age ≥52 years	1.37	0.75–2.50	0.31
FIGO stage I or II disease	0.73	0.32–1.64	0.44
Grade 1 or 2 disease	0.72	0.41–1.29	0.27
Serous adenocarcinoma	1.87	0.85–4.11	0.12
Adjuvant chemotherapy	1.48	0.54–4.04	0.44
Optimal cytoreduction	0.15	0.06–0.34	<0.01
Intermediate or high expression of *AHCYL1*	0.20	0.07–0.55	<0.01

HR, hazard ratio, CI, confidence interval; FIGO, International Federation of Gynecology and Obstetrics

Discussion

In the present study, we first demonstrated that expression of the *AHCYL1* gene increased only in glandular epithelia of cancerous ovaries compared with normal ovaries of hens. Our current results support our hypothesis that AHCYL1 is a critical regulator of growth and development of epithelial-derived ovarian cancer in hens. Chickens are the most relevant animal model to identify biomarkers for patients with EOC because their incessant ovulation increases the possibility of gene mutations by genomic damage to the ovarian surface epithelium, which can lead to ovarian cancer.[29] In addition, various biomarkers such as cancer antigen 125, cytokeratin, epidermal growth factor receptor, Lewis Y and erbB-2 for human ovarian cancer are cross-reactive and expressed in chicken ovarian carcinoma cells, but not in normal ovaries.[30–33] Furthermore, hens have similar tumor-related gene alterations such as p53, ras and HER-2/neu compared with those of women.[34] Indeed, we have reported that SERPINB3 is associated with ovarian carcinogenesis in chickens and it can serve as a prognostic factor for platinum resistance and poor survival in patients with EOC.[35]

Recently, we found tissue-specific expression of *AHCYL1* mRNA and protein in the chicken oviduct to be regulated by estrogen in a tissue and cell-specific manner (Supplementary Figure 2; please see http://ebm.rsmjournals.com/lookup/suppl/doi:10.1258/ebm.2012.011433/-/DC1).

However, the significance of chicken AHCYL1 is poorly understood in ovarian cancer research. Therefore, we investigated expression of AHCYL1 in normal and cancerous ovaries from hens. As illustrated in Figures 1–3, *AHCYL1* mRNA and protein were predominantly found in cancerous ovaries, but there was little or no expression in normal ovaries. These results indicate that AHCYL1 is a potential biomarker that is up-regulated in the glandular epithelium of cancerous chicken ovaries and confirm that the chicken is an appropriate animal model for ovarian cancer research to identify and develop biomarkers for early diagnosis and application of therapeutics to treat the disease.

In the current study, our results demonstrated that AHCYL1 protein is abundant in nuclei glandular epithelial cells of cancerous ovaries as compared with normal ovaries. In addition, bisulfite sequencing results to examine methylation patterns in the promoter region of the *AHCYL1* gene revealed that approximately 30–38% of the three CpG sites are demethylated in ovarian cancerous cells compared with normal ovarian cells. These findings suggest that AHCYL1 plays a role in tumor development and proliferation of ovarian epithelial cells and that it is associated with ovarian carcinogenesis through the activation of transcription factors and increased transcriptional activity via a partial demethylation of the 5′ promoter region of the gene. In spite of the lack of evidence for a direct association between AHYCL1 expression and ovarian carcinogenesis, the *AHYLC1* gene is down-regulated in response to chemotherapeutic drugs based on comparative genomic hybridization in a human malignant melanoma cell line.[36]

Based on the previous reports and findings in the present study, we hypothesize that *AHCYL1* contributes to the development and progression of EOC. However, the pattern of expression of *AHCYL1* was different among patients with EOC. When we considered the strongest expression of AHCYL1 protein in normal ovaries, it was less abundant than that in EOC. Although we expected high expression of AHCYL1 to be associated with poor prognosis in patients with EOC as found in a previous study, intermediate or high expression of AHCYL1 was found to be a favorable prognostic factor for OR (adjusted OR, 7.23; 95% CI, 1.36–38.39) and PFS (adjusted HR, 0.20; 95% CI, 0.07–0.55). Thus, *AHCYL1* is an oncogene for developing ovarian cancer in the chicken model, whereas it acts as a tumor suppressor gene in human EOC. Since the functional role of the *AHCYL1* gene in cancer has not been reported previously, we presumed that it has a paradoxical function of acting as an oncogene in one species and a tumor suppressor gene in another species. Our hypothesis can be supported by previous reports indicating that specific genes have different functions in carcinogenesis. For example, Notch acts paradoxically in various types of tumors. It is an oncogene leading to loss of cellular differentiation in pancreas, breast, cervical and renal cancers,[37–40] but a tumor suppressor gene leading to growth arrest in neuroendocrine and thyroid cancers.[41,42] Furthermore, Mdm2 can act both as an oncogene and a tumor suppressor gene. Mdm2 increases cellular proliferation and tumor development by inactivating p53, but it can also reduce mutant p53 expression to act as a tumor suppressor.[43] Thus, the current study suggests paradoxical functions of *AHCYL1* in ovarian carcinogenesis that is variable among species.

To the best of our best knowledge, results of the current study are the first to indicate expression of *AHCYL1* in ovarian carcinogenesis and to provide evidence for it having paradoxical functions based on results obtained by comparing its expression in hens and in women with EOC. In contrast to the association between *AHCYL1* expression and ovarian carcinogenesis in chickens, a high level of expression of *AHYCL1* was a favorable prognostic factor for tumor response and survival in women with EOC. Thus, *AHCYL1* is likely to be an important target gene related to EOC that requires further evaluation to determine the functional mechanism whereby it affects carcinogenesis.

Author contributions: All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript. WJ, HSK, WL, JK, H-JJ and MAK conducted the experiments. WJ, HSK, FWB and GS wrote the manuscript. JYH contributed to providing all the animals for tissue sampling. YBK, DHS, KK, HHC and YSS contributed to providing all the human tissues. GS and JYH designed the experiments and analyzed experimental data. WJ and HSK contributed equally to this work.

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