# Expression of Adiponectin and Its Receptors Is Altered in Epithelial Ovarian Tumors and Ascites-Derived Ovarian Cancer Cell Lines

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**Objectives:** Recent evidence suggests that higher body mass index is associated with a modest increase in ovarian cancer risk. Reduced serum levels of adiponectin are correlated with obesity and increased cancer risk. The objectives of the present study are to determine if expressions of adiponectin and its receptors, AdipoR1 and AdipoR2, are altered in epithelial ovarian tumors and ascites-derived ovarian cancer cell lines and to determine if plasma adiponectin levels are altered in the chicken model of ovarian cancer.

**Methods:** Adiponectin, AdipoR1, and AdipoR2 mRNA concentrations in ovaries and chicken ovarian cancer (COVCAR) cell lines were determined by quantitative real-time polymerase chain reaction analysis. Existence of adiponectin isoforms in the ovaries and COVCAR cells was identified by nondenaturing gel electrophoresis. Adiponectin, AdipoR1, and AdipoR2 protein amounts were determined by Western blot analysis. Plasma total adiponectin levels were determined by an enzyme immunoassay.

**Results:** Adiponectin, AdipoR1, and AdipoR2 mRNA concentrations were significantly lower in cancerous ovaries and COVCAR cell lines compared with normal ovaries and normal ovarian surface epithelial (NOSE) cells, respectively. Adiponectin in ovary and COVCAR cell lines appeared as a heavy-molecular-weight isoform that is greater than 720-kd mass. In addition, a lower-molecular-weight adiponectin isoform was found in COVCAR cells but not in NOSE cells. Adiponectin and AdipoR1 protein concentrations were not different in COVCAR cell lines compared with NOSE cells. However, AdipoR2 protein concentrations were significantly higher in cancerous ovaries but lower in COVCAR cell lines compared with normal ovaries and NOSE cells, respectively. Plasma adiponectin concentrations were not different in chickens that had ovarian carcinoma compared with control animals.

**Conclusions:** Expression of adiponectin in ovarian tumors and in metastatic ovarian tumor cells is likely to affect cellular metabolism and proliferation through activating AdipoR1 and/or AdipoR2. Plasma adiponectin levels may not be predictive of advanced stages of ovarian tumor in the chicken model.

**Key Words:** Adenocarcinoma, AdipoR1, AdipoR2, Ascites, Enzyme immunoassay, HMW adiponectin isoform, Metastasis

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The American Cancer Society estimates that 21,980 new cases of ovarian cancer will be diagnosed and 14,270 deaths are expected due to ovarian cancer in 2014 in the United States. Ovarian cancer ranks fifth among cancer-related deaths with relatively low 5-year survival rate (44%). One of the modifiable risk factors for several cancers is higher body mass index and obesity, and recent evidence suggests that higher body mass index is also associated with a modest increase in ovarian cancer risk.[1,2] Adipose tissue is considered as an endocrine organ that secretes a variety of hormones, proinflammatory and anti-inflammatory cytokines. Adiponectin, an adipocytokine, is the most abundant protein in the adipose tissue and also the most abundant hormone in serum with levels as high as 3 to 30 μg/mL.[3,4] Adiponectin has been found to exert pleiotropic beneficial effects on carbohydrate and fatty acid metabolism and prevents dyslipidemia, cardiovascular disease, and type 2 diabetes. Unlike other adipose-secreted hormones, adiponectin levels in circulation are inversely correlated to body mass index.[5,6] Furthermore, reduced levels of serum adiponectin are correlated with metabolic syndrome,[7] cardiovascular diseases,[8] diabetes, hypertension,[9] and cancer.[10]

During biosynthesis in adipocytes, adiponectin undergoes several posttranslational modifications and multimerization resulting in secretion of 3 major isoforms denoted as low- (LMW), medium- (MMW), and heavy-molecular-weight (HMW) isoforms.[11–13] The LMW adiponectin isoform represents a homotrimer of 3 adiponectin monomers, whereas the MMW isoform is a hexamer composed of 2 homotrimers, and the HMW adiponectin isoform is likely to contain 18 or more adiponectin monomers.[12] Among the 3 major isoforms, the HMW isoform appears to be more important for preventing metabolic diseases than the MMW and LMW isoforms.[13] Adiponectin exerts its beneficial actions by binding to 2 distinct receptors, AdipoR1 and AdipoR2, which are ubiquitously expressed in various organs including the ovaries.[9]

Recent studies suggest that there is a link between adipose-derived hormones and elevated cancer risk.[14,15] A negative correlation of serum adiponectin levels and cancer risk has been documented in several cancers such as colon, esophageal, liver, prostate, breast, and endometrial cancers.[10] Adiponectin seems to exert its beneficial effects through several mechanisms, such as up-regulation of proapoptotic genes and down-regulation of antiapoptotic genes in an esophageal adenocarcinoma cell line[16] and activation of caspases in hepatocarcinoma cells.[17] Adiponectin treatment was found to suppress breast cancer cell proliferation,[18,19] and to inhibit endothelial cell proliferation and migration. In addition, adiponectin treatment inhibits tumor formation following fibrosarcoma cell xenograft in mice, partly by decreasing neovascularization.[18] Knockdown of AdipoR1 or AdipoR2 using RNA interference in human ovarian granulosa tumor (KGN) cells was found to induce apoptosis and alter progesterone secretion through mitogen-activated protein kinase pathway, respectively.[20] Similarly, adiponectin receptors were found to mediate the antiproliferative and apoptotic effects of adiponectin in MCF7 breast cancer cells.[21] Based on the foregoing, it is important to elucidate the role of adiponectin signaling in ovarian epithelial tumor cells to understand its role in tumorigenesis. However, expression of adiponectin and its receptors in ovarian tumors of women or in any animal model has not been investigated. Therefore, the objectives of the current study are to characterize the expression levels of adiponectin and its receptors in tumorous ovaries and ascites-derived ovarian tumor cells in a naturally developing ovarian cancer model. In addition, we sought to determine if plasma adiponectin concentrations are altered in chickens with ovarian cancer relative to control animals. The chicken model is utilized as it is the most appropriate animal model to study spontaneously developing ovarian tumors.[22,23] We have recently isolated primary ovarian cancer cells from ascites of chickens that had advanced stages of ovarian tumor.[24] Such ascites-derived ovarian cancer (COVCAR) cell lines were found to express several ovary-specific genes and were highly invasive in Matrigel extracellular matrix and readily formed colonies in soft agar. Furthermore, COVCAR cell lines had elevated expression of several genes or proteins such as E-cadherin that are commonly associated with ovarian tumors.[24] Considering the above, we utilized COVCAR cell lines in addition to the ovarian tumor to determine the expression of adiponectin and adiponectin receptors.

## MATERIALS AND METHODS

### Animals

Single-comb white Leghorn chickens were maintained in individual cages at 2 locations (Cornell University, Ithaca, NY, and Pennsylvania State University, University Park, PA). The animals were provided unrestricted access to feed and water at all times and were maintained at 16-hour light and 8-hour dark cycle. All animal procedures were carried out in accordance with the Institutional Animal Care and Use Committee–approved protocol.

### Tissue Collection and Cell Culture

Normal and cancerous ovaries were collected from chickens (3–4 years old; n = 10 each) as described previously.[24] The ovaries were frozen in liquid nitrogen and stored at −80°C for extraction of protein and RNA. Ascites was collected aseptically for isolation and culture of COVCAR cells, as described previously.[24] COVCAR cells lines (C5, C6, C7, C11, and C19) obtained from 5 animals were cultured in MCDB105-M199 cell culture medium at 37°C and 5% $CO_2$. Total RNA and protein were extracted from COVCAR cells (passages 3–4) as described previously.[24] To serve as control for COVCAR cells, normal ovarian surface epithelial (NOSE) cells were collected by gentle scraping of the surface of preovulatory ovarian follicles of healthy chickens. The NOSE cells and COVCAR cell lines were cultured under identical conditions.

### Real-Time Quantitative Polymerase Chain Reaction

Total RNA was extracted from ovary tissue (cancerous and normal, n = 10 animals each) and cell lines (COVCAR and NOSE cells, n = 5 animals) and reverse transcribed as described previously.[24] Briefly, ovarian tissue and cell line cDNA was mixed with 1× PerfeCTa SYBR Green Fastmix (Quanta Biosciences, Gaithersburg, MD) and 300 nM forward

and reverse primers[25] to amplify chicken adiponectin, AdipoR1, AdipoR2 or 18S RNA. Reactions were carried out in a 7500 Fast Real-Time PCR System (Life Technologies) with the following thermal cycle: 95°C for 20 seconds followed by 35 cycles of 95°C for 3 seconds, 55°C for 10 seconds, and 63°C for 30 seconds. At the end of amplification, a melting curve analysis was performed to confirm the presence of a single amplification product. Samples from each ovary tissue and cell line were run in triplicate to obtain average $C_T$ values for adiponectin, AdipoR1, AdipoR2, and 18S mRNA. Adiponectin, AdipoR1, and AdipoR2 mRNA quantity was expressed as a proportion of 18S mRNA quantity following $2^{-\Delta\Delta C}{}_T$ method for converting log-linear $C_T$ values to linear term[26] and analyzed. For negative controls, reverse transcription reactions using 1 μg total RNA with no reverse transcriptase (−RT) were used as template in place of single-stranded cDNA in the quantitative real-time polymerase chain reaction (PCR) (data not shown).

## Competitive Enzyme Immunoassay

Plasma adiponectin levels were measured using an enzyme-linked immunosorbent assay developed by us.[27] Blood was collected from animals before killing and centrifuged for separation of plasma. Adiponectin peptide standards (0–20,000 ng/mL; ≥95% pure; Sigma-Genosys, Woodlands, TX) were applied to 96-well plates coated with goat anti–rabbit immunoglobulin G (IgG) (Pierce). Affinity purified anti–chicken adiponectin IgG (0.2 μg/mL) developed by our laboratory[27] was added to each well and incubated for 2 hours. The wells were washed with 10 mM Tris, 150 mM NaCl, 0.1% Tween-20 (TBST) and incubated with streptavidin-conjugated horseradish peroxidase (GE Healthcare; 1 μg/mL) for 1 hour. Each well was then washed 6 times with TBST, and 3,3′,5,5′-tetramethylbenzidine substrate solution (Pierce) was added and incubated for 30 minutes under mild agitation. Absorbance was read at 450 nm using a Victor[3] 1420 Multilable plate reader (PerkinElmer, Waltham, MA). Plasma adiponectin concentrations in normal and cancerous chickens were determined by comparing with a standard curve generated using known quantities of chicken adiponectin as described previously.[27]

## Western Blot Analysis Under Native Conditions

Western blotting analysis under nonreducing native conditions was performed to determine the presence of adiponectin isoforms in cancerous ovary and COVCAR cell lines. Protein lysates of ovary tissue (cancerous and normal, n = 4–5 each) and cell lines (COVCAR and NOSE cells, n = 5 cell lines each) were prepared as described previously.[24] Samples were prepared by combining 10 μg plasma protein (as positive control) or 15 μg of protein lysate from ovary or COVCAR cell lines with 4× NativePAGE sample buffer (Invitrogen) and 5% G-250 sample additive. Proteins were separated on a 4% to 16% Bis-Tris polyacrylamide gel (Invitrogen) with NativePAGE running buffer under nonreducing conditions and electrotransferred onto polyvinylidene difluoride membranes (0.20 μm). The membranes were incubated in blocking buffer (SuperBlock; Pierce) before incubating overnight in rabbit anti–chicken adiponectin antibody (1:40,000 in blocking buffer) at 4°C. The membranes were washed in TBST followed by incubation in horseradish peroxidase conjugated to goat anti–rabbit IgG (1:10,000; Pierce). The membrane was then washed and treated

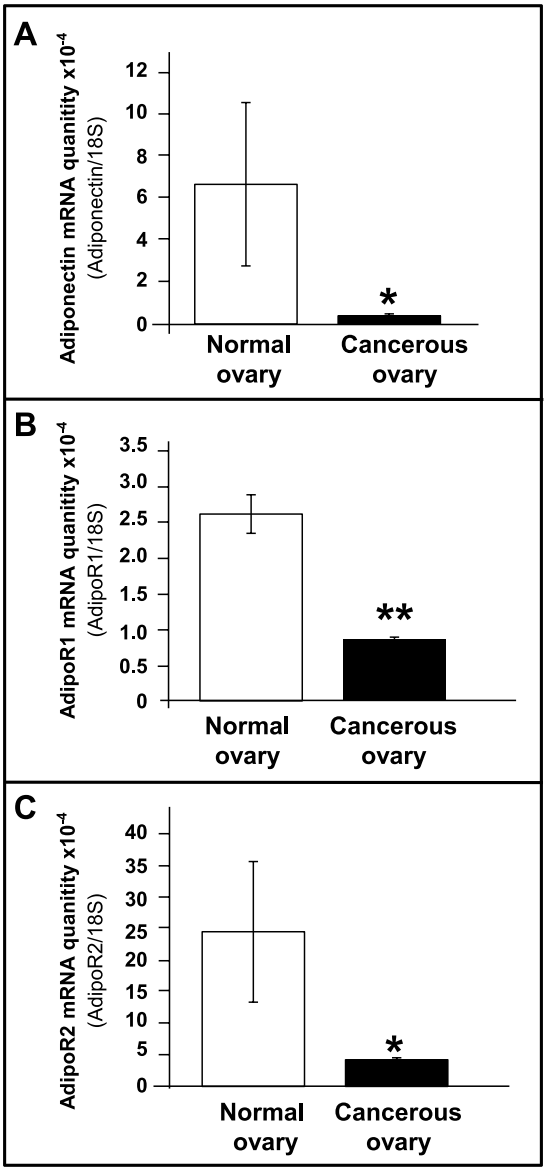


**FIGURE 1.** A–C, Quantification of adiponectin (A), AdipoR1 (B), and AdipoR2 (C) mRNA in normal and cancerous ovaries. Total RNA extracted from normal and cancerous ovaries (n = 10) was DNAse I digested and reverse transcribed. Following reverse transcription, approximately 50 ng of cDNA was used in quantitative real-time PCR using SYBR green as the dye to quantify adiponectin, AdipoR1, AdipoR2 mRNA, and 18S mRNA in separate reactions. Each reaction was run in triplicate per ovary tissue, and the critical threshold ($C_T$) values averaged, normalized to that of 18S mRNA, and converted from log linear to linear term. Adiponectin and AdipoR2 data were further log transformed before Student *t* test analyses. *$P < 0.05$, **$P < 0.01$.

with WesternSure Premium chemiluminescent substrate (LI-COR Biosciences, Lincoln, Neb). Chemiluminescent signals were detected using the CDigit scanner and analyzed using Image Studio software (LI-COR Biosciences). The signal intensity of each band was calculated using a local average background correction. Relative levels of adiponectin in COVCAR cell lines were expressed as a proportion of adiponectin in NOSE cell lines. Western blotting experiment was repeated 3 to 4 times.

## Western Blot Analysis Under Reducing Conditions

Electrophoresis under denaturing conditions was performed to quantify AdipoR1 and AdipoR2 levels in cancerous ovaries and COVCAR cell lines. Protein lysates were prepared by combining approximately 15 μg of ovarian protein extract with reducing agent (Invitrogen) and heating for 10 minutes at 70°C prior to electrophoresis. Proteins were separated on a 4% to 12% Bis-Tris polyacrylamide gel (Invitrogen) under denaturing conditions and blotted as described above. The membranes were immunostained as described above using anti–chicken AdipoR1 (1:1000) or anti–chicken AdipoR2 (1:1,000) as primary antibody. Following image analysis, the membranes were reprobed using mouse anti–α-tubulin or anti–β-actin antibody (0.7 μg/mL antibody; Sigma-Aldrich), incubated in horseradish peroxidase conjugated to goat anti–mouse IgG (1:10,000; Pierce), and imaged as described previously. AdipoR1 or AdipoR2 protein quantities were expressed as a proportion of α-tubulin or β-actin and then compared. Western blotting experiment was repeated 2 to 3 times.

## Statistical Analyses

All data analyses were performed using SigmaPlot version 12 (Systat Software Inc, San Jose, CA). Adiponectin, AdipoR1, and AdipoR2 mRNA or protein concentrations and plasma adiponectin concentrations were analyzed using Student *t* test, and $P < 0.05$ was considered statistically significant.

# RESULTS

## Adiponectin, AdipoR1, and AdipoR2 Gene Expression

Adiponectin, AdipoR1, and AdipoR2 mRNA concentrations were significantly lower in ovarian tumors (Figs. 1A–C), and COVCAR cell lines (Figs. 2A–C) when compared with normal ovaries and NOSE cells, respectively ($P < 0.01$). Melting curve analyses showed the presence of a single PCR product for adiponectin, AdipoR1, AdipoR2, or 18S mRNA, confirming the specificity of the reactions (data not shown).

## Detection of Adiponectin Isoforms in Cancerous Ovaries and COVCAR Cell Lines

To identify adiponectin oligomeric and multimeric isoforms in ovaries and COVCAR cell lines, Western blot analysis under nonreducing and non–heat-denaturing conditions was performed. A strong immunoreactive band with a molecular mass of approximately 720 kd was detected in ovaries (Fig. 3A) as well as in both NOSE and COVCAR cell lines (Fig. 3B),

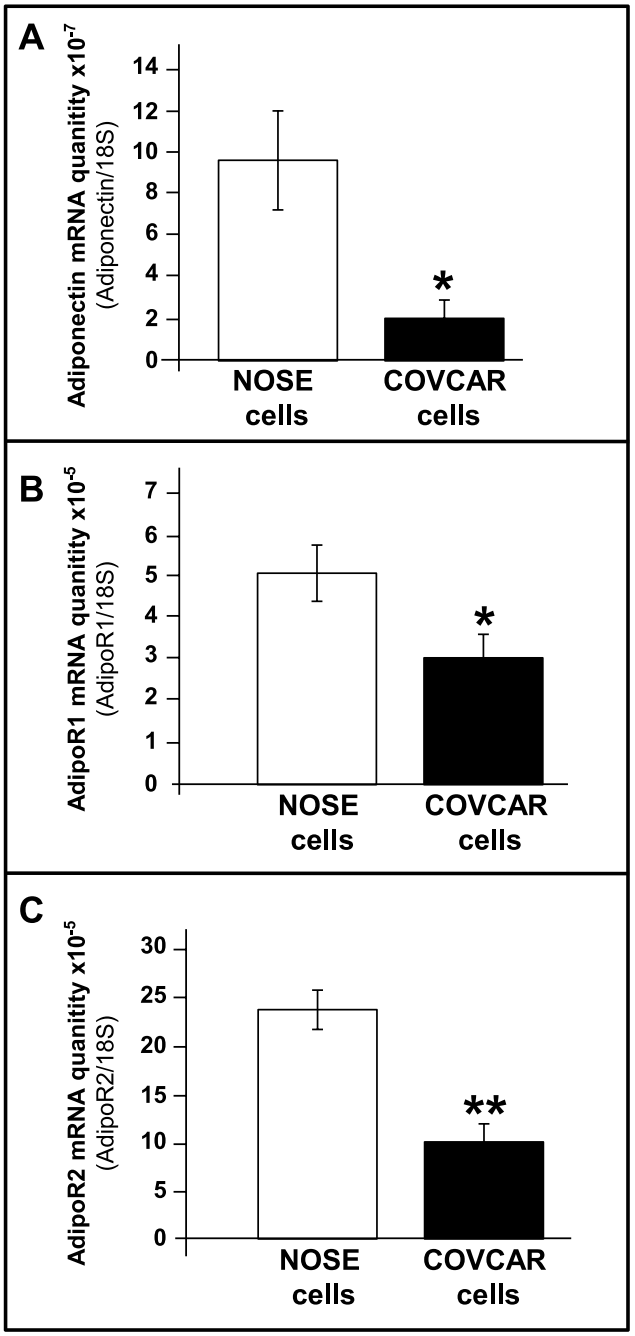


**FIGURE 2.** A–C, Quantification of adiponectin (A), AdipoR1 (B), and AdipoR2 (C) mRNA in chicken ovarian cancer (COVCAR) cells. Total RNA extracted from NOSE and COVCAR cells (n = 5) was DNAse I digested and reverse transcribed. Following reverse transcription, approximately 50 ng of cDNA was used in quantitative real-time PCR using SYBR green as the dye to quantify adiponectin, AdipoR1, AdipoR2 mRNA, and 18S mRNA in separate reactions. Each reaction was run in triplicate per cell line, and the critical threshold ($C_T$) values averaged, normalized to that of 18S mRNA, and converted from log linear to linear term. Data were analyzed by Student *t* test analyses. *$P < 0.05$, **$P < 0.01$.

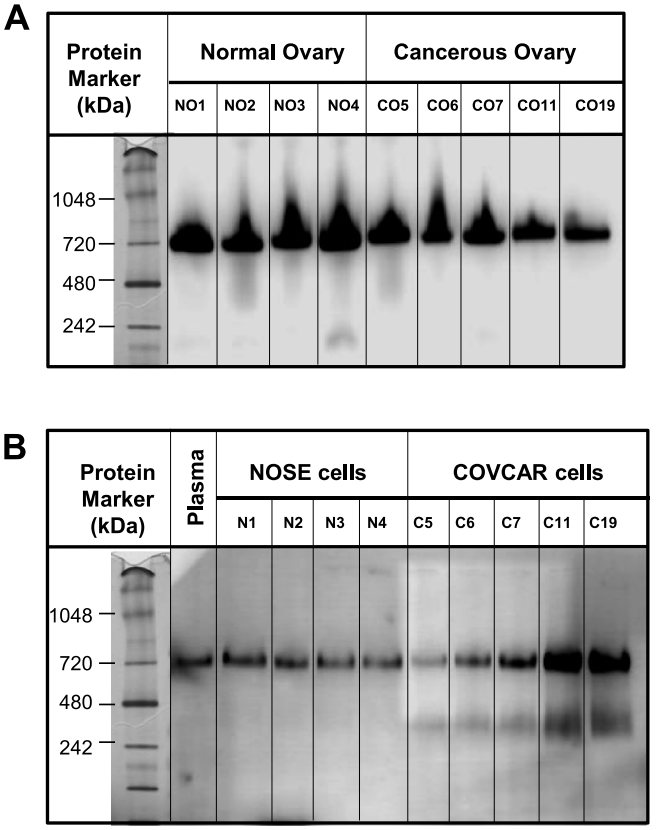


**FIGURE 3.** A and B, Nonreducing and non–heat-denaturing Western blot analysis under native conditions to detect adiponectin in cancerous ovaries (A) and ascites-derived ovarian cancer (COVCAR) cell lines (B). Protein lysates from normal ovaries (NO1, NO2, NO3, NO4), cancerous ovaries (CO5, CO6, CO7, CO11, CO19), NOSE cells (N1, N2, N3, N4), and COVCAR cell lines (C5, C6, C7, C11, C19) were subjected to Western blot analysis under nonreducing and non–heat-denaturing conditions. Adiponectin isoforms were examined by immunostaining using anti–chicken adiponectin antibody and chemiluminescence. Chicken plasma was used as a positive control in B.

representing the HMW adiponectin isoform. In addition to the HMW adiponectin isoform, another adiponectin isoform of approximately 400 kd was detected in the COVCAR cells but not in NOSE cells or ovary tissue (Figs. 3A, B). Preadsorption of the anti–chicken adiponectin antibody with the chicken adiponectin peptide completely abolished immunostaining (data not shown). Adiponectin levels in lysates of COVCAR cell lines did not differ from NOSE cells (data not shown). Quantification of adiponectin in the ovary was not attempted as it is not feasible to measure ovarian adiponectin levels accurately because of presence of blood in the ovary.

## AdipoR1 and AdipoR2 Concentrations

AdipoR1 and AdipoR2 concentrations in ovaries and COVCAR cell lines were quantified by Western Blotting and immunostaining. AdipoR1 concentration in cancerous ovaries and COVCAR cell lines did not differ from normal ovaries and NOSE cell lines, respectively ($P \geq 0.05$; data not shown). However, AdipoR2 concentration was significantly higher in the cancerous ovary compared with normal ovaries ($P < 0.05$; Fig. 4A), but significantly lower in COVCAR cell lines compared with NOSE cell lines ($P < 0.05$; Fig. 4B).

## Plasma Adiponectin Levels

Plasma adiponectin levels were not significantly different in chickens that had ovarian tumor compared with

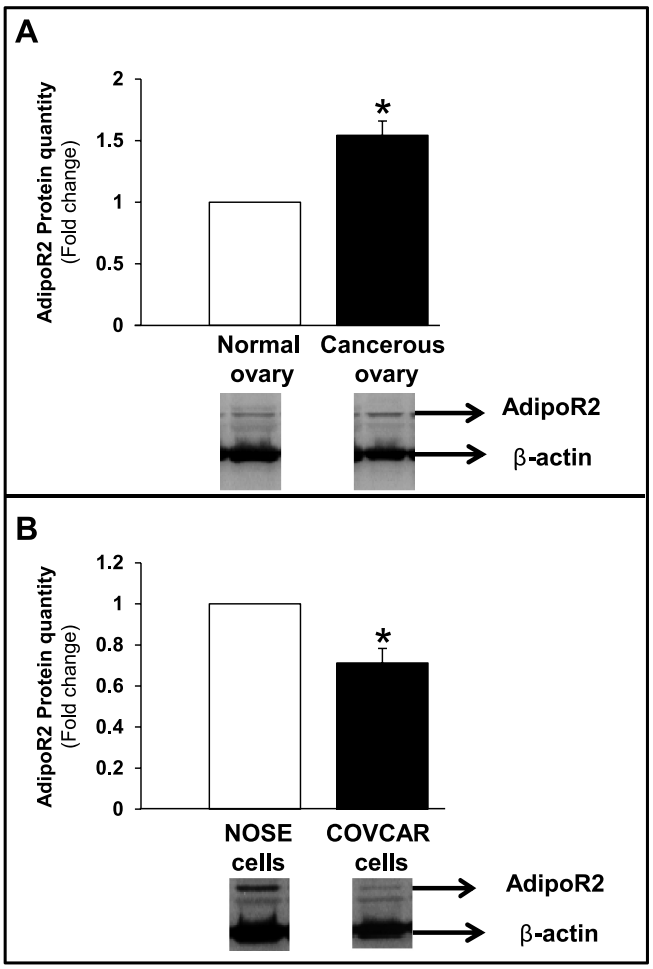


**FIGURE 4.** A and B, Quantification of AdipoR2 protein in ovaries and ascites-derived ovarian cancer cells. Protein extracts from normal and cancerous ovaries (A; n = 5 animals) and COVCAR and NOSE cells (B; n = 4–5 cell lines) were subjected to Western blot analysis under reducing conditions. Chicken AdipoR2 protein was detected by immunostaining using anti–chicken AdipoR2 antibody and chemiluminescence. β-Actin was used as loading control. AdipoR2 concentration in cancer ovaries and COVCAR cells is presented as fold change compared with AdipoR2 concentration in normal ovaries and NOSE cells, respectively. *$P \leq 0.001$ cancerous versus normal ovary or COVCAR versus NOSE cells.

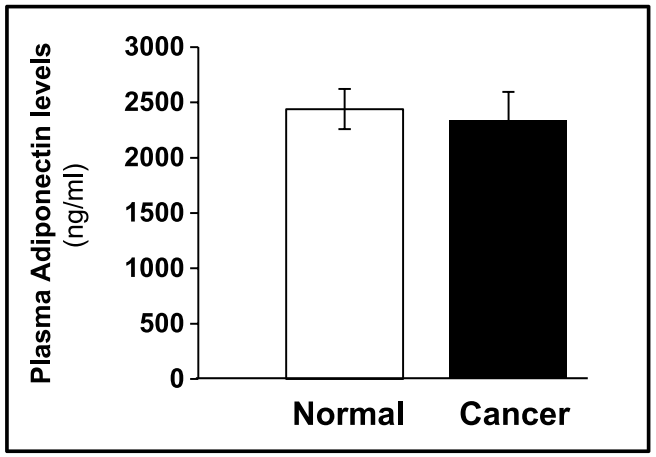


**FIGURE 5.** Quantification of plasma adiponectin levels in chickens with or without ovarian tumor. Plasma adiponectin levels were quantified in normal and cancerous animals by enzyme-linked immunosorbent assay. Data represented as mean ± SEM (n = 10).

chickens without ovarian tumor ($P \geq 0.05$; Fig. 5). Inter-assay coefficient of variation, as determined by quantifying adiponectin in pooled chicken plasma in every plate, was found to be 4.33%, whereas intra-assay coefficient of variation was determined to be 4.48%.

## DISCUSSION

This is the first report documenting expression of adiponectin, AdipoR1, and AdipoR2 in ovarian tumors and in epithelial ovarian adenocarcinoma cell lines. Although expression of adiponectin has been reported in rat[28] and chicken ovary,[29] expression of adiponectin in ovarian tumors has not been investigated in the past. Our data suggest that adiponectin exists as an HMW isoform in both cancerous ovaries and COVCAR cell lines. In addition to the HMW adiponectin isoform, a smaller adiponectin isoform with a mass of approximately 400 kd was found exclusively in COVCAR cell lines. Occurrence of a smaller adiponectin isoform may possibly indicate instability of HMW adiponectin arising from defective multimerization and/or proteolysis due to certain enzyme activity unique to COVCAR cells. The functional significance of adiponectin produced in cancerous ovarian tissue and in COVCAR cells is currently unknown. It is possible that adiponectin produced in ovarian cells may act as a paracrine factor to influence cellular metabolism and survival of neighboring cells. Our data suggest that adiponectin gene expression is down-regulated in cancerous ovaries and COVCAR cell lines compared with controls. Factors influencing the abundance of ovarian adiponectin mRNA are currently unknown. Mean adiponectin protein levels in COVCAR and NOSE cells did not differ because of large variations in protein levels (Fig. 3B).

We found that the expression of AdipoR1 and AdipoR2 mRNA was significantly down-regulated in cancerous ovaries and COVCAR cell lines compared with controls. Consistent with our data, malignant prostate tumor was found to have lower expression levels of adiponectin receptors when compared with benign prostate tumor.[30] Similarly, AdipoR1 and AdipoR2 protein levels were found to be lower in renal cell carcinoma compared with the normal human kidney tissue.[31] However, levels of AdipoR1 and AdipoR2 mRNA in benign and malignant renal cell carcinoma specimens did not differ after adjusting for age and sex.[32] Immunohistochemical staining of archived tumor tissues revealed a pattern of significantly greater expression of adiponectin receptors in cancers strongly associated with obesity compared with cancers that are not associated with obesity, possibly as a result of compensatory mechanism(s) to counteract hypoadiponectinemia observed in obese subjects.[32] Although obesity is considered as one of the risk factors for epithelial ovarian cancer,[2] AdipoR1-immunoreactive cells were detected less frequently in archived human ovarian tumor tissue specimens.[32]

Although AdipoR2 mRNA quantities are lesser in cancerous ovaries compared with normal ovaries, we found that AdipoR2 protein quantities were significantly elevated in cancerous ovaries compared with controls. Such discordance in AdipoR2 mRNA and AdipoR2 protein quantities may suggest that AdipoR2 gene expression in cancerous ovaries is likely to be down-regulated by overabundance of AdipoR2 protein. Intriguingly, AdipoR2 protein quantities were found to be lower in COVCAR cell lines compared with controls. Differences in the abundance of AdipoR2 protein in cancerous ovaries compared with COVCAR cell lines may also arise because of heterogeneity of cancerous ovarian tissue, which may contain some normal tissue. Another possible explanation is that the expression of adiponectin receptor is also influenced by the availability of adiponectin. Adiponectin treatment has been shown to down-regulate AdipoR1 and AdipoR2 gene expression in human MDA-MB 231 breast cancer cell[33] and Barrett adenocarcinoma cell lines.[16] In our study, AdipoR2 mRNA and protein quantities were significantly lower in COVCAR cell lines compared with NOSE cells possibly because of a selective inhibitory effect of adiponectin present in the chicken serum added to the culture medium of COVCAR cell lines. Such selective inhibitory effect may arise from the lower-molecular-weight adiponectin isoform observed exclusively in the COVCAR cells lysates but not in the NOSE cell lysates (Fig. 3B).

We found that the plasma adiponectin levels did not differ in chickens that had ovarian carcinoma compared with chickens that had normal ovary. As in humans and rodent models, increased visceral adiposity is correlated with lower plasma adiponectin levels in chickens.[27] Leghorn chickens are genetically selected for frequent, almost daily, ovulation and higher egg production and are least likely to exhibit obesity-related hypoadiponectinemia. However, monitoring plasma adiponectin levels using a larger sample size over a longer period is necessary to determine if serum adiponectin level is a factor contributing to ovarian tumorigenesis. Although obesity and increased BMI are considered as an overall risk factor for ovarian cancer in women,[2] the relationship between serum adiponectin levels and incidence of ovarian carcinoma has not been investigated. Only 1 study investigated serum adipokine levels in epithelial ovarian cancer subjects who had undergone cytoreductive surgery and platinum-based chemotherapy treatment, and this study

indicated that serum levels of leptin and adiponectin or leptin-adiponectin ratio did not independently predict patient survival.[34] Although the role of adiponectin in ovarian cancer risk is still unclear, serum adiponectin levels were found to be inversely correlated to endometrioid cancer risk.[35] Similarly, cancer risk associated with kidney,[31] prostate,[30] and breast[36] is also inversely correlated to serum adiponectin levels. Therefore, further studies are required to understand the relationship between serum adiponectin levels and ovarian tumorigenesis.

In summary, we provide novel evidence for expression of adiponectin and its receptors in epithelial ovarian carcinoma and in COVCAR cells derived from ascites in the chicken model of ovarian tumor. Future studies should focus on studying expression of adiponectin and its receptors in human ovarian tumors of various histological subtypes to determine if expressions of adiponectin and its receptors are different among serous, mucinous, endometrioid, and clear cell subtypes. In addition, the biological function of adiponectin and its receptors in ovarian tumors should be investigated further to understand the role of adipose-derived factors on ovarian tumorigenesis.

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