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Cellular Distribution of Retinoic Acid Receptor-α Protein in Serous Adenocarcinomas of Ovarian, Tubal, and Peritoneal Origin

Comparison with Estrogen Receptor Status

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Retinoids are effective growth modulators of human ovarian carcinoma cell lines. Their effects are mediated by nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which are transcriptional factors and members of the steroid/thyroid receptor superfamily. To our knowledge, until now, the cellular distribution of RAR proteins in human ovarian tumor specimens is unknown. This study provides new data on the differential cellular localization of RARα protein in 16 serous adenocarcinomas originating from the ovaries, fallopian tubes, and the peritoneum. Using an affinity-purified antiserum specific for RARα and a monoclonal antibody recognizing the full-length estrogen receptor molecule (clone 6F11), we performed immunohistochemistry on frozen tissue sections and examined the relationship between RARα and estrogen receptor protein expression by comparing the percentage of immunostained tumor cells for either receptor. Our findings indicate a strong linear relationship between the percentages of RARα- and estrogen receptor-labeled tumor cells as determined by linear regression analysis ($P < 0.005$, $r = 0.825$). A modest inverse relationship was found between the percentage of RARα-positive tumor cells and histological grade, attesting to a differentiation-dependent trend ($P < 0.04$). No significant relationship was found between RARα-labeled cells and clinical stage ($P = 0.139$), site of tumor origin (ovaries *versus* fallopian tubes *versus* peritoneum) ($P = 0.170$), and primary *versus* metastatic lesion ($P = 0.561$). Thus, serous adenocarcinomas are capable of expressing RARα and estrogen receptor despite high histological grade and advanced stage of neoplastic disease. Compared with the heterogeneous localization of RARα in cancer cells, there was widespread RARα immunoreactivity in tumor-infiltrating lymphocytes, vascular endothelial cells, and stromal fibroblasts, underscoring the value of immunohistochemistry in the accurate determination of RAR/(RXR) content in tumor specimens. ***(Am J Pathol 1998, 153:469–480)***

Ovarian epithelial cancer (adenocarcinoma) is responsible for the largest number of deaths from malignancies of the female genital tract and is the fifth leading cause of cancer death in women.[1] Most ovarian adenocarcinomas are of the serous histological type.[1] Clinically, about two-thirds of serous neoplasms of the ovary present *de novo* as advanced-stage tumors, reflecting their propensity for intra-abdominal/peritoneal spread.[1,2] These tumors arise from transformed cells of the celomic surface epithelium of müllerian origin that accounts for their ontogenetic and phenotypic kinship, histological overlap, and sometimes coexistence with carcinomas of the endometrium and endocervix.[3–5] Occasionally, identical serous neoplasms may arise from the so-called secondary müllerian system[6] involving the pelvic and lower abdominal mesothelium. These extraovarian serous adenocarcinomas, or papillary tumors of the peritoneum, are very closely re-

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This work is dedicated to Dr. Demetrios Katsetos on the occasion of his 70th birthday.

lated to their ovarian counterparts and are different, both in terms of phenotype and clinical behavior, from the mesotheliomas of the peritoneum.[5,7,8] Ovarian carcinomas, like carcinomas of the breast and endometrium, are steroid hormone-dependent epithelial neoplasms. One of the unifying features of the female genital cancer is the presence of steroid receptors in tumor cells, including estrogen, progesterone, and androgen receptors.[9]

Retinoids are metabolites of retinol (vitamin A) and are considered to be important signaling molecules in the modulation of growth and differentiation of normal and neoplastic cells.[10–13] They have been shown to prevent mammary carcinogenesis in rodents,[13] inhibit the growth of human cancer cells *in vitro*,[10–12] and be effective chemopreventive and chemotherapeutic agents in a variety of human epithelial and hematopoietic neoplasms.[14,15] Retinoic acid (RA) has been shown to be an effective growth modulator of human ovarian carcinoma cell lines, imparting an inhibitory effect on ovarian tumor cell growth using RA either alone or in combination with other differentiation-inducing agents.[16–19] The effects of RA on ovarian cancer cells are thought to be mediated by nuclear RA receptors (RARs) and retinoid X receptors (RXRs).[20–22] These nuclear receptors are members of the steroid/thyroid receptor superfamily and can modulate gene transcription through a variety of mechanisms.[23–25] RARs include RARα, RARβ, and RARγ, each of which exhibit high affinity for both all-*trans*-RA and 9-*cis*-RA, whereas RXRs include RXRα, RXRβ, and RXRγ and are activated by 9-*cis*-RA.[26,27] Each RAR subtype comprises various isoforms as a result of different promoters and alternative splicing: the RARα gene contains two promoters transcribing two distinct isoforms, identified as RARα1 and RARα*2* in humans.[28]

Concomitant with ligand binding, the receptor-ligand complexes bind to their respective response elements (RARE and RXRE), located in the regulatory regions of a number of retinoid target genes.[11,21,29] Receptor binding to specific response elements and the resulting gene activation or inhibition occurs through the formation of heterodimers between RARs and RXRs; however, gene activation can also occur through RAR or RXR homodimers.[12] It has been suggested that although RXR ligands mediate transactivation through RXR homodimers, they are largely inactive in mediating transactivation through RAR/RXR heterodimers.[30–32] This raises the possibility that RARs, and not RXRs, are active in the various retinoid-mediated processes.[33]

Despite several previous studies looking at the RAR/RXR signaling pathways in RA-sensitive and RA-resistant human ovarian cancer cell lines, until now, the cellular distribution of RAR proteins in human ovarian tumor specimens is unknown. In the present study, we have evaluated by immunohistochemistry the cellular localization of RARα protein and have examined whether there is a relationship between RARα and estrogen receptor (ER) labeling of tumor cells in surgical specimens of serous adenocarcinoma originating in the ovaries and the secondary müllerian system.

Materials and Methods

Patient Data

Patient data are summarized in Table 1. All 16 patients had undergone exploratory laparotomy, total abdominal hysterectomy, bilateral salpingo-oophorectomy, and debulking surgery. Patient ages ranged from 36 to 80 (median age, 64.5). Fifteen of 16 patients harbored intermediate (grade II) to high grade (grade III) serous papillary adenocarcinomas, and in one patient the tumor contained mixed serous papillary and endometrioid adenocarcinomas (Table 1, case 4). In 11 of 16 patients there was a clearly defined ovarian primary lesion, in 3 patients the tumors were of peritoneal origin (Table 1, cases 5, 15, and 16), and in one instance the origin of the serous adenocarcinoma was traced to the fallopian tube (Table 1, case 11). The majority of patients had advanced Fédération Internationale des Gynaecologistes et Obstetristes (FIGO) stage III/IV disease at presentation (13 of 16 stage III, 1 of 16 stage IV). Two patients with ovarian and tubal serous papillary adenocarcinomas, respectively, were considered to be stage IC (Table 1, cases 3 and 11). In 6 of 16 lesions, the specimens evaluated in this study were derived from metastatic deposits to the contralateral fallopian tube (Table 1, case 9), pelvis (Table 1, case 12), omentum (Table 1, cases 5 and 7), and transverse mesentery (Table 1, case 2).

Tumor Specimens

Ovarian tumor specimens were obtained from the Department of Pathology, Fox Chase Cancer Center, under institutional Internal Review Board approval. All specimens were collected prospectively during a 2-year period (1995 to 1997). The tumor tissue samples procured for this investigational study were microdissected from, and were representative of, the surgical pathology specimens. The presence of viable (nonnecrotic) tumor tissue was determined grossly by the prosector. Surgically resected tumor samples were promptly embedded in ornithine carbamoyltransferase and were kept frozen in −70°C until further processing. Patient characteristics, source of tumor specimens, histopathological diagnosis, tumor grade, and FIGO stage at the time of surgery are summarized in Table 1 (also, see above).

Antibodies

A rabbit polyclonal antibody to RARα and a mouse monoclonal antibody to ER were used. RARα1 (C-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), is an affinity-purified polyclonal antibody raised against a peptide corresponding to amino acids 443 to 462 mapping at the COOH terminus of the RARα1 of human origin, which is identical in sequence to the corresponding region of RARα*2*. C-20 does not cross-react with RARβ or RARγ isoforms. The immunogen of the immunoglobulin (Ig) G1 mouse monoclonal antibody (clone 6F11) to human ER (Novocastra Laboratories Ltd., Newcastle on Tyne, UK) is

Table 1. Patient Characteristics

No.	Age	Source of specimen	Histopathology/Grade	Extent of disease at surgery/Stage	FIGO stage
1	75	Left ovary	Serous papillary CA	Primary: left ovary Metastases to left fallopian tube, right mesovarium, uterine serosa, omentum, left pelvic peritoneum, sigmoid colon and mesentery, and mesentery and serosa of small intestine and hemidiaphragm	IIIB
2	50	Transverse mesentery	Serous papillary CA/II (metastatic)	Primary: N/D Metastases to transverse mesentery, sigmoid colon, vagina, spleen, REIA lymph node, falciform ligament, right upper/diaphragmatic peritoneum	IIIC
3	47	Right ovary	Serous papillary CA/II	Primary: right ovary	IC
4	36	Right ovary	Mixed serous papillary CA, endometrioid CA/II	Primary: right ovary Metastases to fallopian tubes bilaterally, uterine serosa, omentum, sigmoid colon, appendix, and right hemidiaphragm	IIIA/B
5	56	Omentum	Serous papillary CA/III (metastatic)	Primary: c/w peritoneal Metastases to gastrohepatic ligament, transverse mesocolon, and perisplenic region	IIIC
6	66	Left ovary	Serous papillary CA/III	Primary: left ovary Metastases to omentum, left and right fallopian tube and ovary, myometrium, cecum, sigmoid colon, soft tissues around REIA, and cul-de-sac	IIIA/B
7	79	Omentum	Serous papillary CA/III (metastatic)	Primary: left ovary Metastases to ipsilateral fallopian tube, omentum, periumbilical region, and small intestine	IIIB
8	70	Right ovary	Serous papillary CA/II	Primary: right ovary Metastases to right and contralateral (left) fallopian tubes and omentum; serosal metastatic deposits, uterine cervix and corpus uteri; synchronous poorly differentiated intramucosal serous papillary CA of the endometrium	IIIB
9	47	Left fallopian tube	Serous papillary CA/III (metastatic)	Primary: right ovary Metastases to contralateral (left) fallopian tube, left renal, and right para-aortic lymph nodes	IIIC
10	63	Left ovary	Serous papillary CA/III	Primary: left ovary Metastases to right ovary, parametria, uterine wall, omentum, perirenal soft tissues, falciform and round ligaments, right diaphragmatic peritoneum, hemidiaphragm, appendix, rectosigmoid	IIIB
11	80	Right adnexa	Serous papillary CA/III	Primary: right fallopian tube (11.5 cm); focal tumor extension through serosal surface	IC
12	79	Right pelvic mass	Serous papillary CA/III	Primary: right ovary Metastases to the right pelvis/cul-de-sac, myometrium, omentum, transverse colon, appendix, gastrocolic, and REIA lymph nodes (two of two)	IIIC
13	53	Left ovary	Serous papillary CA/III	Primary: left ovary Metastases to left fallopian tube, peritoneum/urinary bladder, sigmoid colon, mesentery; proliferative endometrium with focal endometrial hyperplasia; focal atypical tubal and clear cell metaplasia	IIIB
14	85	Left ovary	Serous papillary CA/II to III	Primary: left ovary Metastases to bladder peritoneum and sigmoid colon; history of breast CA with current new breast CA	IIIA/B
15	49	Omentum	Serous papillary CA/III (metastatic)	Primary: peritoneal Metastases to omentum; transverse colon; descending colon; sigmoid colon; cul-de-sac; and surfaces of the uterus, both ovaries, and right fallopian tube	IIIB
16	72	Peritoneal nodule	Serous papillary CA/III	Primary: peritoneal Metastases to omentum; surface of left ovary; lymphatic invasion of right ovary; serosal surface of anterior uterine wall; left infundibulopelvic ligament; serosal surface of right colon, sigmoid colon, and rectum; liver; lesser gastric curvature; and gastrohepatic ligament	IV

CA, carcinoma; ND, not determined; C/W, consistent with; REIA, right external iliac artery.

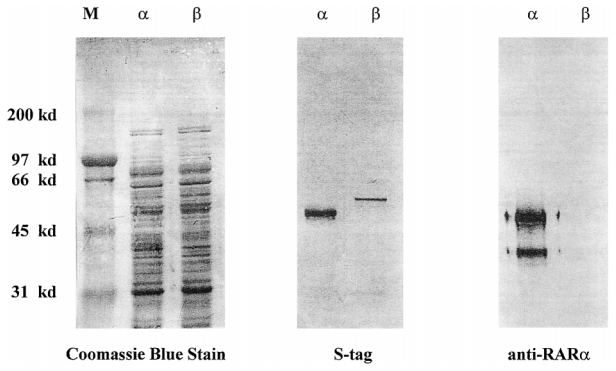

Figure 1. Demonstration of the specificity of the RARα antibody. Bacterial protein extract containing either RARα or RARβ S-Tag fusion protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gels were either stained with Coomassie blue (left) or transferred to polyvinylidene difluoride paper by electroblotting (middle and right). The blots were then probed with either antibody to the S-Tag (middle) or antibody to RARα (right). Protein bands were detected by alkaline phosphatase. Left: Coomassie blue stain of all of the proteins present in the bacterial extracts used in these experiments. Middle: Results when the blot is probed with the anti-S-Tag antibody. It is evident that both RAR proteins are present in the extracts. Right: Results of probing the blot with the anti-RARα antibody. Clearly, the anti-RARα antibody only recognizes the RARα protein and not the RARβ protein. This confirms the specificity of the RARα antibody. The additional band is probably a degraded product that has lost its S-tag.

prokaryotic recombinant protein corresponding to the full-length ER molecule.

Determination of Specificity of the RARα Antibody by Western Blot

Recombinant RARα and recombinant RARβ proteins were prepared as S-Tag fusion proteins as previously described.[34] Briefly, *Escherichia coli* K12 strain BL21(DE3) cells were transformed with the expression of plasmid pET-29a (Novagen, Madison, WI) containing full-length cDNA of mouse RARα or mouse RARβ. Because the cDNAs are cloned in frame with an S-Tag marker, the proteins are expressed as fusion proteins that can be monitored by probing for the presence of the S-Tag. Bacterial extracts containing either recombinant RARα or recombinant RARβ were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride paper by electroblotting. The blots were probed with either S-Tag antibody or RARα antibody (C-20), and protein bands were detected by alkaline phosphatase as described previously.[35] The anti-RARα antibody only recognizes the RARα protein and not the RARβ protein (Figure 1). This confirms the specificity of the RARα antibody used in this study.

Immunoperoxidase Procedure

Cryostat sections (5 μm thick) were air-dried for 1 hour and then fixed in cold acetone at −20°C for 1 hour. Immunohistochemical staining was then performed according to the avidin biotin complex peroxidase method as previously described,[36] using commercially available kits, rabbit IgG and mouse IgG ABC Elite Vectastain

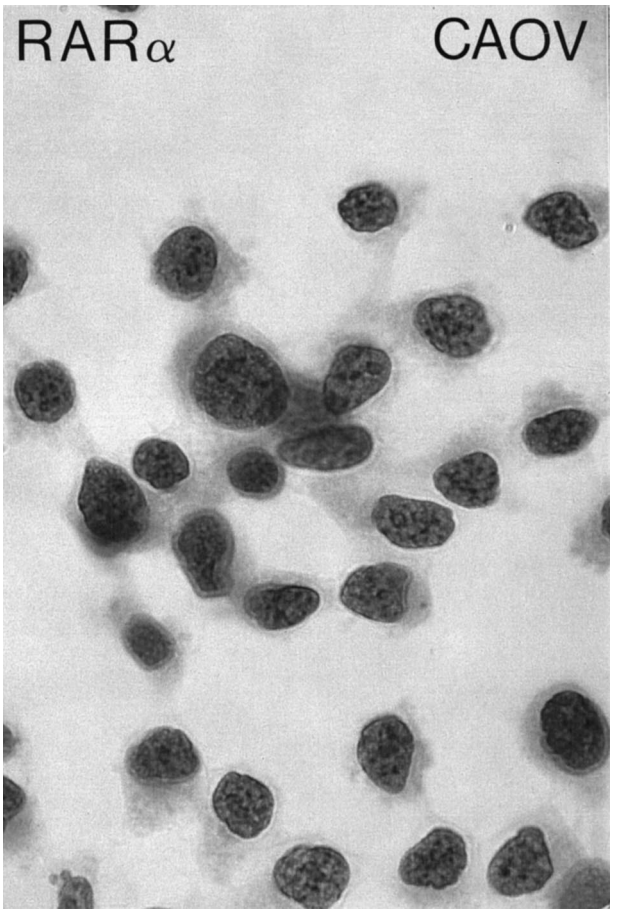

Figure 2. RARα immunostaining of CAOV cells grown as monolayers. CAOV is an RA-sensitive human ovarian carcinoma cell line and has been used in this study as a positive control. Avidin-biotin complex peroxidase method without hematoxylin counterstaining; original magnification, ×1000.

(Vector Laboratories, Burlingame, CA) for polyclonal and monoclonal antibodies respectively. Briefly, sections were incubated with goat (rabbit IgG kit) or horse (mouse IgG kit) sera for 1 hour to reduce nonspecific binding. Sections were incubated with either anti-RARα (dilution 1:50) or anti-ER (dilution 1: 50) antibodies according to the manufacturers' recommendations for 1 hour at room temperature. Antigen-antibody complexes were detected with anti-rabbit (or anti-mouse)-biotinylated avidin-horseradish peroxidase complex. The sections were then developed with 3,3′-diaminobenzidine as the peroxidase substrate. All experiments with the RARα antibody were performed in duplicate. Two complete immunostained sets of slides were generated: one without counterstaining to ensure unambiguous nuclear localization or lack thereof, and the other with light counterstaining with Mayer's hematoxylin (Sigma Chemical Co., St. Louis, MO) to facilitate histological evaluation. The RA-sensitive human ovarian carcinoma cells (CAOV) grown in coverslips served as the positive control for RARα (Figure 2). The CAOV cell line has been shown previously to constitutively express RARα mRNA.[19] Negative controls included normal rabbit IgG (1.25 μg/ml) and nonspecific mouse ascites fluid (Becton Dickinson, Mountain View, CA),

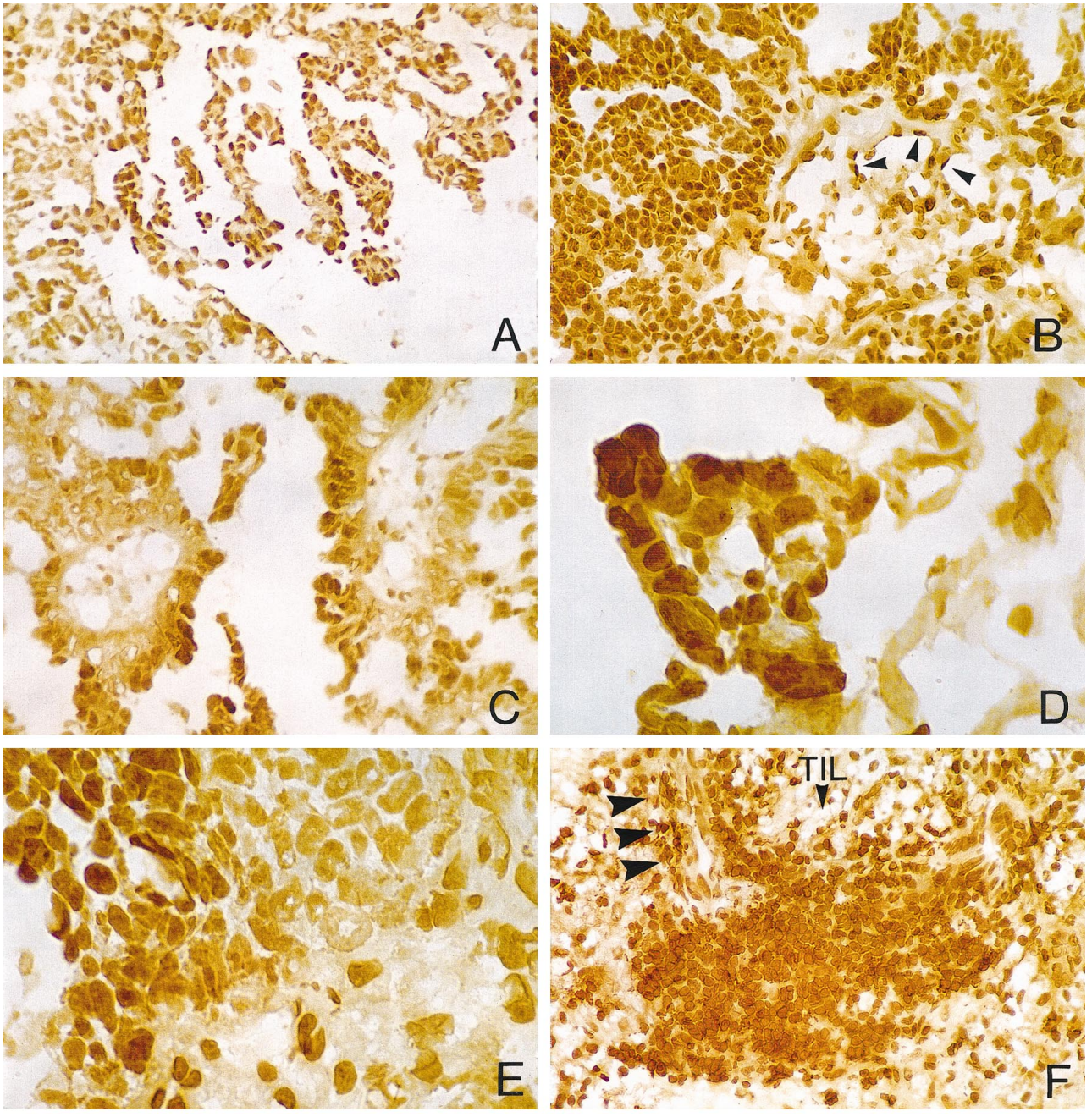

Figure 3. Panel featuring differential cellular localization of RARα in serous adenocarcinomas. A to C: Widespread RARα immunohistochemical staining of neoplastic cells in well to moderately differentiated areas of grade II serous tumors with papillary morphology. B depicts, in addition, RARα staining of endothelial cells in vessels of the fibrovascular core (arrowheads). D and E illustrate, respectively, intratumoral staining heterogeneity associated with better differentiated, albeit abortive, RARα-positive papillary foci (D) or clusters of RARα-positive poorly differentiated tumor cells (E). F: Prominent nodular aggregate of TILs with robust RARα staining. Arrowheads point to a frequently observed perivascular predilection of RARα-positive TILs. Avidin-biotin complex peroxidase method without hematoxylin counterstaining; original magnifications: A and B, ×400; C to F, ×1000.

which were used instead of specific rabbit anti-RARα1 or mouse monoclonal antibody 6F11 to ER, respectively.

Analysis of Staining

Morphological assessment of immunostained tissue preparations and manual cell counting of immunolabeled tumor cells were performed. Initially, representative areas of the histological tumor specimen were selected under low-power magnification. Between 633 and 1271 epithelial tumor cell nuclei were evaluated per case, in 20 representative high-power fields (40×). Only tumor cells with unequivocal nuclear localization, irrespective of intensity of staining, were counted as positive. Staining of the nuclei of tumor-infiltrating lymphocytes (TILs) and the nuclei of nonneoplastic mesenchymal cells, such as fi-

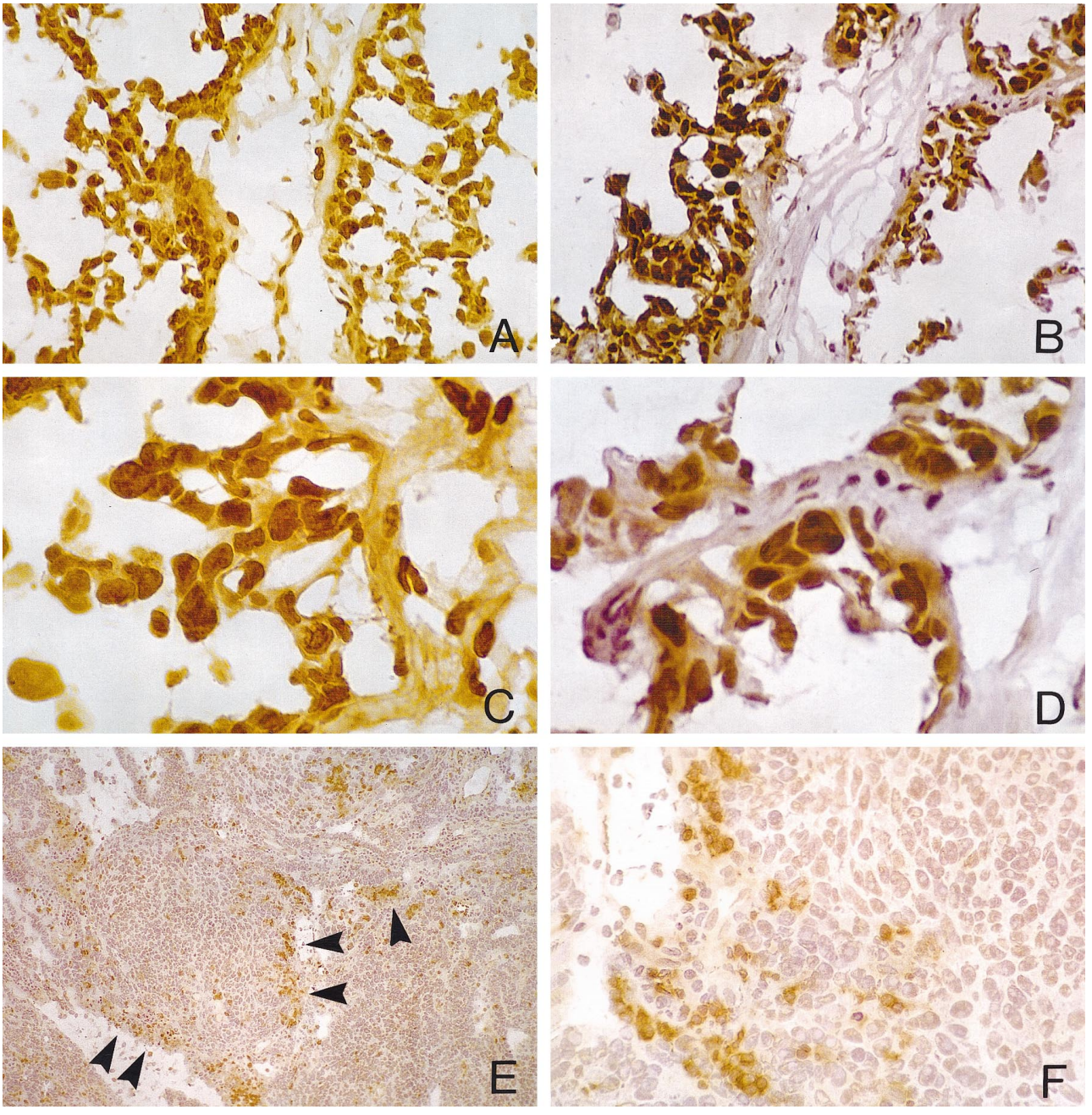

Figure 4. A through D demonstrate a comparison between RARα (A and C) and ER (B and D) nuclear staining in homologous fields from adjacent cryostat sections of a grade II serous adenocarcinoma (C and D are higher magnifications of A and B, respectively). Tumor cell immunoreactivity for both receptors is widespread in papillary areas. Note distinctive nuclear localization of both RARα and ER antibodies (C and D). E and F demonstrate absence or paucity of RARα immunoreactivity in tumor cells of a poorly differentiated (grade III) serous adenocarcinoma of peritoneal origin (F is a higher magnification of E). By contrast, there is robust RARα staining in TILs infiltrating haphazardly the tumor stroma (E, arrowheads, and F). Avidin-biotin complex immunoperoxidase method with (B, D, E, and F) and without (A and C) hematoxylin counterstaining. Original magnifications: A and B: ×400; C and D: ×1000; E: ×100; F: ×400.

broblasts and vascular endothelial cells, was excluded from the cell counts. Immunostained preparations were evaluated by two observers (CDK, IS) independently. The number of positive tumor cells (the numerator) in relation to the total number of tumor cells (the denominator) was recorded as the labeling count for each individual case. The percentage of immunolabeled tumor cells for a given tumor specimen was expressed as a mean labeling percentage based on the number of high-power fields examined. Mean labeling percentages for histological grades II and III were calculated from the total number of representative specimens examined. Interobserver agreement was within 15% ($\kappa = 0.82$).

Statistical Methods

RARα- and ER-labeled cell fractions were expressed in percentages. Overall variations in the percentages of

RARα-labeled cells were evaluated by analysis of covariance using a main effects model for tumor origin, histological tumor grade, clinical (FIGO) stage, and metastasis, with ER percentages as the covariate. Relationships between the percentages of RARα-labeled cells and ER-labeled cells were evaluated by linear regression analysis, the Pearson correlation coefficient, and data plotting; the individual relationships between the percentages of RARα-labeled cells with metastatic source, tumor origin, histological grade, and FIGO stage were evaluated by one-way analysis of variance. A *P* value less than 0.05 was considered to be significant. The general linear model with LSMEANS posthoc testing and the plot procedures of the SAS package (SAS Institute, Cary, NC) were used for these statistical analyses.

Table 2. Regression and Variant Analyses of the Relationship of the Percentage of RARα-Labeled Tumor Cells with Several Continuous and Categorical Variables

Variable*	*P* value
Continuous	
ER percentage†	0.0002
Categorical	
Histological grade	0.038
FIGO stage	0.139
Tumor origin‡	0.170
Metastasis§	0.561

*Also see Figure 5.
†Percentage of ER-positive tumor cells (numerator) relative to total number of tumor cells (denominator).
‡Ovarian, tubal, or peritoneal.
§Tumor specimen derived from an intra-abdominal metastasis (as opposed to the primary site).

Results

Cellular Localization of RARα and ER Proteins

RARα immunohistochemical staining was detected in all specimens, although the degree and cell-type distribution of immunolabeled cells varied widely among tissue specimens. Overall, RARα-positive tumor cells were present in more well differentiated portions of tumor growing as papillary fronts (Figures 3, A, B, and C, and 4, A and C) and much less prominent in poorly differentiated areas of tumor typified by dense cellularity and a predominantly solid pattern of growth (Figures 3E and 4, E and F). The percentage of RARα-labeled tumor cell nuclei was somewhat increased in the moderately differentiated, intermediate-grade (grade II) tumors containing variably prominent tubulopapillary structures, as compared with the less differentiated, high-grade (grade III) serous adenocarcinomas: the mean RARα labeling percentages for grades II and III were 67% (range 58 to 84%) and 30% (range 2 to 66%), respectively. A certain degree of RARα intratumoral staining heterogeneity was present in high-grade tumors, and when present it was usually associated with better differentiated papillary foci (Figure 3D). However, RARα staining was also detected in poorly differentiated areas of tumor, either in scattered, individual cells, or in clusters of tumor cells (Figure 3E).

Robust RARα staining was detected in benign, mononuclear cells infiltrating haphazardly the tumor stroma, known as TILs (Figures 3F and 4, E and F). RARα-positive TILs occurred either as prominent nodular aggregates (Figure 3F) or as scanty stromal mononuclear cell infiltrates (Figure 4, E and F). In one of our cases representing a high-grade, FIGO stage IV serous adenocarcinoma of peritoneal origin (Table 1, case 16), RARα staining was present in TILs but was largely absent in tumor cells (Figure 4, E and F). Also, RARα staining was present in nonneoplastic mesenchymal cells, consistent with fibroblasts of the desmoplastic tumor stroma, in endothelial cells of tumor blood vessels (Figure 3B, arrowheads), and in resident ovarian stromal cells (not shown).

A distinctive ER nuclear staining was detected variously in all specimens. Immunoreactivity among tumor cells was particularly widespread in papillary areas (Figure 4, B and D) but was also present in poorly differentiated areas of tumor (not shown). The number of ER-labeled cells in any given tissue specimen was consistently higher as compared with RARα (the mean ER labeling percentages for grades II and III were 80% (range 71 to 94%) and 53% (range 36 to 74%), respectively), but followed a differentiation-dependent trend similar to that of RARα. A strong linear relationship was found between percentages of RARα- and ER-immunolabeled tumor cells (Table 2, Figure 5) (also, see below).

A deviation of this pattern was observed only in two specimens in which the percentage of ER-positive tumor cells was disproportionately higher as compared with RARα-positive tumor cells (Table 1, cases 7 and 16) (Figure 5): The first, case 7, was derived from an omental metastasis of a grade III/FIGO stage IIIB serous adenocarcinoma of ovarian origin. The second specimen, case

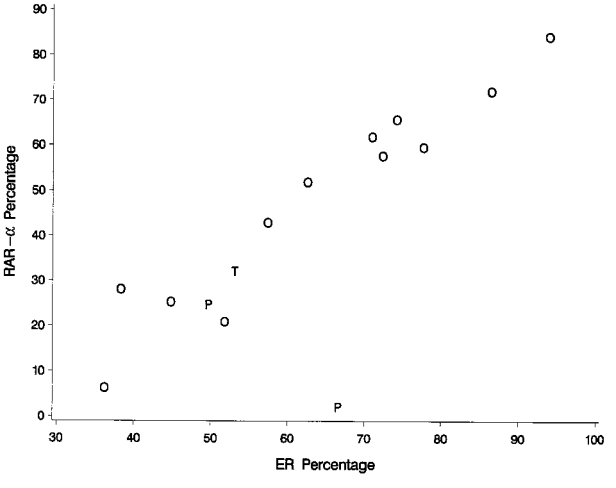

Figure 5. Data plotting depicting a strong linear relationship is found between percentages of RARα- and ER-immunolabeled tumor cells ($r = 0.825$). A significant relationship between tumor origin (O, ovary; T, fallopian tube; P, peritoneum) and the percentage of RARα-positive tumor cells is found only when the covariate effects of ER labeling are taken into account. This relationship appears to derive from the single data point of peritoneal tumor origin (P) that plots below the rest of the data (lower right portion of the graph). However, more data from serous tumors of peritoneal origin are needed before any conclusions may be drawn.

16, was from a grade III/FIGO stage IV serous adenocarcinoma of peritoneal origin (Figure 4, D and F).

TILs and stromal fibroblasts of the tumor were ER negative; however, ovarian stromal cells were ER positive (not shown).

Statistical Analysis

The percentages of ER-labeled cells and the tumor origin were found to be jointly related to the percentages of RARα-labeled cells using analysis of covariance ($P < 0.005$), and on posthoc testing, the percentages of RARα-labeled cells when corrected for the covariate effects of ER labeling in the analysis of covariance model were found to be significantly higher in serous adenocarcinomas of ovarian origin than in homologous tumors of peritoneal origin ($P < 0.01$). Interestingly, when the relationship of RARα and tumor origin was examined by analysis of variance (without the information supplied by the ER labeling), no statistical significance was found (Table 2). Results of statistical analysis of the individual relationships between RARα immunohistochemical labeling percentages of tumor cells and ER percentages, metastatic source of the specimen, tumor grade, and FIGO stage are shown in Table 2. A strong linear relationship was found between the percentages of RARα- and ER-immunostained tumor cells ($r = 0.825$) by linear regression analysis and data plotting (Table 2, Figure 5). A modest relationship ($P < 0.04$) was found between the percentages of RARα-positive tumor cells and histological grade with slightly higher labeling counts noted in grade II as compared with grade III serous tumors. No statistically significant relationship was found, respectively, between RARα immunoreactivity and FIGO stage or specimen sampling from metastatic *versus* primary tumor sites.

Discussion

Cellular Distribution of RARα Protein in Serous Adenocarcinomas

This study provides new data with regard to the cellular distribution of RARα protein in 16 frozen, surgically resected serous adenocarcinoma specimens originating in the ovaries, fallopian tubes and the pelvic peritoneum, ie, the so-called secondary müllerian system. Also, it examines the relationship between RARα and ER protein expression by comparing the percentage of immunostained tumor cells for either receptor in surgical specimens. The relatively small number of cases evaluated notwithstanding, this study indicates a strong linear relationship between the percentages of RARα- and ER-immunostained tumor cells as determined by linear regression analysis (Table 2, Figure 5). RARα and ER immunoreactivities are present in both intermediate- (grade II) and high-grade (grade III) lesions, corresponding, for the most part, to advanced FIGO stage serous ovarian adenocarcinomas. A modest inverse relationship is found between the percentage of RARα-positive tumor cells and histological grade, attesting to a differentiation-dependent trend (Table 2). Because histological grade in ovarian adenocarcinomas is largely a function of differentiation, there is a higher percentage of RARα-positive tumor cells in grade II tumors with papillary areas as compared with the grade III tumors. However, grade III tumors may also contain papillary foci, as well as a relatively small number of poorly differentiated RARα-positive cells. Conversely, no significant relationship is found between RARα-labeled cells and such categorical variables as FIGO stage; site of origin of tumor, ie, from ovary, fallopian tubes, or pelvic peritoneum; and source of specimen from an intra-abdominal metastasis (as opposed to the primary site) (Table 2). Thus, RARα tumor cell labeling is present in specimens from primary ovarian, as well as metastatic tumor implants in the omentum, peritoneum, and parametria, indicating that serous carcinomas are capable of expressing RARα, and also ER, despite high histological grade and advanced clinical stage.

Previous *in vitro* studies have shown that RARα plays a major role in the growth inhibition of mammary cancer cells[37,38] and ovarian cancer cells[39,40] by retinoids in a dose-dependent manner. Still, the presence of RARα in intermediate- to high-grade, advanced-stage serous tumors demonstrated in this study is similar to that described previously in breast carcinomas.[41] van der Leede and collaborators[41] have proposed an apparent uncoupling of RARα expression and proliferation inhibition, offering a threefold explanation for this phenomenon: 1) perturbed transcriptional regulation as part of tumor progression; 2) loss of mechanisms of RARα down-regulation; and/or 3) insufficient retinoid levels to achieve down-regulation, hence culminating in overexpression of RARα.[41] It remains to be determined whether there are alterations in the levels of RARα isoforms in cancer cells because of alterations in the factors regulating RARα gene transcription at the promoter level. In this regard, there is evidence of estrogen-induced expression of RARα1 mRNA, but lack of RARα2 transcripts, in ER-positive breast carcinoma cell lines.[38] Interestingly, serum retinol has been found to be significantly lower in ovarian cancer patients,[42] although the actual content of retinoids in ovarian adenocarcinoma specimens is unknown. Collectively, malignant tumors *in vivo* may exhibit dysregulation of cellular differentiation signaling pathways, which may also involve RARs/RXRs.

To date, there has been only a small number of immunohistochemical studies aimed at the localization of RARs in normal and neoplastic tissues. This may be attributed to several confounding factors. The low concentration of individual RAR epitopes may hinder their detection by immunohistochemistry,[43,44] even in neoplastic cells, such as leukemia cells.[45] At this time, there is only a limited number of commercially available subtype-specific antibodies. Their immunohistochemical performance in chemically fixed tissues is not well defined and may be punctuated by unexpected (or even spurious) localizations. Also, the presence of cellular retinoid binding proteins may hinder the localization of RARs, a problem that has been addressed previously in the context of autoradiography.[46] Thus far, immunohisto-

chemical studies on surgical tumor specimens are limited to the evaluation of RARα in breast carcinomas.[41,47] The latter two studies have used archival, formalin-fixed, paraffin-embedded tissue sections and different antibodies. In one of them, unexpected RARα cytoplasmic staining of tumor cells has been detected in addition to nuclear staining.[41] We have also observed a similar cytoplasmic localization for RARα in a large number of formalin-fixed, paraffin-embedded ovarian tumor specimens, which led us to withdraw these data from the present study. Clearly, an abnormal processing of RARα in tumor cells deserves consideration in this respect;[41] however, in our view, this appears less likely, because cytoplasmic staining has also been detected, by us, in benign epithelial and mesenchymal cells in paraffin, but not in frozen, acetone-fixed sections of ovarian, adnexal, and other unrelated tissues (CDK, Y. Yu, IS, and KJS, unpublished observations).

To minimize potential artifacts introduced either by the use of cross-linking fixatives or by conventional tissue processing and paraffin embedding, we elected to perform immunohistochemical staining only on frozen sections fixed briefly in cold acetone in −20°C. This is a reliable method that renders unambiguous nuclear localizations, at least as evidenced with the RARα antibody used in this study. That the anti-RARα antibody recognizes RARα in bacteria is also supported by the expected nuclear immunolocalizations in human cells *in situ*, thus collectively providing strong evidence for specificity. Additional issues regarding accurate determination of steroid receptors in general and RARs in particular in surgical specimens include tumor cell heterogeneity and receptor expression by nonneoplastic cell types within the tumor, such as TILs, vascular endothelial cells, and stromal fibroblasts. Thus, immunohistochemistry is the single most appropriate and accurate method of determining the cellular source of steroid receptor protein in ovarian tumor specimens.[48]

RARα Localization in TILs

The widespread presence and intensity of RARα staining in TILs is noteworthy. It is detected in >80% of mononuclear inflammatory cells randomly dispersed either in the periphery or within sheets of cancer cells. Although all 16 specimens contain RARα-positive mononuclear cells in varying proportions, 6 of 16 tumors exhibit prominent TILs. A similar RARα staining pattern of the TILs has been described, in passing, in a series of breast carcinomas.[41] Although the immunological significance of TILs in epithelial tumors in general and in ovarian carcinomas in particular is unclear, it has been suggested that they may represent tumor cytolytic oligoclonal T-cell responses.[49]

Retinoids are multicellular immunomodulators both *in vivo* and *in vitro*, including but not limited to various tumor cell types, human and murine thymocytes, fibroblasts, Langerhans' cells, natural killer cells, and T lymphocytes.[50] Both retinol and RA induce vigorous proliferative responses on human peripheral blood mononuclear cells after stimulation with anti-CD3 antibodies, which is specifically mediated through the clonotypic T-cell receptor-CD3 complex and correlates with the up-regulation of surface T-lymphocyte adhesion/activation markers, as well as an augmentation of interleukin-2 and interferon-γ transcripts.[50] It has been shown that RA promotes proliferation and induces RARα gene expression in murine T lymphocytes and that RA and RARα might function in T cells as ligand-inducible transcriptional enhancer factors.[51] It remains to be determined whether the expression of RARα in ovarian carcinoma TILs is related to tumor antigen-specific or oligoclonal T-cell response(s).

Relationship of RARα and ER Immunoreactivity Profiles

Despite the exclusion of RARA, the gene for RARα, as a candidate gene for *brca*-1 (the susceptibility gene for hereditary breast-ovarian cancer),[52] there is nonetheless evidence to suggest that ovarian and breast cancer cells may exploit similar signaling pathways for growth and differentiation, insofar as they are both steroid hormone-dependent neoplasms. Compared with mammary[53] and endometrial cancers[54] in which the ER status is prognostically significant, a linear relationship between ER level and survival has not been established in ovarian carcinomas.[55–57] By biochemical radioligand assays, ovarian carcinomas have been shown to contain ER in the 60% tumor range.[9] Others have reported that this percentage declines to 38% when immunohistochemistry is used as the method of detection.[48] Using solely cryostat sections and monoclonal antibody 6F11, we have found ER mean labeling percentages of 80% for grade II and 53% for grade III serous tumors. Interestingly, Slotman and coworkers[58] have found no correlation between tumor ploidy and histological grade, stage of disease, and ER content in ovarian adenocarcinomas.

There are several lines of evidence suggesting a positive relationship between RARα and ER gene[59] and protein[57] expression in breast carcinomas and human mammary carcinoma cell lines. RARα appears to be required for inhibition of anchorage-independent growth either by natural (all-*trans*-RA) or by conformationally restricted retinoids with agonist activity in the MCF-7 ER-positive breast carcinoma cell line.[60] Moreover, although a statistically significant correlation has been established between RARα and ER mRNA in primary breast tumors, no such correlation has been found to exist between ER levels and expression of either RARβ, RARγ, RXRα, RXRβ, or RXRγ mRNA levels.[61,62] It is believed that the relationship between RARα and ER gene expression is partly caused by estradiol enhancement of RARα gene expression,[59,63] which is mediated through an imperfect half-palindromic estrogen response element and Sp1 motifs.[63] In contrast to RARα, the mechanism responsible for the retinoid sensitivity of breast cancer cells does not involve transcriptional modulation of the RXRs by RA.[64] Most ER-negative breast carcinomas express lower levels of RARα and are largely resistant to the growth-inhibition effects of RA compounds *in vitro*.[65] However, estradiol-independent enhancement of RARα gene ex-

pression has been demonstrated in certain ER-negative breast cancer cell lines, such as SKBR-31 and MDA-MB-435.[65] RA-mediated growth inhibition in these lines is accomplished via a 72-bp fragment of RARα promoter that contains unique *cis* elements.[65] To date, there are conflicting reports with regard to a relationship between RARα and ER in breast tumors at the protein level. Recently, Han and co-workers[47] have reported that RARα expression is significantly increased in ER-positive breast tumors as determined by immunohistochemistry and image cytometry. This is in contrast to a previous immunohistochemical study claiming that no such relationship exists between RARα and ER status.[41]

It has been previously shown that 17-β-estradiol may regulate growth in human ovarian carcinoma lines.[66] 17-β-Estradiol can stimulate the growth of populations of ER-positive ovarian carcinoma cells that may in turn be associated with changes in the cellular levels of steroid hormone receptors.[66] Our findings in serous ovarian carcinomas are consistent with those by Han and collaborators[47] in breast carcinomas, insofar as they support a relationship trend between RARα and ER expression in certain steroid hormone-dependent epithelial neoplasms. However, as alluded to above, a definitive determination in this regard awaits the evaluation of a larger sample of tumors.

Concluding Remarks and Future Directions

In the past, encouraging results have been obtained with RA-based treatment of patients with locally advanced squamous cell carcinoma of the uterine cervix[67] and cisplatin-resistant metastatic endometrial adenocarcinoma.[68] Because RARα plays a major role in retinoid-mediated growth inhibition of breast and ovarian cancer cells *in vitro*, it is also possible that patients with breast[41] and ovarian carcinomas could be responsive to retinoids independently of their responsiveness to antiestrogen regimens. Based on their specific RAR/RXR profile and method of delivery *in vivo*, ovarian carcinomas may be amenable to a variety of therapeutic interventions using conformationally restricted retinoid ligands.[40] This underscores the importance of future studies aiming to elucidate the full RAR/RXR profile in serous adenocarcinomas of the ovary and secondary müllerian system.

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