

Retinoid receptors in ovarian cancer: expression and prognosis

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Background: Ovarian cancer is frequently lethal despite aggressive multimodal therapy, and new therapies are therefore needed. Retinoids are potential candidate drugs: they prevent the development of ovarian carcinoma and enhance the efficacy of cytotoxic drugs in ovarian cancer cells. At present, little is known about the retinoid receptor expression in ovarian cancer.

Patients and methods: The retinoid receptors comprise two classes, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), each with three subclasses, α , β and γ . We investigated the expression of the subtypes RAR α , RAR γ , RXR α and RXR β by immunohistochemistry in ovarian cancers of 80 patients, and assessed their prognostic significance. In addition, we quantified the expression of retinoid receptor mRNA using real-time PCR and correlated the results with clinical characteristics.

Results: RAR α and RXR β were highly expressed in a majority of ovarian cancers, particularly in advanced stages. High expression of RAR α was an independent negative prognostic factor of survival in addition to FIGO stage, age and p53 accumulation. The mRNA expression of retinoid receptors did not correlate with clinical properties of the tumors.

Conclusions: Retinoic acid receptors are frequently and strongly expressed in epithelial ovarian cancer and may be indicators of an adverse prognosis. This study provides the molecular basis for the therapeutic use of retinoids in ovarian cancer.

Key words: nuclear receptors, ovarian neoplasms, prognostic factors, retinoic acid receptors

Introduction

Ovarian cancer has the highest mortality rate among malignancies of the female genital tract and is the fifth leading cause of cancer death in women in the USA [1]. In Switzerland, the incidence and mortality is comparable to other Western countries [2]. By the time of diagnosis, most patients have advanced stages of disease. Despite multimodal therapy, 60% to 85% of these patients will die from recurrent cancer [3]. Therefore, ongoing research focuses on the development of more potent therapies.

Retinoids are potential candidates for new treatment strategies for ovarian cancer. Retinoids are natural and synthetic derivatives of retinol (vitamin A). The naturally occurring retinoids, all-*trans* retinoic acid, 9-*cis* retinoic acid and 13-*cis* retinoic acid, are generated from diet-derived retinol. Retinoids are ligands of cellular receptors of retinoic acid, including retinoic acid receptors (RARs) and retinoid X receptors (RXRs,

retinoid receptors) [4]. These are members of the steroid and thyroid hormone receptor superfamily [5]. Each subtype of both retinoid receptor classes (RAR α , RAR β , RAR γ , and RXR α , RXR β , RXR γ , respectively) is encoded by separate genes. Multiple isoforms of receptor subtypes exist as a result of alternate splicing. The receptors act mainly as RAR–RXR heterodimers, bind to specific DNA sequences (retinoic acid response elements) and act as ligand-dependent transcription factors [6].

The physiological functions of retinoids include the control of proliferation, apoptosis and differentiation in normal cells during growth and development [6]. Retinoids are essential in the maintenance of normal adult epithelial differentiation. Vitamin A-deficient experimental animals have long been known to develop squamous metaplasia and other precancerous lesions. These alterations were reversed by vitamin A repletion [7]. Several subsequent studies demonstrated the effect of retinoids in the chemoprevention of certain malignant tumors [1, 8, 9]. Indeed, high dietary intake of carotenoids, which are precursors of the physiologic retinoids, had a moderate protective effect against ovarian cancer [10–14]. The same effect was observed in a clinical trial evaluating the effect of the atypical retinoid fenretinide on the prevention of

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contralateral breast cancer [15]. Surprisingly, none of the patients in the treatment group developed ovarian cancer during the intervention period. This benefit was lost when the drug was discontinued, suggesting an effect of fenretinide against ovarian cancer. [16]. The growth inhibitory effect of retinoids has also been extensively studied in established ovarian carcinoma cell lines [17]. Several *in vitro* studies revealed that numerous mechanisms are involved such as induction of apoptosis by various mechanisms [6, 18–20], interference with cell cycle control [21] and cross-talk with other signaling molecules such as AP1 [22] and epithelial growth factor [23].

Retinoids enhance the effect of cytotoxic drugs, such as cisplatin [24–26] and docetaxel [27], and of ionizing radiation [28] in ovarian cancer cell lines. The toxic effects of retinoids differ from those of traditional cytotoxic agents. Thus, retinoids are potentially attractive partners for combination therapies with cytotoxic drugs. Certain retinoids are in clinical use, such as all-*trans* retinoic acid in the treatment of acute promyelocytic leukemia [29] and targeetin in the therapy of cutaneous T-cell lymphoma [30], but their clinical application for the therapy of solid tumors such as ovarian cancer is still experimental.

Despite the knowledge of the biological effects of retinoids in ovarian cancer, limited information is available on the expression of retinoid receptors in these tumors [31]. Therefore, the aim of the present study was to determine the expression of RAR α , RAR γ , RXR α and RXR β in paraffin-embedded ovarian cancer tissues using immunohistochemistry, and to measure the mRNA expression of all RAR and RXR subtypes in frozen tissue samples with real-time quantitative PCR.

Patients and methods

Tissue samples

Two tissue banks of ovarian tumors were used: between 1984 and 1996, 172 patients were treated at the University Hospital of Berne for malignant tumors of the ovaries. One hundred and forty-five of these patients had invasive epithelial ovarian adenocarcinoma and were included in a previous study [32]. Of 80 of these patients, paraffin-embedded tissue obtained from surgical resection specimens was available for immunohistochemical analysis after review of the slides by one of the authors (M.K.). All tumors were classified and graded according to standard WHO criteria [33]. Frozen samples from 35 ovarian adenocarcinomas and adenomas were collected in 1999 at operating theatres of the University Hospital of Berne, Switzerland. Tumor samples were selected by the surgeon and by a postdoctoral fellow of our laboratory to exclude macroscopically identifiable non-tumor tissue. The tissue was snap-frozen in liquid nitrogen in the operating theatre and stored at -70°C until further processing.

Patient data

Patient information was collected from clinical records after obtaining the permission of the local ethics committee according to Swiss law; the patient information was immediately anonymized. All 80 patients included in the immunohistochemical study had undergone cytoreductive surgery. Patient ages ranged from 18 to 82 years (median 57.5). Forty-seven

patients (59%) had serous carcinoma, 15 (18%) had mucinous carcinoma and 13 (16%) had endometrioid adenocarcinoma. Two patients (2%) were diagnosed with a clear cell carcinoma and three (4%) had unclassified carcinomas. Sixty-six of 80 patients had grade 2 or 3 carcinomas. The majority of the patients had FIGO stage III/IV disease at the time of diagnosis (40 of 80 stage III, 21 of 80 stage IV).

Patients included in the real-time PCR (RT-PCR) analysis had undergone resection of a tumorous lesion of the ovary. Patient ages ranged from 41 to 83 years (median 61). Thirteen patients (37%) had ovarian adenomas and 22 (63%) had epithelial ovarian adenocarcinoma. Twenty-one of 22 carcinomas (96%) were grade 2 or 3. Seventeen of 22 patients had FIGO stage III/IV disease (10 stage III, seven stage IV). The main characteristics of both patient sets are summarized in Table 1.

Immunohistochemistry

Tissues were prepared according to standard procedures including steps of dewaxing, rehydration and pretreatment by boiling in a pressure cooker (10 mM citrate buffer, pH 6.0), by boiling in a microwave oven (citrate buffer; 100 mM Tris–5% urea, pH 9.5), or by digestion with 1 mg/ml trypsin. The bound antibodies were visualized with biotinylated secondary antibodies (DakoCytomation, Glostrup, Denmark) in conjunction with streptavidin–biotin complex/horseradish peroxidase (Vector, Burlingame, CA, USA) and developed in 0.1% 3,3-diaminobenzidine (Sigma, St Louis, MO, USA) with 0.03% H_2O_2 . The sections were counterstained with hematoxylin. Positive controls were stained in parallel with each series. They included human skin and breast tissue for the retinoic acid receptors, tonsil for bcl-2, bax and MIB-1, and colon cancer for p53 and vimentin.

The monoclonal antibodies used included clone Vim3B4 for vimentin, DO-7 for p53, 124 for bcl-2, MIB-1 for Ki-67 (all from DakoCytomation), 336* for RAR β , 147 for RXR β and 1373* for RXR γ (Neomarkers, Fremont, CA, USA); polyclonal antibodies were used for bax (DakoCytomation): RAR α , RAR β *, RAR γ , RXR α , RXR β * and RXR γ * (all from

Table 1. Patient characteristics

| | | Paraffin | | Frozen | |
|---------------------------------|--------------|----------|-----|--------|----|
| | | n | % | n | % |
| Number of patients | | 80 | | 35 | |
| Age at diagnosis | <50 years | 23 | 29 | 11 | 31 |
| | 51–65 years | 29 | 36 | 10 | 29 |
| | >65 years | 28 | 35 | 14 | 40 |
| Histology ^a | Carcinomas | 80 | 100 | 22 | 63 |
| | Adenomas | 0 | 0 | 13 | 37 |
| | Serous | 48 | 60 | 11 | 50 |
| | Mucinous | 14 | 18 | 2 | 9 |
| | Endometrioid | 13 | 16 | 2 | 9 |
| | Clear cell | 2 | 3 | 1 | 5 |
| Histological grade ^a | NOS | 3 | 4 | 6 | 27 |
| | 1 | 8 | 10 | 1 | 5 |
| | 2 | 19 | 24 | 5 | 23 |
| FIGO stage ^a | 3 | 53 | 66 | 16 | 73 |
| | I&II | 19 | 24 | 3 | 14 |
| | III | 40 | 50 | 10 | 45 |
| | IV | 21 | 26 | 7 | 32 |

^aCarcinomas only.

NOS of otherwise specified.

Santa Cruz, CA, USA). Antibodies marked with an asterisk showed no reactivity when tested on various samples of formalin-fixed, paraffin-embedded tissues and were therefore not used for the study.

The expression of all antigens except p53, Ki-67 and bax was scored semiquantitatively by a modified histoscore method [34, 35]. The proportion score (0, $\leq 5\%$; 1, $\leq 30\%$; 2, $\leq 50\%$; 3, $\leq 70\%$; 4, $\leq 95\%$; 5, $> 95\%$) was added to the intensity score (1, weak; 2, intermediate; 3, strong). Accumulation of abnormal p53 protein was defined as nuclear staining in $> 10\%$ of tumor cells [36]. For Ki-67, a proportion score was used (0, $< 5\%$; 1, $< 10\%$; 2, $< 40\%$; 3, $> 40\%$) [37]. Bax was considered positive if the intensity score was 2 [38]. Vimentin was used to assess the quality of the tissue sections [39]. Each slide was evaluated independently by two observers (P.C.K. and M.K.); divergences were resolved using a double microscope.

cDNA synthesis and RT-PCR

Thirty milligrams of frozen tumor tissue was disrupted with a mortar and pestle with concurrent cooling with liquid nitrogen. Total RNA was extracted using the RNeasy Mini kit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. cDNA was synthesized from 1 μg total RNA in 25 ml reaction buffer using MMLV reverse transcriptase, recombinant RNasin (Promega, Wallisellen, Switzerland) and random primers p(dN)₆ (Roche, Rotkreuz, Switzerland) after DNase digestion with DNase I (Roche, Rotkreuz, Switzerland). The cDNA products were used for RT-PCR in a reaction mixture (25 μl) containing 12.5 μl 2 \times SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and forward and reverse primer at 300 nM. The primers for RAR α , RAR β , RAR γ and RXR α , RXR β , RXR γ were designed according to the NCBI reference sequences for the corresponding genes (Table 2). 7S rRNA was used as reference target sequence (primers: 5'-accaccagggtgcctaagga and 5'-cacgggagtttgacctgct). We used the ABI PRISM 7700 Sequence Detection System for RT-PCR. The following parameters were used for the RT-PCR: initial denaturation (10 min at 95°C) followed by 45 amplification cycles (denaturation for 30 s at 95°C, annealing for 30 s at 55°C and elongation for 15 s at 72°C). We excluded the formation of primer dimers by final melting curve analysis after RT-PCR (temperature slope 0.05°C/s from 40 to 100°C). All samples were measured as duplicates with a no-template control in each run. PCR products of each primer pair were

cloned into plasmids using the TOPO TA Cloning Reaction (Invitrogen, Basel, Switzerland) and calibration curves for each resulting plasmid were established using 10-fold serial dilutions in 50 $\mu\text{g}/\mu\text{l}$ yeast RNA (Ambion, Huntingdon, UK). The concentration of 7S was used as a control for RNA content and for normalization of RNA content of each sample.

Statistical analysis

Group comparisons were based on Pearson's χ^2 -test for nominal and on the linear χ^2 -test for ordinal variables [40]. Correlations were evaluated with the Spearman rank correlation coefficient. The RT-PCR data were analyzed with non-parametric tests as the distributions of the retinoid receptor: 7S ratios were highly skewed; the Mann-Whitney *U* statistic was used for nominal and the Jonckheere-Terpstra test for intrinsically ordered grouping variables. Survival was analyzed by Kaplan-Meier plots [41] and proportional hazards regression [42]. The significant covariates of the univariate analyses were used for the multivariate analysis; log-minus-log plots did not reveal a violation of the proportionality assumption. The patients were dichotomized by histoscore such that approximately half were below and half above the cut-off value. In addition, we performed the same analyses with age and histoscore as continuous variables; the results were virtually identical (not shown). The global model significance was based on the χ^2 method and the significance of individual covariates on the Wald statistic. Two-sided tests were used throughout. All statistical analyses were performed using SPSS 11.5 (SPSS Inc., Chicago, IL, USA).

Results

Immunohistochemical study

Nuclear expression of RAR α , RAR γ , RXR α and RXR β was present in carcinoma cells (Figure 1). Specific immunoreactivity for RAR β and RXR γ was not discernible, either in a selection of ovarian cancers or in other tissues that were supposed to express the antigen. No mRNA for RXR γ was detected in frozen ovarian cancer tissue in contrast to mRNA for RAR β . The expression of RAR α , RAR γ , RXR α and RXR β is summarized in Table 3.

Table 2. Real time PCR primers sequences for RAR α , RAR β , RAR γ and RXR α , RXR β , RXR γ

| Gene | Reference sequence no. | Orientation | Sequence | Positions | Product size (bp) |
|--------------|------------------------|-------------|------------------------------|-----------|-------------------|
| RAR α | NM_000964 | Forward | 5'-cagagcagcagttctgaagagata | 292–315 | 184 |
| | | Reverse | 5'-gacacgtgtacaccatgttctct | 452–475 | |
| RAR β | NM_000965 | Forward | 5'-gtcaccagataagaactgtgtta | 819–812 | 179 |
| | | Reverse | 5'-actcagctgtcatttcatagctctc | 937–997 | |
| RAR γ | NM_000966 | Forward | 5'-ctgtatcatcaacaaggtgacca | 822–844 | 183 |
| | | Reverse | 5'-tggatgatgagctcttctaactgag | 980–1003 | |
| RXR α | NM_002957 | Forward | 5'-tgcttcgtgtaagcaagatacaag | 4699–4723 | 192 |
| | | Reverse | 5'-ctctttatggatctgtcatcctctc | 4866–4890 | |
| RXR β | NM_021976 | Forward | 5'-ccagagtctcttttaccactcacc | 2293–2317 | 182 |
| | | Reverse | 5'-tcttagtcaacctggaaagtacag | 2450–2474 | |
| RXR γ | NM_006917 | Forward | 5'-gatctagaggcagattcctgactaa | 6–30 | 187 |
| | | Reverse | 5'-catgtttactctgcatgttcatttc | 167–191 | |

RAR, retinoid acid receptor; RXR, retinoid X receptor.

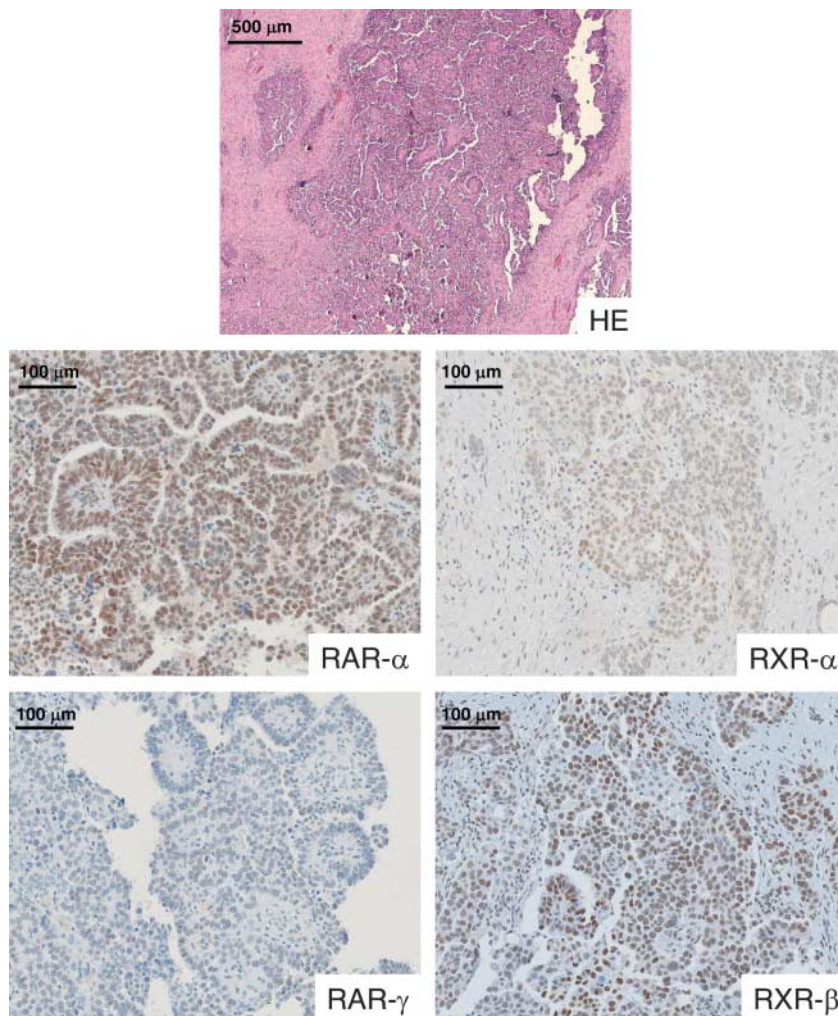


Figure 1. Immunohistochemical staining of retinoid receptors in epithelial ovarian cancer. HE, hematoxylin and eosin; RAR, retinoic acid receptor; RXR, retinoid X receptor.

RAR α was expressed in the majority of the tissue sections, and 48 patients (60%) had a high score of 7 or 8. High RAR α expression was significantly related to advanced FIGO stages ($P=0.04$). The expression of RAR γ was similar in different histological types of ovarian carcinoma. Tumors with lower FIGO stages had lower levels of RAR γ . RXR α was highly expressed in most of the tissue samples; in 36 patients (45%) a score of 7 to 8 was observed. There were no significant correlations of RXR α expression with clinical or morphological features. RXR β was strongly expressed in the majority of the tumors, with 70% scoring 7 to 8. Low FIGO stage tumors showed a trend towards lower expression levels. There was no significant relation between RXR β expression and other clinical or pathological features. Ki-67 and p53 were present in the nuclei, while bcl-2 and bax antigens were distributed in the cytoplasm. Ki-67 was correlated with tumor grade ($P=0.03$), whereas the detection of p53 was not associated with clinical variables. The expression of bcl-2 was low in the majority of the patients and was not correlated with other variables. Bax showed a high expression level in the majority of the tumors, and there was

a trend towards higher bax expression in low-grade tumors ($P=0.07$).

The expression of RAR α and RXR α was positively correlated (Spearman's rank correlation coefficient 0.32; $P=0.004$), as was that of RAR α and RXR β (correlation coefficient 0.26; $P=0.02$). RAR γ was not correlated with the evaluated antigens. RXR α was also positively correlated with bax (correlation coefficient 0.22; $P=0.04$), as was RXR β with bcl-2 (correlation coefficient 0.27; $P=0.014$). No correlation was observed between markers of proliferation (Ki-67), p53 status and apoptosis (bcl-2 and bax).

Survival data are summarized in Figure 2 and Table 4. Patients with FIGO stage III and IV cancers had median survival times of 52 and 14 months, respectively, whereas the median survival was not reached in stage I and II cancers. RAR α and RAR γ were predictors of survival in univariate proportional hazards regression analysis, in addition to age, FIGO stage and p53 status. FIGO stage, p53 status and RAR α expression were independent predictors of survival: high expression of RAR α indicated a higher risk of death from ovarian cancer.

Table 3. Expression of retinoid receptor, ki-67, p53, bcl-2 and bax

| All patients | Age class | | | Histology | | | | | Grade | | | Stage | | |
|----------------------------|-----------|----------|----------|-----------|----------|--------------|--------------|------------|----------|-----------|----------|----------|----------|----------|
| | <50 | 51–65 | >65 | Serous | Mucinous | Endometrioid | Unclassified | Clear cell | 1 | 2 | 3 | FIGO 1&2 | FIGO 3 | FIGO 4 |
| | 23 | 29 | 28 | 48 | 14 | 13 | 1 | 2 | 8 | 19 | 53 | 19 | 40 | 21 |
| Immunohistochemistry score | | | | | | | | | | | | | | |
| RAR α | | | | | | | | | | | | | | |
| 1–3 | 1 (4%) | 2 (7%) | 0 | 1 (2%) | 1 (8%) | 0 | 0 | 1 (50%) | 1 (14%) | 0 | 2 (4%) | 2 (11%) | 0 | 1 (5%) |
| 4–6 | 9 (39%) | 13 (45%) | 6 (22%) | 19 (40%) | 5 (38%) | 3 (23%) | 0 | 1 (50%) | 5 (71%) | 7 (37%) | 16 (30%) | 10 (53%) | 11 (28%) | 7 (33%) |
| 7–8 | 13 (57%) | 14 (48%) | 21 (78%) | 28 (58%) | 7 (54%) | 10 (77%) | 2 (100%) | 0 | 1 (14%) | 12 (63%) | 35 (66%) | 7 (37%) | 28 (72%) | 13 (62%) |
| <i>P</i> * | | 0.11 | | | | 0.13 | | | | | 0.047 | | | 0.036 |
| RAR γ | | | | | | | | | | | | | | |
| 1–3 | 13 (57%) | 10 (34%) | 12 (43%) | 15 (31%) | 9 (64%) | 8 (62%) | 1 (50%) | 2 (100%) | 4 (50%) | 11 (58%) | 20 (38%) | 13 (68%) | 17 (43%) | 5 (24%) |
| 4–6 | 8 (35%) | 15 (52%) | 13 (46%) | 25 (52%) | 5 (36%) | 4 (31%) | 1 (50%) | 0 | 4 (50%) | 7 (37%) | 25 (47%) | 5 (26%) | 16 (40%) | 15 (71%) |
| 7–8 | 2 (9%) | 4 (14%) | 3 (11%) | 8 (17%) | 0 | 1 (8%) | 0 | 0 | 0 | 1 (5%) | 8 (15%) | 1 (5%) | 7 (18%) | 1 (5%) |
| <i>P</i> * | | 0.47 | | | | 0.16 | | | | | 0.11 | | | 0.030 |
| RXR α | | | | | | | | | | | | | | |
| 1–3 | 6 (26%) | 11 (38%) | 7 (25%) | 17 (25%) | 2 (14%) | 4 (31%) | 0 | 1 (50%) | 3 (38%) | 5 (26%) | 16 (30%) | 4 (21%) | 11 (28%) | 9 (43%) |
| 4–6 | 9 (39%) | 4 (14%) | 7 (25%) | 11 (23%) | 3 (21%) | 4 (31%) | 1 (50%) | 1 (50%) | 1 (13%) | 3 (16%) | 16 (30%) | 6 (32%) | 11 (28%) | 3 (14%) |
| 7–8 | 8 (35%) | 14 (48%) | 14 (50%) | 20 (42%) | 9 (64%) | 5 (38%) | 1 (50%) | 0 | 4 (50%) | 11 (58%) | 21 (40%) | 9 (47%) | 18 (45%) | 9 (43%) |
| <i>P</i> * | | 0.52 | | | | 0.56 | | | | | 0.63 | | | 0.40 |
| RXR β | | | | | | | | | | | | | | |
| 1–3 | 2 (9%) | 1 (3%) | 1 (4%) | 2 (4%) | 1 (7%) | 1 (8%) | 0 | 0 | 1 (13%) | 1 (15%) | 2 (4%) | 1 (5%) | 2 (5%) | 1 (5%) |
| 4–6 | 7 (30%) | 7 (24%) | 7 (25%) | 10 (21%) | 5 (36%) | 4 (31%) | 1 (50%) | 1 (50%) | 3 (38%) | 4 (21%) | 14 (26%) | 9 (47%) | 9 (23%) | 3 (14%) |
| 7–8 | 14 (61%) | 21 (72%) | 20 (71%) | 36 (75%) | 8 (57%) | 8 (62%) | 1 (50%) | 1 (50%) | 4 (50%) | 14 (74%) | 37 (70%) | 9 (47%) | 29 (73%) | 17 (81%) |
| <i>P</i> * | | 0.40 | | | | 0.70 | | | | | 0.39 | | | 0.060 |
| Ki-67 | | | | | | | | | | | | | | |
| <5% | 10 (48%) | 15 (52%) | 17 (63%) | 28 (60%) | 8 (67%) | 4 (31%) | 1 (50%) | 0 | 6 (100%) | 12 (63%) | 24 (46%) | 10 (59%) | 18 (46%) | 14 (67%) |
| 5–10% | 2 (10%) | 6 (21%) | 4 (15%) | 8 (17%) | 2 (17%) | 1 (8%) | 0 | 1 (50%) | 0 | 3 (16%) | 9 (17%) | 2 (12%) | 6 (15%) | 4 (21%) |
| 11–40% | 6 (29%) | 6 (21%) | 6 (22%) | 7 (15%) | 2 (17%) | 7 (54%) | 1 (50%) | 1 (50%) | 0 | 2 (11%) | 16 (31%) | 2 (12%) | 13 (33%) | 3 (14%) |
| >40% | 3 (14%) | 2 (7%) | 0 | 4 (9%) | 0 | 1 (8%) | 0 | 0 | 0 | 2 (11%) | 3 (6%) | 3 (18%) | 2 (5%) | 0 |
| <i>P</i> * | | 0.10 | | | | 0.18 | | | | | 0.030 | | | 0.34 |
| p53 | | | | | | | | | | | | | | |
| Negative | 5 (22%) | 2 (7%) | 11 (41%) | 10 (22%) | 4 (29%) | 2 (17%) | 2 (100%) | 0 | 3 (38%) | 0 | 15 (29%) | 3 (16%) | 11 (28%) | 4 (21%) |
| Positive | 18 (78%) | 25 (93%) | 16 (59%) | 36 (78%) | 10 (71%) | 10 (83%) | 0 | 2 (100%) | 5 (63%) | 18 (100%) | 36 (71%) | 16 (84%) | 28 (72%) | 15 (79%) |
| <i>P</i> * | | 0.10 | | | | 0.40 | | | | | 0.56 | | | 0.62 |

Table 3. (Continued)

| All patients | Age class | | | Histology | | | | Grade | | | Stage | | | | |
|--------------|-----------|----------|----------|-----------|----------|--------------|--------------|------------|---------|----------|----------|----------|----------|----------|--------|
| | <50 | 51-65 | >65 | Serous | Mucinous | Endometrioid | Unclassified | Clear cell | 1 | 2 | 3 | FIGO 1&2 | | FIGO 3 | FIGO 4 |
| | | | | | | | | | | | | 19 | 40 | | |
| bol-2 | 23 | 29 | 28 | 48 | 14 | 13 | 1 | 2 | 8 | 19 | 53 | 19 | 40 | 21 | |
| 1-3 | 15 (65%) | 24 (83%) | 20 (71%) | 36 (75%) | 11 (79%) | 10 (77%) | 1 (50%) | 1 (50%) | 7 (88%) | 11 (58%) | 41 (77%) | 15 (79%) | 30 (75%) | 14 (67%) | |
| 4-6 | 6 (26%) | 3 (10%) | 4 (14%) | 7 (15%) | 3 (21%) | 1 (8%) | 1 (50%) | 1 (50%) | 1 (13%) | 4 (21%) | 8 (15%) | 1 (5%) | 8 (20%) | 4 (19%) | |
| 7-8 | 2 (9%) | 2 (7%) | 4 (14%) | 5 (10%) | 0 | 2 (15%) | 0 | 0 | 0 | 4 (21%) | 4 (8%) | 3 (16%) | 2 (5%) | 3 (14%) | |
| P* | 1.00 | | | 0.31 | | | | 0.80 | | | 0.76 | | | | |
| bax | | | | | | | | | | | | | | | |
| Negative | 7 (30%) | 10 (34%) | 9 (32%) | 17 (35%) | 4 (29%) | 4 (31%) | 0 | 1 (50%) | 2 (25%) | 2 (11%) | 22 (42%) | 5 (26%) | 14 (35%) | 7 (33%) | |
| Positive | 16 (70%) | 19 (66%) | 19 (68%) | 31 (65%) | 10 (71%) | 9 (69%) | 2 (100%) | 1 (50%) | 6 (75%) | 17 (89%) | 31 (58%) | 14 (74%) | 26 (65%) | 14 (67%) | |
| P* | 1.00 | | | 0.82 | | | | 0.07 | | | 0.59 | | | | |

*Linear χ^2 -test; histology; exact Pearson's χ^2 -test. RAR, retinoic acid receptor; RXR retinoid X receptor.

RNA expression study

The results of mRNA quantitation of the major classes of retinoid receptors are summarized in Table 5. All types of receptors were expressed in ovarian adenomas and carcinomas except for RXR γ . We did not observe significant associations between the mRNA expression of retinoid receptors and clinical features.

Discussion

The present study is the first to systematically investigate the expression of retinoid receptors in epithelial ovarian cancer tissue. We demonstrate that retinoid receptors are highly expressed in a large proportion of ovarian cancers, and that high expression of RAR α independently predicts an unfavorable prognosis in addition to established prognostic factors such as stage and age. The population in this study comprised all patients treated at our institution between 1984 and 1996 for whom tissues blocks were available. Although no standardization of the measurement, assessment and reporting of retinoid receptors exists, the methodology used in the present study is in accordance with usual procedures for the quantitation and reporting of nuclear hormone receptors by immunohistochemistry [34].

Despite substantial improvement of the surgical and medical therapy, ovarian cancer continues to be a highly lethal disease. Even with state-of-the-art surgery and modern chemotherapy, the probability of long-term survival is \sim 30% for the majority of patients with stage III and IV disease [43-45].

Retinoids have numerous biological effects: depending on the cellular context, they induce differentiation, inhibit the cellular proliferation or cause apoptosis [46]. All-trans retinoic acid was one of the first examples of 'targeted' therapy: it induces remissions in a majority of patients with promyelocytic leukemia. Moreover, retinoids prevent the development of solid tumors [47] including ovarian carcinoma [15], and preliminary data indicate that retinoids are also effective in the therapy of certain solid tumors. In a recently reported phase II trial, fenretinide was shown to be active in ovarian cancer [48]. Retinoids interact *in vitro* with conventional cytotoxic drugs such as cisplatin [24-26] and the taxanes paclitaxel and docetaxel [27, 49, 50] to lower the threshold of apoptosis. The clinical side-effects of retinoids do not overlap with the typical toxicity of cytotoxic chemotherapeutic agents. Thus, clinical and *in vitro* evidence make retinoids attractive investigational agents for the combination with conventional cytotoxic chemotherapy.

Most biological effects of retinoids are mediated through retinoid receptors; previous work has established that retinoid receptors are expressed in a variety of ovarian cancer cell lines. However, the expression of retinoid receptors has hardly been investigated in clinical ovarian cancer tissues. In the present study we observed that the retinoid receptors RAR α , RAR γ , RXR α and RXR β were present in a majority of ovarian cancer tissues, especially in advanced-stage tumors and those with

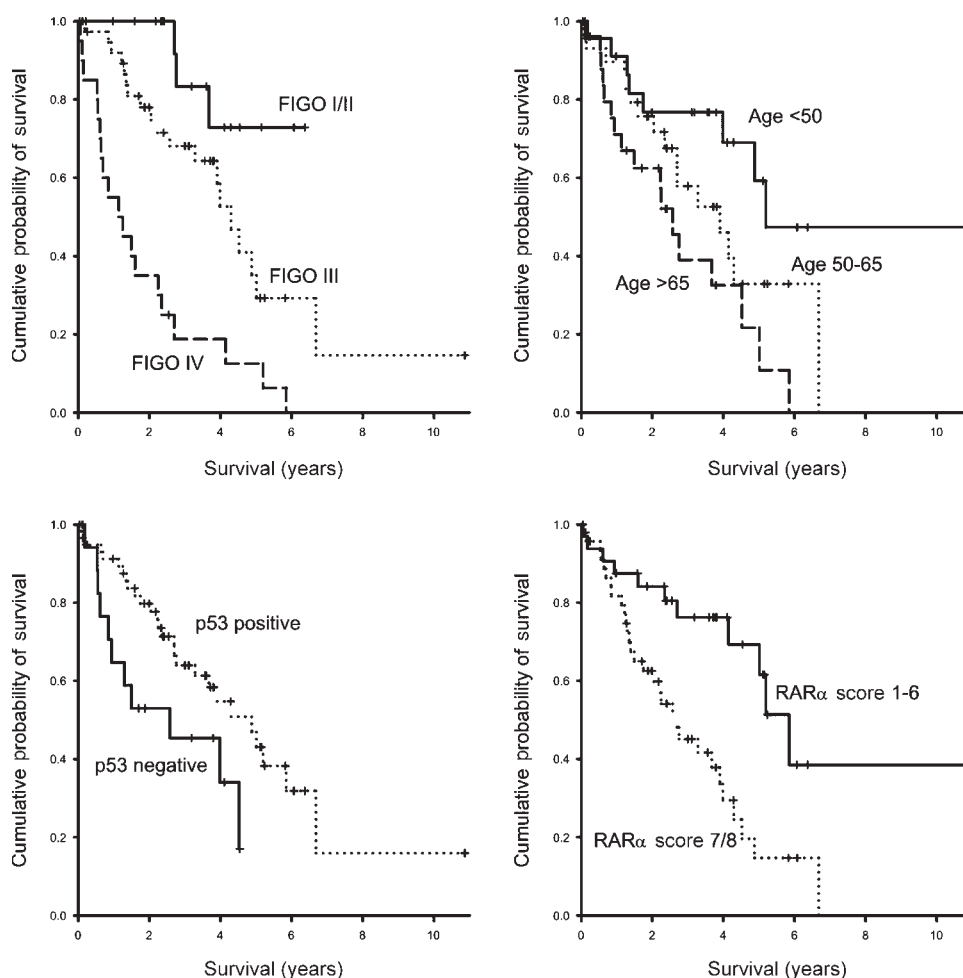


Figure 2. Overall survival by FIGO stage, age, p53 immunohistochemistry and retinoic acid receptor α (RAR α) immunohistochemistry score.

poor differentiation. It is thus not surprising that the high expression of RAR α was a negative prognostic factor of survival. The lack of apparent correlation between the expression of mRNA and protein by immunohistochemistry may be explained by the impossibility of cross-calibration of antibody binding affinity and by the unavailability of a suitable antibody for RAR β . Similar observations were made in oral squamous cell carcinoma, where RAR α was an independent indicator of a poor prognosis [51]. RAR α was correlated with unfavorable prognostic factors such as high grade and high proliferation rate in prostate [52] and breast [53] cancer. Considering the role of retinoids in normal epithelial cell differentiation, the biological mechanisms underlying this observation are not obvious. At least two potential explanations have been proposed. (i) In human cell systems including MCF7 breast cancer cells, retinoic acid enhances the ubiquitylation of RAR α and induces its degradation in the proteasome [54, 55]. Thus, in conditions of low concentrations of retinoic acid at the receptor, the degradation of RAR α might be inhibited; alternatively, the post-transcriptional regulation of RAR α could be deranged by an unknown mechanism. (ii) Unliganded RAR α molecules may inhibit the retinoic acid-dependent processes such as differentiation [56] and apoptosis, perhaps by inhibiting the effects of RAR β ; the expression of the latter is induced by

liganded RAR α and is lost as an early event in the carcinogenesis of various tumors [57].

The presence of RXRs, although not of independent prognostic value in this study, may also be important: RXRs are obligatory dimerization partners for other members of the steroid receptor superfamily such as other retinoid receptor subtypes, the thyroid hormone receptor, the peroxisome proliferator-activated receptor gamma (PPAR γ) and the vitamin D receptor. PPAR γ has very recently been shown to be expressed in ovarian cancer and may play a role in the development of epithelial ovarian carcinoma [58]. Thus, the expression of retinoic acid receptors may be a predictive marker for the therapeutic utility of retinoids and PPAR γ ligands similar to the current use of estrogen receptor assessment for the prediction of endocrine responsiveness in breast cancer. Given the complexity of the retinoid signaling pathways it appears likely that retinoid receptors are required though not sufficient to predict the action of retinoids. For instance, the expression of RAR β predicted the response to isotretinoin (13-*cis* retinoic acid) in a proportion of patients with renal cell cancer [59]. Moreover, the RXR agonist bexarotene is active in about one-fifth of patients with metastatic breast cancer; unfortunately, it has not been investigated whether the expression of RXR is correlated

Table 4. Prognostic factors of survival

| | Univariate analysis | | Multivariate analysis | |
|----------------------|-----------------------|----------|-----------------------|----------|
| | Hazard ratio (95% CI) | <i>P</i> | Hazard ratio (95% CI) | <i>P</i> |
| Age years | | 0.015 | | 0.81 |
| <50 | Reference | | Reference | |
| 50–65 | 2.02 (0.86–4.8) | | 1.35 (0.50–3.6) | |
| 65+ | 3.52 (1.49–8.3) | | 1.32 (0.49–3.5) | |
| Grade | | 0.26 | | |
| 1 | Reference | | Reference | |
| 2 | 3.36 (0.73–15.4) | | | |
| 3 | 3.24 (0.77–13.6) | | | |
| FIGO stage | | <0.001 | | <0.001 |
| I/III | Reference | | Reference | |
| III | 3.48 (1.02–11.8) | | 2.02 (0.56–7.3) | |
| IV | 12.0 (3.55–40.9) | | 13.1 (3.4–50) | |
| Immunohistochemistry | | | | |
| p53 | | 0.04 | | 0.050 |
| Negative | Reference | | Reference | |
| Positive | 0.47 (0.23–0.97) | | 0.41 (0.17–1.00) | |
| Ki-67 (score) | 0.96 (0.72–1.27) | 0.35 | | |
| Bcl-2 (score) | 0.98 (0.84–1.13) | 0.75 | | |
| Bax | | 0.82 | | |
| Negative | Reference | | | |
| Positive | 0.92 (0.48–1.80) | | | |
| RAR α | | 0.002 | | 0.003 |
| Score 1–6 | Reference | | Reference | |
| Score 7–8 | 2.99 (1.49–6.03) | | 3.77 (1.56–9.1) | |
| RAR γ | | 0.02 | | 0.95 |
| Score 1–4 | Reference | | Reference | |
| Score 5–8 | 2.15 (1.12–4.12) | | 0.975 (0.42–2.3) | |
| RXR α | | 0.26 | | |
| Score 1–5 | Reference | | | |
| Score 6–8 | 1.45 (0.76–2.79) | | | |
| RXR β | | 0.10 | | |
| Score 1–6 | Reference | | | |
| Score 7–8 | 1.91 (0.88–4.17) | | | |

CI, confidence interval, RAR, retinoid acid receptor; RXR, retinoid X receptor.

with the responsiveness to bexarotene [60]. In ovarian cancer, fenretinide induced a clinically worthwhile benefit in a minority of patients with ovarian cancer, even though the patients were not selected for retinoid receptor expression [48]. It is conceivable that with a more selective use of retinoids exclusively in tumors that express retinoic acid receptors, a substantially better benefit to toxicity ratio may be achieved in combination with conventional chemotherapy.

Although the present investigation is limited by the small number of samples analyzed and the virtual lack of

information on RAR β protein, it reveals that certain retinoid receptors are present in human epithelial ovarian cancer. RAR α may be associated with an adverse prognosis independently of established prognosticators such as FIGO stage. This finding will have to be verified in a larger independent set of tumor samples. The present study provides the molecular basis for clinical trials to evaluate the efficacy of retinoids in ovarian cancer; this prospect is promising in view of preclinical data that show a synergic interaction with platinum drugs and taxanes.

Table 5. mRNA expression of retinoid receptors (copies of retinoid receptor mRNA/copies of 7S rRNA)

| | RAR α | | | RAR β | | | RAR γ | | | RXR α | | | RXR β | | RXR γ | |
|--------------|--------------|------------------|-------------|-------------|------------------|-------------|--------------|------------------|-------------|--------------|------------------|-------------|-------------|------------------|--------------|-------|
| | Median | 25–75 percentile | P^a | Median | 25–75 percentile | P^a | Median | 25–75 percentile | P^a | Median | 25–75 percentile | P^a | Median | 25–75 percentile | | P^a |
| All patients | 0.007 | 0.003–0.014 | | 0.188 | 0.04–0.287 | | 0.048 | 0.023–0.121 | | 0.259 | 0.1–0.479 | | 0.177 | 0.023–0.314 | | |
| | Not detected | 2 | | 3 | | | 0 | | | 3 | | | 4 | | 31 | |
| Age (years) | <50 | 0.013 | 0.006–0.021 | 0.06 | 0.194 | 0.071–0.287 | 0.75 | 0.083 | 0.031–0.168 | 0.14 | 0.374 | 0.242–0.543 | 0.24 | 0.246 | 0.015–0.333 | 0.40 |
| | 51–65 | 0.007 | 0.003–0.036 | | 0.177 | 0.094–0.221 | | 0.049 | 0.039–0.173 | | 0.308 | 0.21–0.518 | | 0.196 | 0.096–0.265 | |
| | >65 | 0.006 | 0–0.009 | | 0.225 | 0.034–0.348 | | 0.038 | 0.023–0.107 | | 0.265 | 0.096–0.366 | | 0.157 | 0.025–0.431 | |
| Histology | Adenoma | 0.007 | 0.002–0.009 | 0.22 | 0.223 | 0.066–0.291 | 0.65 | 0.045 | 0.038–0.057 | 0.17 | 0.335 | 0.142–0.516 | 0.82 | 0.165 | 0.35–0.222 | 0.18 |
| | Carcinoma | 0.008 | 0.003–0.017 | | 0.180 | 0.41–0.322 | | 0.100 | 0.018–0.20. | | 0.255 | 0.12–0.519 | | 0.240 | 0.064–0.431 | |
| Grade | Borderline | 0.015 | | 0.22 | 0.002 | | 0.21 | 0.018 | | 0.48 | 0.015 | | 0.95 | 0.01 | | 0.66 |
| | Grade 1 | 0.08 | | | 0.083 | | | 0.031 | | | 0.243 | | | 0.200 | | |
| | Grade 2 | 0.013 | 0.05–0.025 | | 0.172 | 0.014–0.863 | | 0.130 | 0.601–0.404 | | 0.382 | 0.169–1.127 | | 0.333 | 0.148–0.756 | |
| | Grade 3 | 0.011 | 0.004–0.015 | | 0.194 | 0.167–0.287 | | 0.168 | 0.032–0.246 | | 0.251 | 0.15–0.511 | | 0.392 | 0.0840–0.461 | |
| | Unknown | 0.004 | 0–0.016 | | 0.360 | 0.34–0.684 | | 0.073 | 0.017–0.205 | | 0.238 | 0.142–0.693 | | 0.146 | 0.062–0.376 | |
| Stage | FIGO I&II | 0.015 | 0.000–0.021 | 0.92 | 0.284 | 0.001–0.570 | 0.62 | 0.054 | 0.11–0.332 | 0.76 | 0.259 | 0.011–0.806 | 0.97 | 0.246 | 0.001–0.468 | 0.74 |
| | FIGO III | 0.008 | 0.006–0.013 | | 0.188 | 0.083–0.287 | | 0.130 | 0.032–0.237 | | 0.271 | 0.242–0.382 | | 0.296 | 0.177–0.428 | |
| | FIGO IV | 0.007 | 0.003–0.025 | | 0.110 | 0.023–0.863 | | 0.049 | 0.013–0.404 | | 0.217 | 0.095–1.127 | | 0.114 | 0.041–0.709 | |

^aJonckheere–Terpstra test, except histology: Mann–Whitney *U*-test.

RAR, retinoic acid receptor; RXR retinoid X receptor.

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