Original article

Annals of Oncology 16: 1477–1487, 2005
doi:10.1093/annonc/mdi265
Published online 12 July 2005

Retinoid receptors in ovarian cancer: expression and prognosis

P. C. Kaiser[1,2], M. Körner[3], A. Kappeler[3] & S. Aebi[1,2]*

[1]*University Hospital Berne, Department of Medical Oncology, Bern;* [2]*University of Bern, Department of Clinical Research, Bern;* [3]*University of Bern, Institute of Pathology, Bern, Switzerland*

Received 21 March 2005; revised 13 April 2005; accepted 14 April 2005

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 RARα, RARγ, RXRα and RXRβ 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: RARα and RXRβ were highly expressed in a majority of ovarian cancers, particularly in advanced stages. High expression of RARα 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 occuring 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, rexinoid receptors) [4]. These are members of the steroid and thyroid hormone receptor superfamily [5]. Each subtype of both retinoid receptor classes (RARα, RARβ, RARγ, and RXRα, RXRβ, RXRγ, 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

**Correspondence to:* Dr S. Aebi, Department of Medical Oncology, University Hospital Berne, Inselspital PT2C, 3010 Berne, Switzerland. Tel: +41-31-632-4114; Fax: +41-31-382-1237; E-mail: stefan.aebi@insel.ch

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 targretin 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 RARα, RARγ, RXRα and RXRβ 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 RARβ, 147 for RXRβ and 1373* for RXRγ (Neomarkers, Fremont, CA, USA); polyclonal antibodies were used for bax (DakoCytomation): RARα, RARβ*, RARγ, RXRα, RXRβ* and RXRγ* (all from

Table 1. Patient characteristics

		Paraffin		Frozen	
		n	%	*n*	%
Number of patients		80		35	
Age at diagonisis	<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
	NOS	3	4	6	27
Histological grade[a]	1	8	10	1	5
	2	19	24	5	23
	3	53	66	16	73
FIGO stage[a]	I&II	19	24	3	14
	III	40	50	10	45
	IV	21	26	7	32

[a]Carcinomas 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, ≤5%; 1, ≤30%; 2, ≤50%; 3, ≤70%; 4, ≤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×SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and forward and reverse primer at 300 nM. The primers for RARα, RARβ, RARγ and RXRα, RXRβ, RXRγ were designed according to the NCBI reference sequences for the corresponding genes (Table 2). 7S rRNA was used as reference target sequence (primers: 5′-accaccaggttgcctaagga and 5′-cacgggagttttgacctgct). We used the ABI PRISM 7700 Sequence Detection System for RT–PCR. The following parameters were for used 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 μg/μl 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 RARα, RARγ, RXRα and RXRβ was present in carcinoma cells (Figure 1). Specific immunoreactivity for RARβ and RXRγ was not discernible, either in a selection of ovarian cancers or in other tissues that were supposed to express the antigen. No mRNA for RXRγ was detected in frozen ovarian cancer tissue in contrast to mRNA for RARβ. The expression of RARα, RARγ, RXRα and RXRβ is summarized in Table 3.

Table 2. Real time PCR primers sequences for RARα, RARβ, RARγ and RXRα, RXRβ, RXRγ

Gene	Reference sequence no.	Orientation	Sequence	Positions	Product size (hp)
RARα	NM_000964	Forward	5′-cagagcagcagttctgaagagata	292–315	184
		Reverse	5′-gacacgtgtacaccatgttcttct	452–475	
RARβ	NM_000965	Forward	5′-gtcaccgagataagaactgtgtta	819–812	179
		Reverse	5′-actcagctgtcatttcatagctctc	937–997	
RARγ	NM_000966	Forward	5′-ctgtatcatcaacaaggtgacca	822–844	183
		Reverse	5′-tggtgatgagctcttctaactgag	980–1003	
RXRα	NM_002957	Forward	5′-tgcttcgtgtaagcaagtacataag	4699–4723	192
		Reverse	5′-ctctttatggatctgtcatcctctc	4866–4890	
RXRβ	NM_021976	Forward	5′-ccagagtctctttttacacttcacc	2293–2317	182
		Reverse	5′-tcttagtcaacctgggaaagtacag	2450–2474	
RXRγ	NM_006917	Forward	5′-gatctagaggcagattcctgactaa	6–30	187
		Reverse	5′-catgtttactcgtcagttcatgttc	167–191	

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

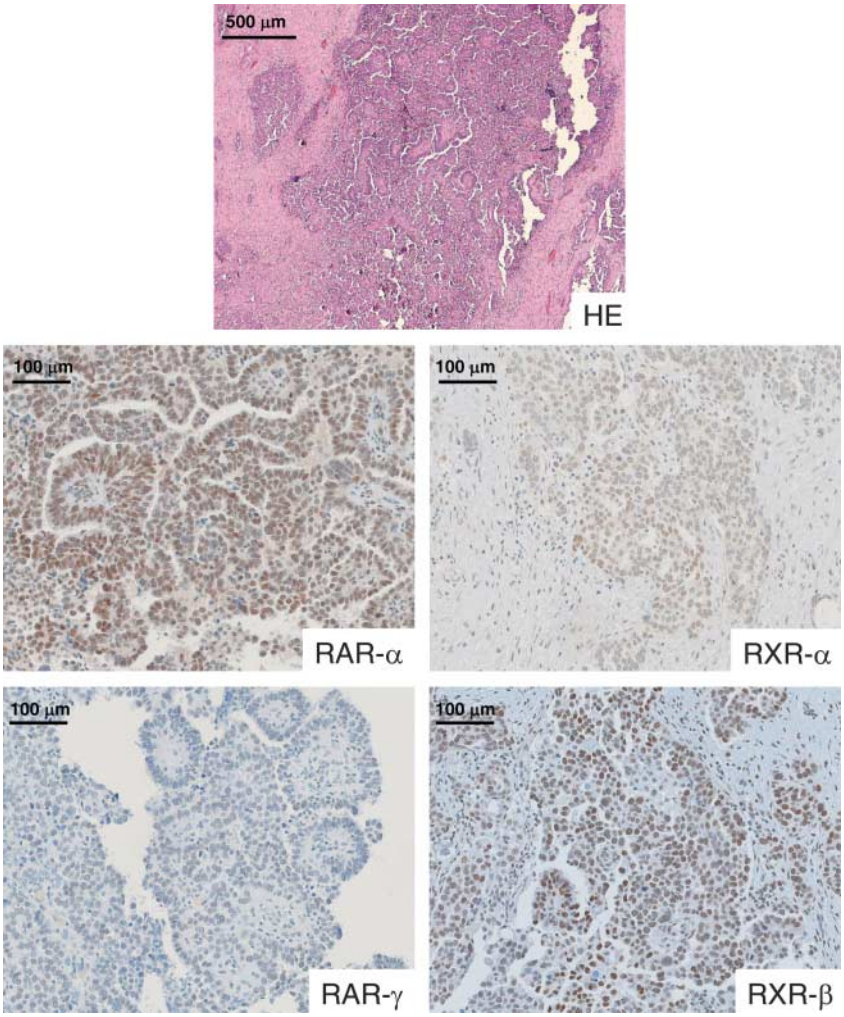

Figure 1. Immunohistochemical staining of retinoid receptors in epithelial ovarian cancer. HE, hematoxylin and eosin; RAR, retinoic acid receptor; RXR, retinoid X receptor.

RARα was expressed in the majority of the tissue sections, and 48 patients (60%) had a high score of 7 or 8. High RARα expression was significantly related to advanced FIGO stages ($P = 0.04$). The expression of RARγ was similar in different histological types of ovarian carcinoma. Tumors with lower FIGO stages had lower levels of RARγ. RXRα 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 RXRα expression with clinical or morphological features. RXRβ 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 RXRβ 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 RARα and RXRα was positively correlated (Spearman's rank correlation coefficient 0.32; $P = 0.004$), as was that of RARα and RXRβ (correlation coefficient 0.26; $P = 0.02$). RARγ was not correlated with the evaluated antigens. RXRα was also positively correlated with bax (correlation coefficient 0.22; $P = 0.04$), as was RXRβ 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. RARα and RARγ were predictors of survival in univariate proportional hazards regression analysis, in addition to age, FIGO stage and p53 status. FIGO stage, p53 status and RARα expression were independent predictors of survival: high expression of RARα 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	Endometroid	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%)
*P**		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%)
*P**		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%)
*P**		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%)
*P**		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
*P**		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%)
*P**		0.10				0.40				0.56			0.62	

Table 3. (*Continued*)

All patients	Age class			Histology				Grade			Stage		
	<50	51–65	>65	Serous	Mucinous	Endometroid	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
bcl-2														
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 RXRγ. 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 RARα 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 ~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 RARα, RARγ, RXRα and RXRβ were present in a majority of ovarian cancer tissues, especially in advanced-stage tumors and those with

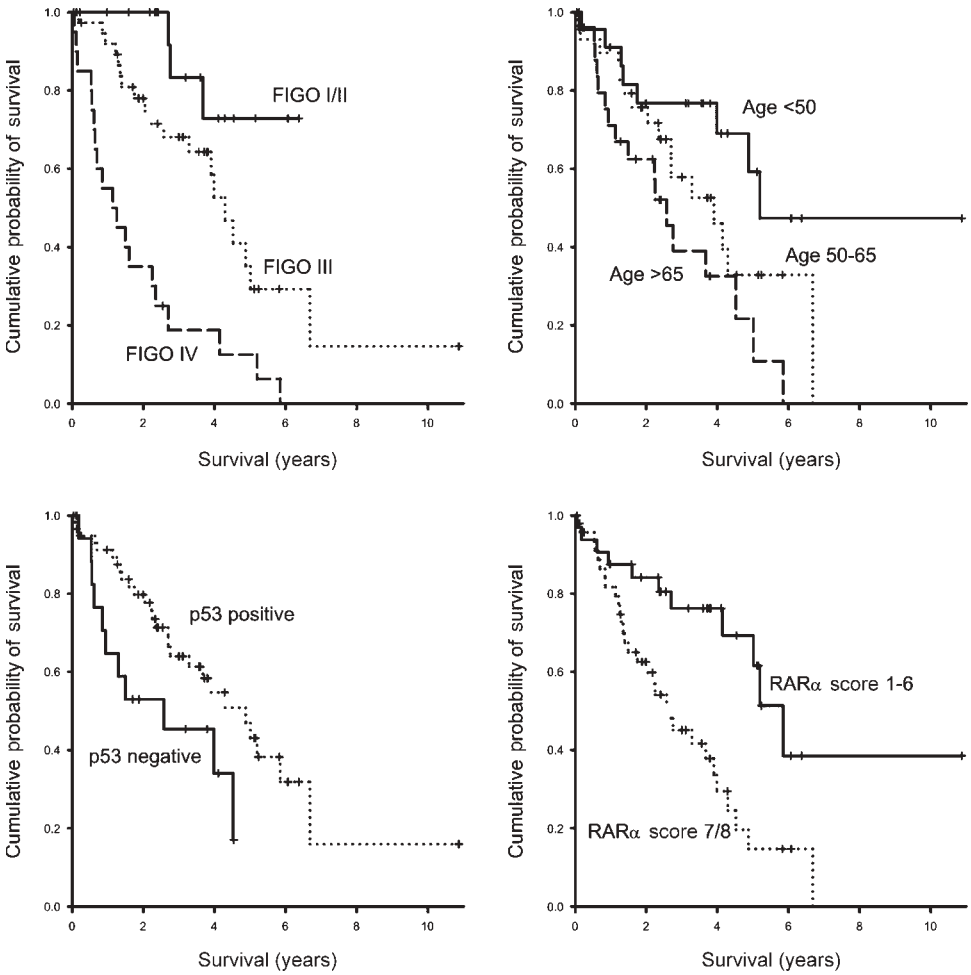

Figure 2. Overall survival by FIGO stage, age, p53 immunohistochemistry and retinoic acid receptor α (RARα) immunohistochemistry score.

poor differentiation. It is thus not surprising that the high expression of RARα 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 RARβ. Similar observations were made in oral squamous cell carcinoma, where RARα was an independent indicator of a poor prognosis [51]. RARα 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 RARα and induces its degradation in the proteasome [54, 55]. Thus, in conditions of low concentrations of retinoic acid at the receptor, the degradation of RARα might be inhibited; alternatively, the post-transcriptional regulation of RARα could be deranged by an unknown mechanism. (ii) Unliganded RARα molecules may inhibit the retinoic acid-dependent processes such as differentiation [56] and apoptosis, perhaps by inhibiting the effects of RARβ; the expression of the latter is induced by liganded RARα 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 (PPARγ) and the vitamin D receptor. PPARγ 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 PPARγ 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 RARβ 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)	*P*	Hazard ratio (95% CI)	*P*
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 RARβ protein, it reveals that certain retinoid receptors are present in human epithelial ovarian cancer. RARα 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		

[a]Jonckheere–Terpstra test, except histology: Mann–Whitney *U*-test.
RAR, retinoic acid receptor; RXR retinoid X receptor.

Acknowledgements

We thank Professor H. J. Altermatt, Berne, for providing tumor tissues. This study was supported by a grant from the Swiss Cancer League (SKL 986-02-2000).

References

1. Jemal A, Tiwari RC, Murray T et al. Cancer statistics. CA Cancer J Clin 2004; 54: 8–29.
2. Schüler G, Bopp M. Ovar und Adnexe. In: Schüler G, Bopp M (eds): Atlas der Krebsmortalität in der Schweiz. Basel: Birkhäuser 1997; 151–154.
3. Vaidya AP, Curtin JP. The follow-up of ovarian cancer. Semin Oncol 2003; 30: 401–412.
4. Mangelsdorf D, Umesono K, Evans R. The retinoid receptors. In Sporn M, Roberts A, Goodman D (eds): The Retinoids, 2 edn. New York: Raven Press 1994; 319–350.
5. Evans R. The steroid and thyroid hormone receptor superfamily. Science 1988; 13: 889–895.
6. Altucci L, Gronemeyer H. The promise of retinoids to fight against cancer. Nat Rev Cancer 2001; 1: 181–193.
7. Wolbach S, Howe P. Tissue changes following deprivation of fat-soluble A-vitamin. J Exp Med 1925; 42: 753–778.
8. Dragnev KH, Rigas JR, Dmitrovsky E. The retinoids and cancer prevention mechanisms. Oncologist 2000; 5: 361–368.
9. Einspahr JG, Stratton SP, Bowden GT, Alberts DS. Chemoprevention of human skin cancer. Crit Rev Oncol Hematol 2002; 41: 269–285.
10. Bertone ER, Hankinson SE, Newcomb PA et al. A population-based case–control study of carotenoid and vitamin A intake and ovarian cancer (United States). Cancer Causes Control 2001; 12: 83–90.
11. Huncharek M, Klassen H, Kupelnick B. Dietary beta-carotene intake and the risk of epithelial ovarian cancer: a meta-analysis of 3,782 subjects from five observational studies. In Vivo 2001; 15: 339–343.
12. Cramer DW, Kuper H, Harlow BL, Titus-Ernstoff L. Carotenoids, antioxidants and ovarian cancer risk in pre- and postmenopausal women. Int J Cancer 2001; 94: 128–134.
13. Salazar-Martinez E, Lazcano-Ponce EC, Lira G et al. Nutritional determinants of epithelial ovarian cancer risk: a case–control study in Mexico. Oncology 2002; 63: 151–157.
14. Zhang M, Lee AH, Binns CW. Reproductive and dietary risk factors for epithelial ovarian cancer in China. Gynecol Oncol 2004; 92: 320–326.
15. Veronesi U, De Palo G, Marubini E et al. Randomized trial of fenretinide to prevent second breast malignancy in women with early breast cancer. J Natl Cancer Inst 1999; 91: 1847–1856.
16. De Palo G, Mariani L, Camerini T et al. Effect of fenretinide on ovarian carcinoma occurrence. Gynecol Oncol 2002; 86: 24–27.
17. Zhang D, Holmes WF, Wu S et al. Retinoids and ovarian cancer. J Cell Physiol 2000; 185: 1–20.
18. Kalli KR, Devine KE, Cabot MC et al. Heterogeneous role of caspase-8 in fenretinide-induced apoptosis in epithelial ovarian carcinoma cell lines. Mol Pharmacol 2003; 64: 1434–1443.
19. Holmes WF, Soprano DR, Soprano KJ. Elucidation of molecular events mediating induction of apoptosis by synthetic retinoids using a CD437-resistant ovarian carcinoma cell line. J Biol Chem 2002; 277: 45408–45419.
20. Holmes WF, Soprano DR, Soprano KJ. Early events in the induction of apoptosis in ovarian carcinoma cells by CD437: activation of the p38 MAP kinase signal pathway. Oncogene 2003; 22: 6377–6386.
21. Vuocolo S, Purev E, Zhang D et al. Protein phosphatase 2A associates with Rb2/p130 and mediates retinoic acid-induced growth suppression of ovarian carcinoma cells. J Biol Chem 2003; 278: 41881–41889.
22. Mehta K. Retinoids as regulators of gene transcription. J Biol Regul Homeost Agents 2003; 17: 1–12.
23. Crowe DL, Kim R, Chandraratna RA. Retinoic acid differentially regulates cancer cell proliferation via dose-dependent modulation of the mitogen-activated protein kinase pathway. Mol Cancer Res 2003; 1: 532–540.
24. Jozan S, Lafon C, Mathieu C et al. Enhancement of cisplatin cytotoxicity by all-trans retinoic acid in a human ovarian carcinoma cell line (OVCCR1) is characterized by apoptosis. Proc Am Assoc Cancer Res 1996; 37: 414 (Abstr).
25. Aebi S, Kröning R, Cenni B et al. All-trans retinoic acid enhances cisplatin-induced apoptosis in human ovarian adenocarcinoma and in squamous head and neck cancer cells. Clin Cancer Res 1997; 3: 2033–2038.
26. Jozan S, Paute S, Courtade-Saidi M et al. All trans retinoic acid enhances CDDP-induced apoptosis: modulation of the CDDP effect on cell cycle progression. Int J Oncol 2002; 20: 1289–1295.
27. Wang Q, Wieder R. All-trans retinoic acid potentiates Taxotere-induced cell death mediated by Jun N-terminal kinase in breast cancer cells. Oncogene 2004; 23: 426–433.
28. Scribner DR Jr, Benbrook DM. Retinoids enhance cisplatin-based chemoradiation in cervical cancer cells in vitro. Gynecol Oncol 2002; 85: 223–225.
29. Sanz MA, Martin G, Diaz-Mediavilla J. All-trans-retinoic acid in acute promyelocytic leukemia. N Engl J Med 1998; 338: 393–394.
30. Farol LT, Hymes KB. Bexarotene: a clinical review. Expert Rev Anticancer Ther 2004; 4: 180–188.
31. Katsetos CD, Stadnicka I, Boyd JC et al. Cellular distribution of retinoic acid receptor-alpha protein in serous adenocarcinomas of ovarian, tubal, and peritoneal origin: comparison with estrogen receptor status. Am J Pathol 1998; 153: 469–480.
32. Balli S, Fey MF, Hanggi W et al. Ovarian cancer. an institutional review of patterns of care, health insurance and prognosis. Eur J Cancer 2000; 36: 2061–2068.
33. Tavassoli FA, Devilee P. Pathology and Genetics of Tumours of the Breast and Female Genital Organs. Lyon: IARC Press 2003.
34. Allred DC, Clark GM, Elledge R et al. Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. J Natl Cancer Inst 1993; 85: 200–206.
35. Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod Pathol 1998; 11: 155–168.
36. Plisiecka-Halasa J, Karpinska G, Szymanska T et al. P21WAF1, P27KIP1, TP53 and C-MYC analysis in 204 ovarian carcinomas treated with platinum-based regimens. Ann Oncol 2003; 14: 1078–1085.
37. Cina SJ, Richardson MS, Austin RM, Kurman RJ. Immunohistochemical staining for Ki-67 antigen, carcinoembryonic antigen, and p53 in the differential diagnosis of glandular lesions of the cervix. Mod Pathol 1997; 10: 176–180.
38. Skirnisdottir I, Sorbe B, Seidal T. P53, bcl-2, and bax: their relationship and effect on prognosis in early stage epithelial ovarian carcinoma. Int J Gynecol Cancer 2001; 11: 147–158.
39. Battifora H. Assessment of antigen damage in immunohistochemistry. The vimentin internal control. Am J Clin Pathol 1991; 96: 669–671.
40. Agresti A. Categorical Data Analysis, 2nd edn. Hoboken, NJ: Wiley-Interscience 2002.
41. Kaplan EL, Meier P. Nonparametric estimation from incomplete observation. J Am Stat Assoc 1958; 53: 457–481.
42. Cox DR. Regression models and life-tables (with discussion). J R Stat Soc Ser B 1972; 34: 187–220.

43. McGuire WP, Hoskins WJ, Brady ME et al. Cyclophosphamide and cisplatin compared with paclitaxel and cisplatin in patients with stage III and stage IV ovarian cancer. N Engl J Med 1996; 334: 1–6.
44. Piccart MJ, Bertelsen K, James K et al. Randomized Intergroup trial of cisplatin–paclitaxel versus cisplatin–cyclophosphamide in women with advanced epithelial ovarian cancer: three-year results. J Natl Cancer Inst 2000; 92: 699–708.
45. Paclitaxel plus carboplatin. Paclitaxel plus carboplatin versus standard chemotherapy with either single-agent carboplatin or cyclophosphamide, doxorubicin, and cisplatin in women with ovarian cancer: the ICON3 randomised trial. Lancet 2002; 360: 505–515.
46. Sun S-Y, Hail N Jr, Lotan R. Apoptosis as a novel target for cancer chemoprevention. J Natl Cancer Inst 2004; 96: 662–672.
47. Lippman SM, Lee JJ, Karp DD et al. Randomized phase III intergroup trial of isotretinoin to prevent second primary tumors in stage I non-small-cell lung cancer. J Natl Cancer Inst 2001; 93: 605–618.
48. Garcia AA, Morgan R, McNamara M et al. Phase II trial of fenretinide (4-HPR) in recurrent ovarian and primary peritoneal carcinoma: A California Cancer Consortium trial. Proc Am Assoc Cancer Res 2004; 23: 461.
49. Wang Q, Yang W, Uytingco MS et al. 1,25-Dihydroxyvitamin D3 and all-trans-retinoic acid sensitize breast cancer cells to chemotherapy-induced cell death. Cancer Res 2000; 60: 2040–2048.
50. Nehme A, Varadarajan P, Sellakumar G et al. Modulation of docetaxel-induced apoptosis and cell cycle arrest by all-trans retinoic acid in prostate cancer cells. Br J Cancer 2001; 84: 1571–1576.
51. Chakravarti N, Mathur M, Bahadur S et al. Retinoic acid receptor-alpha as a prognostic indicator in oral squamous cell carcinoma. Int J Cancer 2003; 103: 544–549.
52. Gyftopoulos K, Perimenis P, Sotiropoulou-Bonikou G et al. Immunohistochemical detection of retinoic acid receptor-alpha in prostate carcinoma: correlation with proliferative activity and tumor grade. Int Urol Nephrol 2000; 32: 263–269.
53. van der Leede BM, Geertzema J, Vroom TM et al. Immunohistochemical analysis of retinoic acid receptor-alpha in human breast tumors: retinoic acid receptor-alpha expression correlates with proliferative activity. Am J Pathol 1996; 148: 1905–1914.
54. Zhu J, Gianni M, Kopf E et al. Retinoic acid induces proteasome-dependent degradation of retinoic acid receptor alpha (RARalpha) and oncogenic RARalpha fusion proteins. Proc Natl Acad Sci USA 1999; 96: 14807–14812.
55. Tanaka T, Rodriguez de la Concepcion ML, De Luca LM. Involvement of all-trans-retinoic acid in the breakdown of retinoic acid receptors alpha and gamma through proteasomes in MCF-7 human breast cancer cells. Biochem Pharmacol 2001; 61: 1347–1355.
56. Kastner P, Lawrence HJ, Waltzinger C et al. Positive and negative regulation of granulopoiesis by endogenous RARalpha. Blood 2001; 97: 1314–1320.
57. Soprano DR, Qin P, Soprano KJ. Retinoic acid receptors and cancers. Annu Rev Nutr 2004; 24: 201–221.
58. Zhang GY, Ahmed N, Riley C et al. Enhanced expression of peroxisome proliferator-activated receptor gamma in epithelial ovarian carcinoma. Br J Cancer 2005; 92: 113–119.
59. Berg WJ, Nanus DM, Leung A et al. Up-regulation of retinoic acid receptor beta expression in renal cancers in vivo correlates with response to 13-cis-retinoic acid and interferon-alpha-2a. Clin Cancer Res 1999; 5: 1671–1675.
60. Esteva FJ, Glaspy J, Baidas S et al. Multicenter phase II study of oral bexarotene for patients with metastatic breast cancer. J Clin Oncol 2003; 21: 999–1006.