Oestrogen receptor 1 mRNA is a prognostic factor in ovarian cancer patients treated with neo-adjuvant chemotherapy: determination by array and kinetic PCR in fresh tissue biopsies

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Abstract

Oestrogen receptors (ESRs) regulate the growth and differentiation of normal ovarian epithelia. However, to date their role as biomarkers in the clinical setting of ovarian cancer remains unclear. In view of potential endocrine treatment options, we tested the role of ESR1 mRNA expression in ovarian cancer in the context of a neo-adjuvant chemotherapy trial. Study participants had epithelial ovarian or peritoneal carcinoma unsuitable for optimal upfront surgery and were treated with neo-adjuvant platinum-based chemotherapy before surgery. RNA was isolated from frozen tumour biopsies before treatment. RNA expression of ESR1 was determined by microarray and reverse transcriptase kinetic PCR technologies. The prognostic value of ESR1 was tested using univariate and multivariate Cox proportional hazards models, Kaplan–Meier survival statistics and the log-rank test. ESR1 positively correlates with proliferation markers and histopathological grading. ESR1 was a significant predictor of survival as a continuous variable in the univariate Cox regression analysis. In multivariate analysis, elevated baseline ESR1 mRNA levels predicted prolonged progression-free survival ($P=0.041$) and overall survival ($P=0.01$) after neo-adjuvant chemotherapy, independently of pathological grade and age. We conclude that pretreatment ESR1 mRNA is associated with tumour growth and is a strong prognostic factor in ovarian cancer, independent of the strongest clinical parameters used in clinical routine. We suggest that ESR1 mRNA status should be considered in order to minimize possible confounding effects in ovarian cancer clinical trials, and that early treatment with anti-hormonal agents based on reliable hormone receptor status determination is worth investigating.

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Introduction

Ovarian cancer is the most lethal gynaecological cancer and the main cause of female cancer deaths with 204 449 new cases and 124 860 deaths worldwide in the year 2002. In Europe, ~64 000 women are diagnosed with ovarian cancer every year (Ferlay et al. 2004). Unfortunately, most patients (70%) are diagnosed at an advanced stage and the majority of them will relapse, and ultimately die of the disease. Major improvements in ovarian cancer therapy are warranted and the development of new biological agents strengthens the need for the discovery of new prognostic and predictive factors in order to tailor anticancer therapies.

Neo-adjuvant chemotherapy, different from the adjuvant setting, permits the evaluation of effect of anticancer treatment on primary tumours. It is therefore an experimental model to investigate the biology of cancer in patients with different outcomes in order to develop novel biomarkers. While being established as a standard of care in locally advanced and early breast cancer, the role of neo-adjuvant chemotherapy in the routine treatment of ovarian cancer is still debated (Schwartz et al. 1999, Vergote et al. 2000, Mazzeo et al. 2003, Bristow & Chi 2006). A recently presented phase III randomized trial comparing upfront surgery versus neo-adjuvant chemotherapy (Vergote 2008) has demonstrated similar survival in the two arms, but reduced morbidity in primary chemotherapy.

In the present study, we assessed oestrogen receptor (ESR) expression by analysing total RNA from fresh ovarian cancer samples obtained before neo-adjuvant chemotherapy. The results obtained on fresh tissues by microarray technology were validated by reverse transcriptase kinetic PCR- (RT-kPCR) based analysis. The prognostic value of ESR1 was further validated in formalin-fixed paraffin-embedded tissue samples from an independent population-based cohort of ovarian cancer patients. Data on ESR1 expression from fresh tissue analysis are presented in this article, while data from fixed tissue analysis are presented in the preceding article published in this issue of the journal (Darb-Esfahani et al. 2009).

Patients and methods

Forty-five newly diagnosed patients with histologically confirmed FIGO stage III–IV epithelial ovarian or peritoneal carcinoma unsuitable for optimal upfront surgery and thus candidates for neo-adjuvant chemotherapy were enrolled in the study between September 2004 and December 2007. Other inclusion

criteria were age >18 years, haematological, renal, hepatic and cardiac function adequate for platinum-based chemotherapy. Exclusion criteria were a Karnofsky performance status (KPS) lower than 70%, a history of other malignancies and contraindications for surgery. The possibility of optimal debulking surgery was excluded at baseline by open laparoscopy.

All the patients were treated at S.Orsola-Malpighi Hospital, Bologna, Italy. The local ethical committee approved the study and a written informed consent was obtained from all the patients. The initial study population of 45 patients was restricted to 35, after excluding nine patients whose biopsy samples were not adequate for the microarray analysis and one patient found to be ineligible because of diagnosis of peritoneal mesothelioma after histological revision. Patient characteristics are listed in Table 1. A standard regimen of carboplatin AUC 5 and paclitaxel 175 mg/m² over 3 h every 3 weeks was administered as neo-adjuvant treatment for six cycles. In three patients who were older than 75 years and in one patient with poor performance status (KPS 70%), single-agent carboplatin was preferred to the combination chemotherapy.

Table 1 Clinical and pathological characteristics of the study population

	No. of patients	Percent
Total	35	100
Age (years)		
Median (range)	67 (43–78)	
Karnofsky performance status		
Median (range)	90 (70–100)	
FIGO stage		
IIlb	1	3
IIlc	19	54
IV	15	43
Grade		
1	1	3
2	2	6
3	29	83
NA	3	8
Histology		
Papillary serous	26	74
Endometrioid	2	6
Undifferentiated	7	20
Tumour site		
Ovarian	30	86
Primary peritoneal	5	14
Neo-adjuvant chemotherapy		
Carboplatin/paclitaxel	31	89
Carboplatin single agent	4	11
Serum CA125 at baseline (U/ml)		
Median (range)	842 (29–6000)	

Evaluation of pathological response to neo-adjuvant chemotherapy

Histopathological response was evaluated after surgery, with surgical samples analysis. To date, no histopathological criteria have been firmly established to describe treatment response after neo-adjuvant chemotherapy in ovarian cancer. According to the literature concerning response to primary chemotherapy in ovarian (Le *et al.* 2007, Sassen *et al.* 2007) and breast cancer (Ogston *et al.* 2003), we considered complete pathological response as the absence of cancer cells in surgical specimens, and as very good partial remission the persistence of only small clusters (< 1 cm) or individual cancer cells and no macroscopic residual after surgery. Partial pathological remission was defined as a tumour burden reduction between 30 and 90% at surgery, while stable disease was defined as no tumour burden reduction or reduction lower than 30% at surgery, compared with initial diagnostic laparoscopy. Only patients with complete and very good partial remissions were considered as pathological responders, while all the other cases were considered as pathological non-responders.

RNA isolation and mRNA analysis

For array-based mRNA detection, tissues collected were snap frozen and stored in liquid nitrogen until analysis. Approximately 20–100 mg of frozen ovarian tumour tissue was crushed in liquid nitrogen. RNA was extracted using commercial kits (Qiagen), RNA integrity was assessed on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), cDNA was synthesized from 1 µg of total RNA using Invitrogen kits (Invitrogen Corp.) and analysed on Affymetrix HG-U133A microarrays (Affymetrix Inc., Santa Clara, CA, USA) as described elsewhere (Ihnen *et al.* 2008). For validation purposes, RT-kPCR was applied to the total RNA isolated from identical fresh tissue biopsies as described above to validate the array data by an independent technical approach. Gene specific TaqMan-based Primer/Probe sets for the assessment of the expression of ESR gene (ESR1) were used. Forty cycles of nucleic acid amplification were applied on an ABI 7900 instrument (Applied Biosystems, Foster City, CA, USA) using the Super Script III One-Step qRT-PCR Kit (Invitrogen) according to the manufacturer's instructions. PCR conditions were as follows: 30 min at 60 °C for reverse transcription and 2 min at 95 °C for Taq activation followed by 40 cycles of 95 °C for 30 s and 60 °C for 60 s. All measurements were performed in triplicates. Inclusion criteria of samples for statistic analysis were

as follows: 1) the cycle threshold (C_T) values for RPL37A were between 25 and 30; 2) at least a duplicate measurement was available for each gene and sample; 3) the single C_T values were not more than 1 C_T apart from each other and 4) C_T values > 40 were excluded from analysis. C_T values were normalized by subtracting the C_T value of the housekeeping gene RPL37A from the C_T value of the target gene (ΔC_T). Virtual copy numbers were calculated using a $2^{(40-\Delta C_T)}$ method, which correlates proportionally with the mRNA expression level of the target gene.

The primer/probe sets used for amplification of the target genes were the following:

ER probe	ATGCCCTTTTGCCGATGCA
Forward primer	GCCAAATTGTGTTTGATGG- ATTAA
Reverse primer	GACAAAACCGAGTCACAT- CAGTAATAG

Statistical analysis

ESR1 mRNA expression in fresh tissue biopsies was determined by microarray technology. In the analysis setting, the global scaling procedure was chosen which multiplied the output signal intensities of each array to a mean target intensity of 500. Samples with suboptimal average signal intensities (i.e. scaling factors > 25) or GAPDH 3'/5' ratios > 5 were relabelled and rehybridized on new arrays. For validation purposes, the relative mRNA expression levels of ESR1 were determined from the same biopsies by RT-kPCR after adjustment to the housekeeping gene RPL37A using a $2^{(40-\Delta C_T)}$ method. Correlations between ESR1 mRNA expression and histological or clinical tumour characteristics were calculated by χ^2 tests using the SPSS 13.0 (SPSS, Chicago, IL, USA) and JMP 5.0.1.2 software (SAS, Cary, NC, USA). In addition to tumour grading, mRNA expression of KI67 and TOP2A were also used as well known proliferation markers in the Spearman correlation analysis. Univariate as well as multivariate hazards and P values were obtained for the respective risk factors using the Cox proportional hazards model as implemented in the SPSS and SAS software packages. Therefore, the ESR1 mRNA expression levels were log transformed to account for scaling differences with clinical variables. To investigate the prognostic and predictive value of ESR, a univariate Cox regression was performed using the ESR1 mRNA expression levels as continuous covariate. Resulting hazards (based on Cox regression

Table 2 Pathological response to neo-adjuvant chemotherapy

Response	n	%	Residual tumour after surgery (cm)			Surgery not performed ^a
			0	<1	>2	
Complete	5	14	5	0	0	0
Very good partial (>90%)	13	37	13	0	0	0
Partial (30–90%)	6	17	2	4	0	0
No change	9	26	0	0	4	5
Disease progression	2	6	0	0	0	2

^aClinical evaluation.

coefficient) and respective *P* values are presented. For multivariate analysis, we compared histological grade G1/G2 versus G3 and age <65 versus 65 years or older. Due to the limited number of patients and as all patients but one were FIGO stage IIIc or IV, the tumour stage was not included in multivariate analysis. Overall survival (OAS) was computed from the date of diagnosis to the date of death. Kaplan–Meier analysis was performed using GraphPad Prism 4.01 software (San Diego, CA, USA). As an objective cut-off, the median of the data distribution was tested. All tests were performed at a significance level of *P*=0.05. All *P* values are two-sided, and no corrections for multiple testing were applied.

Results

Response to neo-adjuvant chemotherapy

Thirty-three patients (94%) completed six courses of neo-adjuvant chemotherapy, while two stage IV patients stopped chemotherapy after five courses (disease progression and poor compliance). After neo-adjuvant chemotherapy, 28 out of 35 patients (80%) were operated on, while in seven cases (six stage IV and one stage IIIC) a debulking surgery was not possible even after chemotherapy. Overall, in 20 (57%) patients the goal of no residual tumour after surgery was achieved (see Table 2). After a median follow-up of 21 months (range 7–43 months) 21 patients have progressed (median progression-free survival (PFS),

15 months) and 10 patients have died. Interestingly, no correlation was observed between pathological response to neo-adjuvant chemotherapy and the gene expression levels of ESR1 (Spearman’s ρ 0.15; *P*=0.38).

Positive correlation of ESR1 mRNA expression with tumour proliferation

ESR1 gene expression levels were correlated with tumour grade and with the proliferation markers MKI67 and TOP2A in the available datasets of 35 patients. Spearman’s correlation of the pretreatment gene expression levels and histological grade revealed a high positive correlation of grading with MKI67 and TOP2A, which are known to be highly expressed in proliferative tissues (Fig. 1). Particularly mRNA expression of KI67, the most important indicator of cellular proliferation on a protein level, highly correlated with pathological grade (Spearman *r*=0.52 *P*=0.001). Importantly, also ESR1 expression levels positively correlated with TOP2A, KI67 and pathological grade (Spearman *r*=0.39 *P*=0.019; *r*=0.36 *P*=0.036 and *r*=0.37 *P*=0.030).

Effect of ESR1 mRNA expression on survival

ESR1 expression revealed a broad spectrum of gene expression levels over 2.5 logs (Fig. 2A). Univariate Cox regression analysis using ESR1 mRNA expression levels as a continuous covariate revealed a significant association for progression free survival

Figure 1 Spearman’s correlation of the pretreatment candidate genes expression levels and histological grade.

Figure 2 ESR1 expression, Affymetrix data distribution. (A) Distribution of the mRNA levels in the study population. Cut-offs for categorization to positive or negative ESR1 expression: median (solid line) and quartiles (scattered lines). (B) Survival status according to ESR1 baseline expression. P : Mann–Whitney test.

($\exp(B)=0.31$; 95% confidence interval 0.11–0.88; $P=0.03$) and for OAS ($\exp(B)=0.21$; 95% confidence interval 0.05–0.83; $P=0.02$). This demonstrates a dose-dependent relevance of ESR1 expression for the survival of the ovarian cancer patients. To test whether baseline ESR1 mRNA expression was an independent predictor of survival in the context of this neo-adjuvant chemotherapy setting, we performed a multivariate analysis using the Cox Proportional Hazards model. Age and pathological grading were included as covariates in multivariate analysis (Table 3).

ESR1 mRNA expression was an independent predictor of progression free survival in multivariate analysis ($\exp(B)=0.30$; 95% confidence interval 0.09–0.95; $P=0.041$). In addition, age below or above 65 years trended to be significant ($\exp(B)=0.46$; 95% confidence interval 0.18–1.20; $P=0.11$) and pathological grade was not significant ($\exp(B)=0.99$;

95% confidence interval 0.41–2.35; $P=0.98$). ESR1 mRNA expression remained significant in multivariate analysis also for OAS ($\exp(B)=0.12$; 95% confidence interval 0.02–0.62; $P=0.01$). Age below or above 65 years was not significant in univariate analysis ($\exp(B)=0.45$; 95% confidence interval 0.13–1.62; $P=0.22$), while displaying a trend towards significance in multivariate analysis ($\exp(B)=0.27$; 95% confidence interval 0.06–1.15; $P=0.08$). By contrast, pathological grade was not significant either in univariate analysis ($\exp(B)=1.08$; 95% confidence interval 0.34–3.42; $P=0.90$) nor in multivariate analysis, including these covariates ($\exp(B)=2.36$; 95% confidence interval 0.45–12.44; $P=0.31$).

Additionally, in the absence of an *a priori* cut-off for ESR1 gene expression, the median was chosen as objective cut-off for categorization of tumours as positive or negative (solid and scattered lines in Fig. 2A). Comparison of the survival status (alive versus dead) revealed a significant difference, when applying non-parametric Mann–Whitney test (Fig. 2B). This difference remained significant when only patients with a minimum follow-up of at least 12 or 24 months were taken into account (data not shown). Median ESR1 expression in survivors was 2578 versus 1363 in patients who have died ($P=0.018$).

Furthermore, in the univariate Kaplan–Meier survival analysis the expression levels of ESR1, as determined by Affymetrix array analysis from fresh tissue biopsies, were significant for patient's OAS when taking the median as objective cut-off (see Fig. 3A). High baseline expression of ESR1 mRNA was a positive prognostic factor for patient survival after neo-adjuvant chemotherapy. Univariate Kaplan–Meier survival analysis for progression free survival also revealed a trend towards prognostic value of ESR1 mRNA expression, when taking the median as objective cut-off (see Fig. 3A). As expected, patients with tumour residual after surgery <1 cm survived significantly longer than patients with residual >2 cm

Table 3 Multivariate survival analysis (Cox regression)

	Hazard	P value
Progression-free survival		
ESR1	0.30	0.04
Grade	0.99	0.98
Age	0.46	0.11
Overall survival		
ESR1	0.12	0.01
Grade	2.36	0.31
Age	0.27	0.08

Figure 3 Overall survival and progression-free survival. (A) Affimetrix analysis from fresh tissue biopsies: overall and progression-free survival. (B) Validation by RT-kPCR: overall and progression-free survival. *P*: log-rank test. OAS, overall survival; PFS, progression-free survival; ESR1, oestrogen receptor 1 mRNA; RT-kPCR, reverse transcription kinetic PCR.

(median not reached versus 20 months, log-rank *P* 0.005, and no differences in ESR1 expression levels were observed between these two groups (median 2403 vs 2329)).

Validation of predictive role of ESR1 expression by RT-kPCR

To exclude technical biases and to further validate the prognostic value of ESR1 mRNA expression for the survival of ovarian cancer patients by an independent technology, RT-kPCR analysis was performed using tumour tissue from the same fresh tissue biopsies collected before treatment. To compare with the microarray analysis described above, the median of the data distribution was again chosen as being objective and adequate given the sample size. ESR1 remained a significant prognostic factor, when using this alternative, more dynamic detection method, for both overall and PFS (Fig. 3B).

Discussion

Our study found that high baseline ESR1 mRNA expression positively correlates with tumour proliferation in therapy naïve tumours and with a better

outcome in patients treated with neo-adjuvant chemotherapy for stages III and IV epithelial ovarian cancer. Importantly, ESR1 expression turned out to be significant in univariate Cox regression analysis using ESR1 mRNA expression as continuous covariate. Both progression-free and OAS were significantly longer in patients with higher ESR1 mRNA expression and independent of grade (I and II versus III), and age (<65 vs ≥ 65 years). Therefore, in the context of neo-adjuvant chemotherapy, high ESR1 mRNA baseline expression is a strong prognostic factor for advanced ovarian carcinoma in terms of progression-free and OAS. Interestingly, pretreatment ESR1 mRNA expression was not related to the response to neo-adjuvant chemotherapy.

Based on our observation, a validation study was performed on an independent cohort of 114 patients treated with platinum-based adjuvant chemotherapy after surgery for stages I–IV ovarian carcinoma (Darb-Esfahani *et al.* 2009). This study confirmed in a different clinical setting the relevance of ESR1 mRNA expression as a prognostic factor. Interestingly, while we assessed fresh tissue biopsies, formalin fixed tissue was analysed in the validation study, thus allowing an easier future clinical application.

Baseline ESR1 expression positively correlated with the proliferative activity of the tumour. This is in line with observations that oestrogens stimulate ovarian cancer *in vitro* and *in vivo* (O'Donnell *et al.* 2005). ESR1 could thus be a surrogate for the proliferative activity. However, the prognostic power of baseline ESR1 mRNA expression was independent of histological grade and outperformed the value of established proliferation markers such as MKI67 and TOP2A. Clearly the prognostic value of ESR1 goes beyond proliferation and it is reasonable to assume that hormonal activities control not only proliferation, but also invasive and differentiation processes. It is well known, that ovarian functions are tightly controlled by hormonal activities. Elevated ESR1 expression in ovarian cancer may indicate tumours having conserved some kind of proliferation control and/or less invasive capacity. In contrast, reduced ESR1 expression may indicate tumours which have become independent of hormonal control, due to genetic alterations that primarily drive the aggressiveness of the tumour including proliferation, invasive and anti-apoptotic properties.

Our finding that ESR1 mRNA expression is of high prognostic value for chemotherapy treated ovarian cancer patients also suggests reconsidering the potential role of hormonal therapy in this disease. The ovary is an endocrine organ and it has been demonstrated in experimental models both *in vitro* and *in vivo* that a moderate to high expression of ESRs is associated with a growth response to oestrogens and with a growth inhibition after treatment with anti-oestrogens (Langdon *et al.* 1990, 1994).

The clinical studies with anti-oestrogens and with aromatase inhibitors involved only a limited number of patients, and their results are inconclusive (Rao & Miller 2006). However, a subset (15–20%) of unselected patients respond to tamoxifen (Hatch *et al.* 1991, Ahlgren *et al.* 1993, Tropè *et al.* 2000) and to aromatase inhibitors (Bowman *et al.* 2002, del Carmen *et al.* 2003, Papadimitriou *et al.* 2004). In the GOG trial (Hatch *et al.* 1991), the largest so far reported, a 17% objective response rate (including 6% in complete remission) was observed in 105 patients treated with tamoxifen after first relapse following chemotherapy, and no differences were observed between platinum sensitive and platinum resistant patients (Markman *et al.* 1996). Similarly, in the Mid-Atlantic Oncology Program experience (Ahlgren *et al.* 1993) a 17% remission rate, including two (7%) responses exceeding 5 years in duration, was described in chemorefractory patients treated with tamoxifen.

In most cases, the patients included in the phase II clinical trials had bulky disease and were heavily pretreated with chemotherapy. Even in metastatic breast cancer, where the role of hormonal therapy is well established, the remission rates reported with second-line endocrine therapy are in the range of 12–25% (Buzdar *et al.* 1998, Dombernowsky *et al.* 1998, Kaufmann *et al.* 2000, Buzdar *et al.* 2001, Howell *et al.* 2002). Moreover, in breast cancer the effect of hormonal therapy is diluted if patients are not selected on the basis of ESR status (Early Breast Cancer Trialists' Collaborative Group 2005). By contrast, the characteristics of ovarian tumours responding to hormonal therapy have not been defined yet, nor has a predictive marker for endocrine response been identified. In particular, it has never been consistently (Hatch *et al.* 1991, Bowman *et al.* 2002, del Carmen *et al.* 2003, Papadimitriou *et al.* 2004) demonstrated that the ESR protein expression measured by immunohistochemistry has any prognostic or predictive value for epithelial ovarian cancer patients. As a consequence, at the present time, hormone receptor determination of ovarian carcinoma is not routinely assessed and no hormonal therapy is approved by the US Food and Drug Administration or by European Agency for the Evaluation of Medicinal Products for the treatment of epithelial ovarian carcinoma.

Our data, confirmed by the results obtained in the independent validation cohort, suggest that ESR1 mRNA expression is a strong prognostic factor for ovarian cancer patients treated with platinum-based neo-adjuvant or adjuvant chemotherapy. We propose to consider ESR1 mRNA status in ovarian cancer, in order to minimize biological confounding effects in clinical trials. Furthermore, our results indicate that studies to investigate whether ESR1 mRNA expression is also a predictive factor for response to endocrine therapy are warranted. We have designed a multicentre prospective trial in order to verify this hypothesis.

Declaration of interest

Ralph M Wirtz and Elke Veltrup are employees of Siemens Healthcare Diagnostics, Cologne, Germany. According to all other authors, there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Claudio Zamagni and Ralph M Wirtz were responsible for the study design, study execution, data analysis and writing of the manuscript. Pierandrea De Iaco and Andrea Angelo Martoni contributed to the study execution and to the data analysis. Marta Rosati, Elke Veltrup, Federica Rosati, Elisa Capizzi, Nicoletta Cacciari, Carlo Alboni, Alessandra Bernardi, Francesco Massari, Sara Quercia, Antonietta D'Errico Grigioni, contributed to the study execution. Manfred Dietel, Jalid Sehoul and Carsten Denkert contributed to data interpretation. All authors were involved in manuscript preparation and consented to the submission of the present version of the manuscript.

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