Estrogen receptor 1 mRNA is a prognostic factor in ovarian carcinoma: determination by kinetic PCR in formalin-fixed paraffin-embedded tissue

Silvia Darb-Esfahani*, Ralph M Wirtz¹*, Bruno V Sinn*, Jan Budczies, Aurelia Noske, Wilko Weichert, Areeg Faggad, Susanne Scharff¹, Jalid Sehouli², Guelten Oskay-Özcelik², Claudio Zamagni³, Pierandrea De Iaco⁴, Andrea Martoni³, Manfred Dietel and Carsten Denkert

Institute of Pathology, Charité Universitätsmedizin Berlin, Charitéplatz 1, 10117 Berlin, Germany

¹Siemens Healthcare Diagnostics, Cologne, Germany

²Department of Gynaecology and Obstetrics, Charité Universitätsmedizin Berlin, Berlin, Germany

³Medical Oncology Unit, S.-Orsola-Malpighi Hospital, Bologna, Italy

⁴Obstetrics and Gynaecology Unit, University of Bologna, Bologna, Italy

(Correspondence should be addressed to S Darb-Esfahani; Email: silvia.darb-esfahani@charite.de)

*(S Darb-Esfahani, R M Wirtz and B V Sinn contributed equally to this work)

Abstract

Epidemiological and cell culture studies indicate that ovarian carcinoma growth is dependent on estrogen stimulation. However, possibly due to the lack of a reliable biomarker that helps to select patients according to prognostically relevant estrogen receptor (ER) levels, clinical trials using anti-estrogenic therapeutics in ovarian carcinoma have had inconsistent results. Therefore, we tested if ER expression analysis by a quantitative method might be useful in this regard in formalin-fixed paraffin-embedded (FFPE) tissue. In a study group of 114 primary ovarian carcinomas expression of estrogen receptor 1 (ESR1) mRNA was analyzed using a new method for RNA extraction from FFPE tissue that is based on magnetic beads, followed by kinetic PCR. The prognostic impact of ESR1 mRNA expression was investigated and compared to ER α protein expression as determined by immunohistochemistry. In univariate survival analysis the expression level of ESR1 mRNA was a significant positive prognostic factor for patient survival (hazard ratio (HR) 0.230 (confidence interval (CI) 0.102–0.516), $P=0.002$). ER α protein expression was correlated to ESR1 mRNA expression ($P=0.0001$); however, ER α protein expression did not provide statistically significant prognostic information. In multivariate analysis, ESR1 mRNA expression emerged as a prognostic factor, independent of stage, grade, residual tumor mass, age, and ER α protein expression (HR 0.227 (CI 0.078–0.656), $P=0.006$). Our results indicate that the determination of ESR1 levels by kinetic PCR may be superior to immunohistochemical methods in assessment of biologically relevant levels of ER expression in ovarian carcinoma, and is feasible in routinely used FFPE tissue.

Endocrine-Related Cancer (2009) 16 1229–1239

Introduction

Ovarian carcinoma is the fifth most common cause of cancer death in women in the US (Jemal *et al.* 2008). Since this type of tumor is usually detected at an advanced stage, most patients require an extensive surgical therapy with the goal of maximal tumor

reduction as well as an adjuvant chemotherapy approach (Winter *et al.* 2008). The standard chemotherapy strategy is a combination therapy of carboplatin and paclitaxel (Harries & Gore 2002). Large clinical studies have suggested that the addition of other chemotherapeutic substances does not lead to

improved clinical outcome (Bookman 2006). Initial response rates to treatment are high; however, most patients finally relapse and die of disease. Salvage chemotherapies show only limited responses and progression-free intervals are generally short. Additionally, classic side effects of these therapies can compromise on the quality of life in these incurable patients. Therefore, new therapeutic strategies for treatment of ovarian carcinoma are urgently needed.

Estrogen receptor (ER) is the prototype of a predictive biomarker. Its expression is used to plan the adjuvant therapy with tamoxifen or aromatase inhibitors in human breast cancer where detection of ER expression by immunohistochemistry is the standard approach (Harvey et al. 1999). Several studies have shown that tumors with high expression of ER show an improved response to tamoxifen or aromatase inhibitors (Fisher et al. 1983, Jakesz et al. 2005).

Estrogens are important regulators of normal ovarian function and there is ample evidence from cell culture studies that ovarian carcinomas are also dependent on estrogen stimulation (Langdon et al. 1994). Furthermore, several epidemiological studies have shown that the risk of ovarian carcinoma is increased after estrogen replacement therapy (Rodriguez et al. 2001, Lacey et al. 2002, 2006, Danforth et al. 2007). However, despite these data from basic research and epidemiology, most clinical trials using antihormonal strategies in patients with recurrent ovarian cancer have yielded disappointing results (Ahlgren et al. 1993, Markman et al. 1996, Karagol et al. 2007, Wagner et al. 2007; Williams CJ 2001 Tamoxifen for relapse of ovarian cancer. *Cochrane Database of Systematic Reviews* Issue 1 Art No CD001034, www.cochrane.org/reviews/en/ab001034.html). In a recent study by Smyth et al. (2007), a response rate of 17% has been observed with anti-estrogenic therapy in ovarian cancer. In a meta-analysis of several clinical trials including 647 patients, an overall objective response rate of 11% has been determined, however, there was a wide variation of response rates between 0 and 56% in the various trials (Tropé et al. 2000). This finding is strongly suggestive of the contribution of tumor-related factors that may be different in the various clinical cohorts. However, so far, there has been no significant correlation between clinical response and the expression of ER (Hatch et al. 1991).

Based on these disappointing results and the clinical need for new therapeutic approaches in ovarian cancer, we have developed the hypothesis that the measurement of ER expression by standard immunohistochemistry, which is the gold standard in breast cancer, may not be suitable for ovarian cancer. Immunohistochemical methods have the disadvantage that the evaluation is

only semiquantitative at best and that there is some subjective observer-based component. Therefore, for those types of tumors where only very small differences in biomarker expression may be relevant for biological behavior, the determination of the marker by a more quantitative and highly sensitive method is necessary. Reverse transcription (RT)-PCR is a quantitative method that can easily be performed on small samples; however, its use in formalin-fixed paraffin-embedded (FFPE) tissue has been very limited so far. Nevertheless, several groups have suggested methods to extract RNA from FFPE tissue and to perform a reliable RT-PCR (Paik et al. 2004, Chang et al. 2008).

In this project, we investigated if the determination of ER expression by quantitative RNA-based methods might be a better prognostic factor in FFPE tissue compared to standard immunohistochemistry. To test this hypothesis, we used a study group of 139 ovarian carcinomas. For all tumors, expression of estrogen receptor 1 (ESR1, estrogen receptor alpha, ESRA) was determined by immunohistochemistry. For a subset of 114 tumors, we isolated RNA from FFPE tissue using a new method that is based on magnetic beads and studied ESR1 mRNA expression by kinetic PCR.

Patients, materials, and methods

Study population and histopathological examination

The study group included 139 patients who underwent radical surgery for primary ovarian cancer in the Department of Gynecology and Obstetrics of the Charité University Hospital, Berlin, Germany from 1991 to 2005. Patients were included in the study based on the availability of tissue and follow-up data in the database. Resection specimens of all patients were examined and diagnosed at the Institute of Pathology, Charité University Hospital; Berlin, Germany. Histology and grade were re-evaluated by an experienced gynecopathologist (C D). Hematoxylin and eosin (H&E) stained sections were cut from paraffin embedded blocks and used for histopathological evaluation; grading of tumors was assessed using the Silverberg grading system (Shimizu et al. 1998). Patients were managed with extensive cytoreductive surgery followed by standard combination chemotherapy; the majority of patients were treated with a platinum-based regimen. Data on intraoperative residual tumor were available for 96 patients. Of these patients 21 (21.9%) had a postoperative residual tumor of ≤ 2 cm, 13 (13.5%) of > 2 cm, and 62 patients (64.4%) had no macroscopic residual tumor.

All clinical and pathological characteristics are comparable to the subset of 114 tumors used for mRNA analysis. Follow-up data were available for all patients in the study group. The date of last follow-up was either the date of the event (death or first clinical evidence of disease relapse) or the date of the last follow-up examination without event. Last update of the follow-up data was in October 2007. Follow-up examination was performed every 3 months during the first 3 years after surgery, every 6 months 4 to 5 years after surgery, and annually after the sixth year after surgery. The study has been approved by the institutional review board of the Charité Hospital.

Slide review and all experimental procedures were carried out blinded to the survival status of the patients.

Immunohistochemical staining and evaluation

Immunohistochemical staining was performed on tissue microarrays, which were constructed by selection of representative tumor areas, which were marked on the routine H&E stained histological sections. For each case four tissue cores (1.5 mm diameter) from different tumor areas of the sample tissue blocks were punched using a tissue micro-arrayer (Beecher Instruments; Woodland, CA, USA) and embedded into a new paraffin array block.

Immunohistochemical staining was performed using a Ventana Discovery XT instrument (Ventana Medical Systems Inc., Tucson, AZ, USA) according to the manufacturer's instructions. A polyclonal rabbit antibody directed against the C-terminus of human ESR1, diluted 1:50, was applied (Labvision, Fremont, CA, USA). ER immunostaining in tumor cells was evaluated without prior knowledge of clinico-pathological parameters and patient outcome. Nuclear immunoreactivity was evaluated according to the percentage of positive cells. As used in the evaluation of breast carcinomas, ER-positive cases were defined as tumors with more than 10% stained nuclei.

RNA extraction

A 10- μ m thick section was cut from each paraffin block and put in a 1.5 ml tube. All tumor samples included in the study contained at least 50% malignant cells as evaluated by H&E staining. RNA was extracted using a technique based on magnetic beads developed by Siemens Healthcare Diagnostics (Cologne, Germany) according to a standard protocol provided by the manufacturer. In brief, the FFPE section was deparaffinized in xylol and rehydrated in graded solutions of ethanol. The pellet was allowed to dry at room temperature for 10 min, lysed in lysis buffer and

SDS-PAGE for 5 min at 95 °C. Proteinase K was added and incubation at 56 °C with shaking for 2 h was done. A binding buffer and the magnetic beads were added and nucleic acids were allowed to bind to the beads for 15 min at room temperature aided by shaking. On a magnetic rack the supernatant was removed and the beads were washed several times with washing buffers. After addition of elution buffer and incubation for 10 min at 70 °C with strong shaking, the supernatant was transferred to a new tube without touching the beads. This was followed by DNase I treatment for 30 min at 37 °C then inactivation of DNase I, using TURBO DNA-free kit according to the procedure provided by the manufacturer (Ambion, Austin, TX, USA); after a short centrifugation the supernatant was taken carefully, kept at –80 °C and used for kinetic RT-PCR.

Kinetic RT-PCR

Intron-spanning primers and TaqMan fluorogenic probes for ESR1 gene and the housekeeping gene RPL37A were provided by Siemens Healthcare Diagnostics. All reactions were performed on an ABI Prism 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 and a reaction volume of 10 μ l on 384-well plates. PCR conditions were as follows: 30 min at 60 °C for RT 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. Cycle threshold (C_t) values, which indicate the (interpolated) number of PCR cycles until the fluorescence reached its threshold, were determined. After initial measurement of RPL37A, its C_t value was adjusted to 26 by dilution of each sample. Raw C_t values were measured in triplicates. Each gene was measured three times independently for each tumor sample. ΔC_t values, which is the difference between mean of the target gene C_t values and the mean of the housekeeping gene C_t values of each sample, were calculated, and $\Delta\Delta C_t$ values ($40 - \Delta C_t$) were used for statistical analysis, which thereby correlate proportionally to the mRNA expression level of the target gene (Monney *et al.* 2002, Fiala *et al.* 2007).

Inclusion criteria of samples for the statistical analysis were as follows: 1) the C_t value for RPL37A lay between 22 and 28, 2) at least a duplicate measurement was available for each gene and sample, 3) the single C_t values were not more than one C_t apart from each other. In our study on ESR1 expression 12 samples were excluded: in ten samples the mean C_t for RPL37A was > 29 ; in two samples the C_t for RPL37A was > 1 between different measurements.

Statistical evaluation

Statistic analyses were computed using SPSS 15 (SPSS, Chicago, IL, USA), and GraphPad Prism 4 statistical software (GraphPad Software, San Diego, CA, USA). The Mann–Whitney and Kruskal–Wallis tests were used for correlation with clinical and pathological features. For grouped data, Fisher’s exact test was performed. Patient survival in dependence of ESR1 expression was analyzed by the Cox regression model as well as the Kaplan–Meier method and the logrank test. The graph showing hazard ratios (HRs) in dependence of cut-off points was generated with the R package survival (www.r-project.org). In general, *P* values <0.05 were considered as statistically significant (two-tailed tests).

Results

Clinical and pathological features of the study group

A total of 139 cases were studied. The median age of the patient at diagnosis was 57 years with a range between 33 and 81 years. The median follow-up time for event-free patients was 38 months (range 2–118). During this follow-up period, 36 patients (25.9%) died.

Data on postoperative chemotherapy were available for 128 patients (92.1%). The majority of patients (119; 85.6%) had received a platinum-based chemotherapy, three patients had received another type of chemotherapy, and six patients, who had been staged FIGO I, had not received chemotherapy. The remaining clinical data are shown in Table 1.

Prognostic value of ESR1 mRNA expression in ovarian carcinoma

RNA was isolated from a total of 126 paraffin-embedded tumor samples and expression of ESR1 was determined by kinetic RT-PCR. Data of 114 cases (90.5%) met the quality requirements for statistical analysis; distribution of clinicopathological data and significance of classical prognostic factors were similar as in the whole study group of 139 patients (Table 1). ESR1 mRNA expression ($\Delta\Delta C_t$) ranged from 29.00 to 36.42 with a median of 34.39 $\Delta\Delta C_t$. The distribution of ESR1 gene expression in the study group is shown in Fig. 1A. $\Delta\Delta C_t$ ESR1 was a favorable prognostic factor in our study group. Separation of the study group by quartiles of ESR1 mRNA expression showed a significant survival difference between patients with low (>50% of study group) as well as high (<50% of study group) ESR1 mRNA expression in

Table 1 Clinical and pathological features of the study group

Parameter	Protein analysis		RNA analysis	
	Case number	Percent (%)	Case number	Percent (%)
Total	139	100	114	100
Age at surgery (years)				
≤60	81	58.3	66	57.9
>60	58	41.7	48	42.1
Histological type				
Serous	87	62.6	78	68.4
Mucinous	7	5.0	4	3.5
Endometrioid	18	13.0	14	12.3
Clear cell	6	4.3	2	1.8
Transitional cell	7	5.0	5	4.4
Undifferentiated	14	10.1	11	9.6
FIGO stage				
I	27	19.4	20	17.5
II	12	8.6	9	7.9
III	91	65.5	79	69.3
IV	9	6.5	6	5.3
pT				
pT1	29	20.9	22	19.3
pT2	15	10.8	11	9.6
pT3	95	68.3	81	71.1
pN				
pN0	55	39.6	44	38.6
pN1	56	40.3	44	38.6
pNX	28	20.1	26	22.8
Silverberg grade				
G1	22	15.8	15	13.1
G2	58	41.7	49	43.0
G3	59	42.5	50	43.9
Residual tumor after surgery				
None	62	44.6	51	44.8
≤2 cm	21	15.1	19	16.7
>2 cm	13	9.4	11	9.6
Missing	43	30.9	33	28.9
Chemotherapy (CTX)				
Platinum-based	119	85.6	98	86.0
Other CTX	3	2.2	2	1.7
No CTX	6	4.3	4	3.5
Missing	11	7.9	10	8.8

Kaplan–Meier analysis (*P*=0.013; Fig. 1B). To determine an optimum cut-off point for the study group, HRs were plotted against all possible cut-off points. As shown in Fig. 1C, a wide range of cut-offs yielded significant dichotomizations of the study group (upper confidence interval (CI) lower than 1). The lowest HR (0.230) with the narrowest CI (0.102–0.516) was observed by using a cut-off level of 34.10 $\Delta\Delta C_t$. Using this cut-off point, a highly significant separation of the study group in patients with a good and an unfavorable prognosis was achieved (*P*<0.0001; Fig. 1D). However, it should be stated that the validity of the *P* value is limited by the fact that the cut-off point had not been chosen *a priori*. A Cox regression

Figure 1 (A) Distribution of the mRNA levels in the study group. Dotted line: median. (B) Survival rate in dependence of quartiles of ESR1 mRNA expression ($\Delta\Delta C_t$). ESR1 highest, 75–100%; ESR1 high, 50–75%; ESR1 low, 25–50%; ESR1 lowest, 1–25% of study group. *P*, logrank test. (C) Hazard ratios in dependence of possible cut-off points (solid line). Dotted lines: 95% confidence interval (CI); vertical-dashed line, optimal cut-off. (D) Survival rate in dependence of optimal cut-off. High ESR1, $\leq 34.10 \Delta\Delta C_t$, Low ESR1, $> 34.10 \Delta\Delta C_t$. *P*, logrank test.

analysis with ESR1 mRNA expression as a continuous variable showed similar results (HR 0.756 (95% CI 0.634–0.903) per $\Delta\Delta C_t$ ($P=0.002$)).

Classical prognostic factors and evaluation of the prognostic effect of ESR1 mRNA in ovarian carcinoma subgroups

To see if the study group is representative of typical ovarian carcinomas, we evaluated other established prognostic factors. We found that Silverberg grading, presence of residual tumor, as well as the patient age were significant prognostic factors, as known for ovarian carcinoma (not shown). ESR1 mRNA expression was higher in non-serous than in serous and undifferentiated carcinomas ($P=0.049$), no associations were found with other clinicopathological factors (Fig. 2A).

We evaluated the prognostic role of the ESR1 mRNA in the clinically important subgroups of high-stage and high-grade tumors, as well as cases with no residual tumor. In the light of previous studies that showed a differential biomarker expression in different histological types of ovarian carcinoma (Köbel *et al.* 2008), we further investigated the largest histological subgroup of serous and undifferentiated carcinomas. In all subgroups, we applied the cut-off of $34.10 \Delta\Delta C_t$. As shown in Fig. 2B, the prognostic effect was retained in those major subgroups ($P<0.05$).

Determination of ER expression by protein analysis

Using immunohistochemistry, we evaluated ER α protein expression in ovarian carcinoma. We found a highly significant correlation between ER-protein expression and the results of the quantitative PCR ($P<0.0001$; Kruskal–Wallis test); however, there was a high variation of the mRNA levels within the different groups for ER α expression (Fig. 3A).

Neither Cox regression analysis using the percentage of stained tumor cells nor Kaplan–Meier analysis testing multiple different dichotomizations of ER α expression data revealed a significant prognostic effect of ER protein expression (Fig. 3B: optimal dichotomization of the study cohort using a cut-off point of 10% of stained tumor cells; $P=0.1552$; logrank test).

Multivariate analysis

In the multivariate analysis all parameters that were significant on univariate analysis were included. Cox regression analysis was performed using the optimal cut-off for ESR1 mRNA expression (Table 2A). In this setting, ESR1 mRNA expression was identified as a prognostic factor independent of age, grade, and residual tumor mass. Patients whose tumors expressed high levels of ESR1 had more than fivefold diminished

Figure 2 (A) Associations between ESR1 mRNA expression and clinicopathological parameters. Boxes, interquartile range; solid lines, median; whiskers, 10th–90th percentile, *P*, Mann–Whitney test. (B) Prognostic value of ESR1 mRNA expression in important ovarian carcinoma subgroups. The study group was separated by the use of the optimal cut-off point of 34.10 $\Delta\Delta C_t$, hazard ratios of ESR1 low expression is set as 1. Dots, hazard ratio of high ESR1 expression; whiskers, 95% confidence interval (CI), *P*, logrank test.

risk of death than patients with low-level ESR1 expression (HR 0.186 (95% CI 0.068–0.515), *P*=0.001). Furthermore, patient age and tumor grading were independent prognostic factors in our study group. Similar data were generated using ESR1 mRNA expression as a continuous parameter (*P*=0.003, HR 0.720 per $\Delta\Delta C_t$ (95% CI 0.580–0.895), not shown).

In a variant multivariate analysis including all traditional prognostic factors as well as ER α protein expression, ESR1 mRNA expression remained a significant prognostic factor (HR 0.227 (CI 0.078–0.656), *P*=0.006) while ER α protein expression did not reveal prognostic significance (HR 0.716 (CI 0.264–1.944), *P*=0.512, Table 2B).

Discussion

Our study evaluates a new quantitative method for the analysis of ER expression using a new method for isolation of mRNA from FFPE tissue followed by kinetic RT-PCR. By comparing the kinetic RT-PCR

with conventional immunohistochemistry, we demonstrate that the mRNA expression levels are clearly prognostic over a wide range of different cut-offs, while the protein-based method is not able to detect a prognostically significant expression difference.

This is the first time that the new bead-based RNA-extraction method has been tested in samples of ovarian cancer routine FFPE tissue for ER expression. Other studies have investigated hormone receptor expression by immunohistochemistry in ovarian cancer, and found that these receptors were expressed in a large subset of ovarian carcinoma. However, concerning the prognostic role the results were discordant. Several groups report a prognostic effect of progesterone receptor, but not of ER (Kommos *et al.* 1992, Münstedt *et al.* 2000, Hornung *et al.* 2004, Lee *et al.* 2005). Some groups reported a positive prognostic effect of ER expression measured by immunohistochemistry (Isola *et al.* 1990, Høgdall *et al.* 2007), while other studies did not find any prognostic effect (Scambia *et al.* 1995, Lu *et al.* 2006).

Figure 3 (A) Correlation between ER α protein and mRNA expression. *P*, Kruskal–Wallis test; solid lines, medians; ESR1 immunoreactivity refers to the rate of positive cells. (B) Survival rate in dependence of ER α protein expression. *P*, logrank test.

These results from previous studies suggest that immunohistochemistry may not be the ideal method to evaluate ER expression in ovarian cancer, and that quantitative mRNA-based methods may be more suitable for the evaluation of this marker. Two groups have investigated ER expression on the RNA level using fresh frozen ovarian tumor tissue. [Chan *et al.* \(2008\)](#) found a prognostic impact of ESR2 but not ESR1 mRNA expression; however, the significance of these results is limited by the fact that epithelial and non-epithelial malignant ovarian tumors as well as borderline tumors were evaluated together. [Fujimoto *et al.* \(2000\)](#) found the ratio ESR2 to ESR1 mRNA to be prognostic for ovarian cancer patients but did not evaluate ER mRNA expression quantitatively and used a very small sample size (28 patients) for the analysis.

Interestingly, using the same method for RNA extraction and quantification from FFPE samples of a large, randomized, multicenter clinical trial on breast cancer, we found that the optimal ESR1 mRNA cut-off to separate ovarian cancer patients according to outcome is very similar to the naturally occurring cut-off of ESR1 mRNA in breast cancer ([Pentheroudakis *et al.* 2009](#)). These data indicate that ESR1 expression levels are similar in both tumor entities.

Estrogens are important regulators of normal ovarian function. Human ovarian surface epithelial (HOSE) cells surround the ovary and play an important role in the repair of the cyclic rupture of the Graafian follicle during ovulation ([Murdoch 1996](#), [Katabuchi & Okamura 2003](#)). HOSE cells are sensitive to estrogen produced by granulosa cells in the developing follicle due to the expression of ER ([Lau *et al.* 1999](#)). In accordance with the function of HOSE cells in the normal ovary, estrogen stimulation results in the

Table 2 Multivariate survival analysis (Cox regression model) for overall survival of patients with invasive ovarian carcinomas

	Hazard ratio (HR)	95% CI of HR	<i>P</i> value
A			
$\Delta\Delta C_t$ ESR1 (optimal cut-off)	0.186	0.068–0.515	0.001
Grade (Silverberg)			
G1–2	1.000		0.039
G3	2.975	1.057–8.371	
Residual tumor			
0–2 cm	1.000		0.410
> 2 cm	1.539	0.552–4.287	
Age (per year)	1.055	1.009–1.103	0.020
B			
$\Delta\Delta C_t$ ESR1 (optimal cut-off)	0.227	0.078–0.656	0.006
ER α protein			
≤ 10%	1.000		0.512
> 10%	0.716	0.264–1.944	
Age (per year)	1.056	1.004–1.111	0.033
Grade (Silverberg)			
G1–2	1.000		0.054
G3	2.842	0.981–8.235	
Residual tumor			
0–2 cm	1.000		0.258
> 2 cm	1.824	0.643–5.170	
FIGO stage			
FIGO I–II	1.000		0.655
FIGO III–IV	1.599	0.204–12.559	
Histological type			
Serous + undifferentiated	1.000		0.333
Non-serous	1.892	0.521–6.869	

CI, confidence interval.

expression of genes regulating proliferation, cell cycle regulation, and energy balance (Syed *et al.* 2005). Under the hypothesis that recurrent injury of the surface epithelium during ovulation might lead to the accumulation of genetic alterations, which might result in neoplastic transformation, HOSE cells are suggested to constitute the cells of origin of epithelial ovarian cancer (Godwin *et al.* 1992). However, HOSE cells differ remarkably from ovarian cancer cells in the expression profile of estrogen-regulated genes, i.e. genes with tumor-suppressing functions are upregulated by estrogen stimulation in HOSE cells and downregulated in ovarian cancer cells (Syed *et al.* 2005). The biological functions of estrogens are mediated by the nuclear ERs that bind to estrogen-responsive elements that are located in the promoter region of target genes. In our study, we focused on ESR1. This ER isoform has been identified as the main isoform in ovarian cancer and an upregulation of ESR1 is a key feature of ovarian carcinomas (O'Donnell *et al.* 2005), while a loss of ESR2 (estrogen receptor β , ESRB) has been described in this tumor type (Bardin *et al.* 2004). Therefore, based on the results obtained with the determination of ESR1 mRNA by array and kinetic PCR in fresh tissue biopsies, we have chosen ESR1 as the main type of ER for our study. In cell culture models, it has been shown that proliferation of ovarian cancer cells is stimulated by estrogens and inhibited by tamoxifen and that this effect is related to the expression level of ER (Langdon *et al.* 1990). Furthermore, the anti-estrogenic treatment might be synergistic to current standard therapies, since it has been shown in ovarian carcinoma cell lines that the development of resistance to cisplatin is delayed by tamoxifen and antiproliferative effects are increased (McClay *et al.* 1994, Ercoli *et al.* 1996).

It may be expected that anti-estrogenic agents are effective predominantly in ovarian carcinomas with active ERs similar to hormone-receptor-positive breast cancers. Considering the fact that several clinical trials have resulted in inconclusive results on the therapeutic benefit of anti-estrogenic medications, a better system for biomarker assessment in ovarian cancer is definitely needed in order to identify the patients who would benefit from such medication. In the early clinical trials, it has been suggested that a semi-quantitative immunohistological scale could be used as an inclusion criterion for clinical trials (Bowman *et al.* 2002, Smyth *et al.* 2007). However, ER expression level is a continuous parameter; therefore, it is difficult to identify the relevant threshold for biologically important ER expression. In our study, ER protein expression was highly correlated to mRNA expression,

yet, within the different groups for the immunoreactive score measuring the protein expression, there was a considerably high variation of the mRNA levels, indicating that for single cases the protein expression may not measure the complete range of receptor expression. This may explain the fact that we found mRNA expression but not protein expression to show a prognostic impact although both parameters were correlated.

Interesting research topics for future studies will be the evaluation of the mechanisms of ESR1 mRNA overexpression, such as ESR1 gene amplification that has been reported in breast and endometrial carcinoma (Holst *et al.* 2007, Lebeau *et al.* 2008). Similarly, progesterone receptor expression might be helpful in the prediction of patient prognosis and therapy response and should be investigated further.

In this study, we demonstrate that the determination of ER levels by kinetic PCR yields better results with respect to survival prediction than immunohistological methods in assessment of ER expression in ovarian carcinoma and may be a valuable method for the identification of those cases where ER expression is of biological significance. Most importantly, this analysis is feasible in routine FFPE tissue. Therefore, further clinical studies should use this new technique to measure ER expression and to define quantitative inclusion criteria for anti-estrogenic therapies. This might be an approach to reduce the high variability of response rates that has been observed in the various clinical trials and a contribution to a more individualized therapy in ovarian cancer.

Declaration of interest

Ralph M Wirtz and Susanne Scharff 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.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Acknowledgements

We would like to thank Ms Lisa Glanz and Ms Petra Wachs for their excellent technical assistance and Ms Martina Eickmann for her assistance in editing the manuscript.

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