Lack of relationship between EGFR-1 immunohistochemical expression and prognosis in a multicentre clinical trial of 93 patients with advanced primary ovarian epithelial cancer (*GINECO* group)

Clinical

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Epidermal growth factor receptor 1 (EGFR-1) overexpression is usually described as linked with a worse prognosis in a variety of tumours of epithelial origin. However, its role in ovarian cancer is still controversial. The aim of the present study was to analyse the prognostic impact of EGFR-1 in a retrospective series of 93 stage III–IV primary ovarian epithelial tumours. All patients, enrolled in a multicentre GINECO prospective clinical trial, were treated with the same platinum-based combination chemotherapy, and were followed up with a median of 69 months. Epidermal growth factor receptor 1 plasma membrane expression, assessed by immunohistochemistry on paraffin-embedded tissues, was correlated with clinical parameters as well as immunohistochemical expression results of HER-2 (c-erbB-2), BAX, BCL-2, p53 and anti-Ki-67, previously studied in the same series of patients. Positive immunostaining for EGFR-1 was seen in 31 of the 93 analysed cases (33%). No correlation was found between EGFR-1 expression and clinical parameters. No correlation was found between EGFR-1 expression and other biological markers, except for HER-2, which was limit for significance. Indeed, among the EGFR-1-negative cases, 10.3% expressed HER-2, whereas the HER-2-expressing tumours accounted for 27.6% of EGFR-1-positive cases ($P = 0.06$). Epidermal growth factor receptor 1 overexpression had no prognostic impact on both overall and progression-free survival through univariate and multivariate analyses. The potential effect of EGFR-1 and HER-2 co-expression on targeted therapy against EGFR-1 and/or HER-2 molecules has to be further analysed.
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Ovarian cancer is known to have one of the highest mortality rates of all gynaecological malignancies. Due to the lack of reliable tumour markers, most patients present an advanced stage of the disease at the time of diagnosis. Despite a good initial response to first-line chemotherapy, prognosis remains poor due in part to the development of resistance to chemotherapy. According to the International Federation of Gynecology and Obstetrics (FIGO), clinical stage, histological grade and postoperative residual tumour mass are all identified as the most important prognostic factors for survival in patients with ovarian cancers. Clinical factors as well as prognostic models derived from these clinical factors (Clark *et al*, 2001) remain insufficient to predict accurately the outcome for a specific patient. New markers are needed in order to identify groups of patients who may benefit from different therapeutic options. In this paper, we analyse the prognostic role of EGFR-1 immunohistochemical overexpression.

Recently, the interest of the scientific community has increased for receptors with tyrosine kinase activity, since they constitute potential therapeutic targets. Among them are HER-2 and EGFR-1, two of the four known members of the EGFR family. Epidermal growth factor receptor family receptors (ErbB family) play an important role in regulating a wide variety of cellular functions, including regulation of cell cycle, cell death, angiogenesis and cell differentiation (Alper *et al*, 2001). After binding the ligand (epidermal growth factor or transforming growth factor-alpha), EGFR-1 oligomerises with other EGFR-1 molecules or other members of EGFR family (e.g. HER-2). Activation of the intrinsic receptor tyrosine kinase promotes an intracellular pathway, leading to DNA replication and cell division (Carpenter, 1984).

Abnormal expression of EGFR-1 and its ligands has been shown in several human cancers, such as lung (Tateishi *et al*, 1994), gastric (Yonemura *et al*, 1992), oral (Storkel *et al*, 1993; Maurizi *et al*, 1996), breast (Hainsworth *et al*, 1991) and colorectal (Steele *et al*, 1990; Kluftinger *et al*, 1992; De Jong *et al*, 1998) cancers. The overexpression of EGFR-1 in ovarian carcinoma and cell lines derived from this tumour has also been reported. However, the prognostic impact of EGFR-1 expression in ovarian cancers still

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remains controversial. In a previous work (Camilleri-Broët *et al*, 2003), we showed a poor prognostic impact of HER-2 over-expression, whereas other tested biological markers (Ki-67, p53, BCL-2 and BAX) were not of prognostic significance. In the present study, we analysed EGFR-1 immunohistochemical expression in a series of homogeneous patients with advanced ovarian tumours in order to evaluate its prognostic impact.

PATIENTS AND METHODS

Patients

All cases were included in the CEP trial of the GINECO group, which included from February 1994 through June 1997 164 patients with advanced ovarian cancer (range: 18–70 years). All patients had a histological proven epithelial ovarian cancer, stage III or IV according to the FIGO guidelines, a World Health Organisation (WHO) performance status less than 3, no previous chemotherapy and no major organ failure. Chemotherapy regimen consisted of a combination of i.v. cisplatin ($75 \, mg \, m^{-2}$), epirubicin ($50 \, mg \, m^{-2}$) and cyclophosphamide regimen (CEP) that patients received for six cycles, with a 21-day interval between each cycle. The cyclophosphamide regimen was randomised between a standard dose of $500 \, mg \, m^{-2}$ and a higher dose of $1800 \, mg \, m^{-2}$ with G-CSF support. Following the six-cycle chemotherapy course, patients were regularly monitored at 3 months interval during the first 3 years and every 6 months thereafter. Second-look laparotomy was performed in 114 patients (69.5%).

At the time of our analysis, 134 patients (87%) showed a disease progression, and 117 (71%) deaths have been recorded for the entire population. The median progression-free survival duration was 15 months (95% confidence interval (CI): [13.1–16.5]) and the median overall survival was 32 months (95% CI: [27.7–36.2]). Response to chemotherapy was evaluated either clinically or with a second-look laparotomy, defined as a 50% or greater reduction in the product obtained from the measurement of each lesion and no appearance of new lesions. Response to chemotherapy was observed in 74 patients (88%).

There was no statistical difference in response rate, progression-free survival and overall survival, according to the cyclophosphamide dose in the two treatment arms. For the biological study, we therefore analysed all patients as a homogeneous population. Of the 164 patients included in the clinical trial, slides from 93 tumours obtained from first surgery were available for further immunohistochemical analysis. Clinical and biological variables were not different when comparing the group to the overall population (detailed in Table 1). Age was categorised into two groups: (a) ⩽60 years and (b) >60 years.

Material

For each case, one sample chosen from the first surgery was reviewed, for histological type and grade, by a panel of pathologists unaware of the initial diagnosis (for more details, see our previous work in the same series of patients) (Camilleri-Broët *et al*, 2003).

Out of the 93 cases reviewed, 57 cases (61.3%) were diagnosed as serous or mixed epithelial carcinoma, two (2.2%) were clear cell carcinoma, seven (7.5%) endometrioid and 26 (28.0%) were undifferentiated carcinoma. In non-clear cell carcinoma, a clear cell constituent was found in 10 (10.8%) cases. Among the 93 cases with primary ovarian tumour samples, nine (9.7%) were considered as grade 2, 38 (40.9%) cases as grade 3 and 26 (28%) cases as grade 4 (Table 1).

Immunohistochemistry

Immunohistochemical study was performed with the monoclonal antibody raised against EGFR-1 (1/10 dilution, NCL-EGFR, clone

Table I Patient characteristics

Characteristics	Clinical trial		Biological study	
	N = 164	%	N = 93	%
Age				
Median	59		60	
Range	23–74		23–70	
WHO performance status				
0	41	25.0	22	23.7
I	98	59.8	56	60.2
2	15	9.1	9	9.7
Missing value	10	6.1	6	6.5
FIGO stage				
IIIa	5	3.0	2	2.2
IIIb	30	18.4	16	17.2
IIIc	90	54.9	55	59.1
IV	34	20.7	20	21.5
Missing value	5	3.0	0	0
Ascitis				
No	86	52.4	46	49.5
Yes	70	42.7	46	49.5
Missing value	8	4.9	1	1.1
Residual tumour after first laparotomy				
No residual disease	20	12.2	11	11.8
Residual disease <2 cm	51	31.1	28	30.1
Residual disease >2 cm	89	54.3	54	58.1
Missing value	4	2.4	0	0
Histological type				
Serous or mixed epithelial	74	45.1	57	61.3
Clear cell	4	2.4	2	2.2
Endometrioid	9	5.5	7	7.5
Undifferentiated	30	18.3	26	28.0
Missing value	47	28.7	1	1.1
Histological grade				
Grade 1	0	0	0	0
Grade 2	15	9.1	9	9.7
Grade 3	47	28.7	38	40.9
Grade 4	33	20.1	26	28.0
Missing value	69	42.1	20	21.5

EGFR.113; Tebu) on slides obtained from fixed and paraffin-embedded tissue. Thin $4 \, \mu m$ slices were deparaffinised in a routine manner, followed by a microwave pretreatment in tampon urea ($4 \, M$, pH 7) and 2 h incubation with the primary antibody. A standard streptavidin–biotin–peroxidase method was applied using a commercially available kit (ABCYS Biospa, Milano), including 30 min incubation for each step, and nuclei were counterstained with haematoxylin. The immunohistochemical study was performed in a single laboratory.

All slides were examined by two pathologists without any knowledge of the clinical data, using a double-headed microscope. Epidermal growth factor receptor 1-positive expression was defined as a plasma membrane positivity in more than 10% of tumour cells. Cytoplasmic staining was considered as nonspecific. A highly EGFR-1-expressing squamous cell lung carcinoma was used as a positive control.

Statistical analysis

The relationship between EGFR-1 status and the categorical variables described just below were tested using the χ^2 test or Fisher's exact test, whichever was appropriate: patient

characteristics (age, WHO performance status, FIGO stage, residual tumour volume and ascitis), histological subtype, tumour grade and immunohistochemical results issued from our previous study (Ki-67, BCL-2, BAX, HER-2 expressions).

Overall survival was calculated from the date of surgery to death or last follow-up examination. Progression-free survival was calculated from the date of surgery to progression or last follow-up examination. Survival curves were derived from Kaplan–Meier (Kaplan and Meier, 1958) estimates. Univariate Cox model analysis (Cox, 1972) was performed to estimate and test the prognostic influence of clinical variables and biological markers/immunohistochemical labelling data. Prognostic impact of EGFR-1 overexpression, adjusted for the other prognostic factors, was assessed in multivariate analyses by using the Cox proportional hazards regression model (Cox, 1972) from a backward stepwise selection procedure. In this setting, we have selected for the started model variables associated with prognosis, with a *P*-value less than or equal to 0.20 in univariate analyses. Hazard ratios (HRs) associated with overall survival or progression-free survival are given with their 95% CI. Statistical significance was considered as *P*-values less than 0.05. All these analyses were carried out using the S-Plus software package.

RESULTS

Expression of EGFR-1 according to clinical and other biological variables

Epidermal growth factor receptor 1 membrane expression has been found in more than 10% of tumour cells in 31 out of the 93 cases tested (33.3%). As we observed previously for HER-2 overexpression in ovarian carcinomas, some cases showed an important heterogeneity of expression (Figure 1).

No relationship was found between EGFR-1 expression and all tested clinical parameters, as well as the previously studied biological parameters: p53, BAX, BCL-2 and the rate of Ki-67 positivity (Table 2). Among the 87 patients who were tested for both EGFR-1 and HER-2 expression, eight cases (8.6%) co-expressed EGFR-1 and HER-2. Among the EGFR-1-negative cases, six out of 58 (10.3%) expressed HER-2, whereas the HER-2-expressing tumours accounted for eight of the 29 EGFR-1-positive cases (27.6%). This link between EGFR-1 and HER-2 over-expressions was of limited significance ($P = 0.06$).
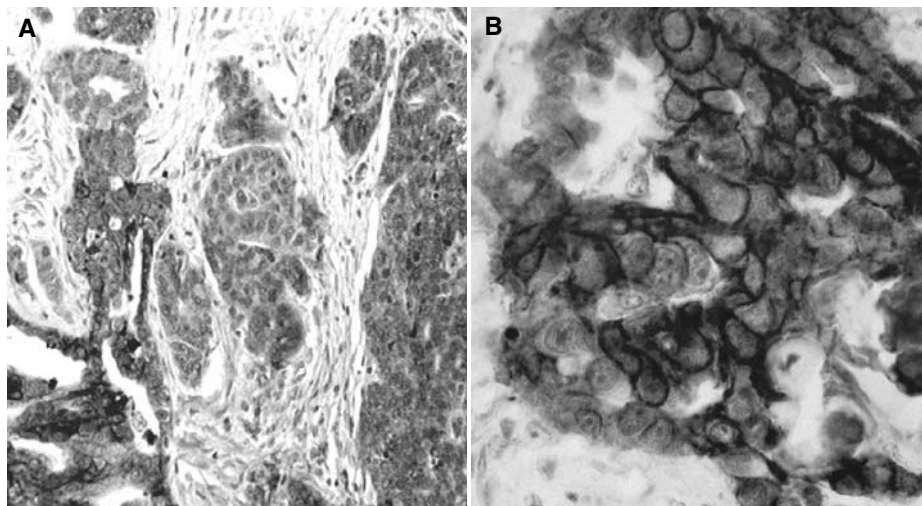
Patient's outcome and response to chemotherapy

Response to chemotherapy was observed in 50 patients of the 59 EGFR-1-negative patients (85%) and in 24 of the 25 EGFR-1-positive patients (96%). This difference was not significant ($P = 0.27$).

At the time of our analysis, out of the 93 patients included in our retrospective series, 80 patients showed a disease progression (86.0%) and 74 patients died (79.6%). The median follow-up was 69 months (95% CI: [58.8–79.0]). Within EGFR-1-overexpressing tumours (31 cases), 28 patients (90.3%) had shown a disease progression, while 24 (77.4%) died.

In univariate analysis, EGFR-1 expression had no prognostic impact on progression-free survival ($P = 0.80$). The median progression-free survival time was 14.3 months (95% CI: [12.9–20.2]) in tumours with EGFR-1-negative cases, whereas it was 15.3 months (95% CI: [13.0–27.0]) in EGFR-1-positive cases (Figure 2A). In contrast, the presence of an ascitis ($P = 0.02$) and HER-2 overexpression by tumour cells ($P = 0.01$) were significantly associated with shorter progression-free interval.

When focusing on overall survival, EGFR-1 expression still had no significant prognostic impact ($P = 0.45$). The median overall survival duration was 25.6 months (95% CI: [22.6–44.6]) in EGFR-1 negative cases, whereas it was 34.3 months (95% CI: [29.0–38.9]) in EGFR-1-positive cases (Figure 2B). The presence of an ascitis ($P = 0.03$), a high performance status ($P = 0.03$) and HER-2 overexpression ($P = 0.03$) were significantly associated with shorter overall survival.

In the final Cox model for multivariate analysis (Table 3), presence of ascitis ($P = 0.008$) and HER-2 overexpression ($P = 0.001$) both retained a poor prognostic impact on progression-free survival. Regarding overall survival, FIGO stage ($P = 0.043$), presence of ascitis ($P = 0.030$) and HER-2 overexpression ($P = 0.008$) were linked with a poor overall survival. When including EGFR-1 expression variable in the previous multivariate Cox models, it still did not show any prognostic impact (data not shown).

DISCUSSION

Epidermal growth factor receptor 1 overexpression is thought to be linked with a poor prognosis in several common solid tumours. However, regarding ovarian carcinomas, its prognostic value still remains debated in the literature, showing for some studies a poor

Figure I Immunohistochemical labelling results with anti-EGFR-1 antibody-1. (**A**) Heterogeneous plasma membrane expression for EGFR-1 in tumour cells. (**B**) Strong and homogeneous plasma membrane expression for EGFR-1 in tumour cells.

Table 2 Univariate analysis for overall survival and progression-free survival of clinical, histopathological and immunohistochemical parameters

Prognostic factor	Overall survival		Progression free survival	
	HR [95% CI]	P-values	HR [95% CI]	P-values
EGFR-1 (% of tumour cells)	0.83	0.45	0.94	0.81
⩾10% vs <10%	[0.51; 1.35]		[0.60; 1.50]	
Age	1.07	0.76	1.31	0.23
⩾60 years vs <60 years	[0.68; 1.70]		[0.84; 2.03]	
Performance status	1.95	0.03	1.50	0.15
1/2 vs 0	[1.08:3.52]		[0.87; 2.59]	
Stage	1.48	0.19	1.10	0.73
IIIc/IV vs IIIa/IIIb	[0.83; 2.66]		[0.63; 1.91]	
Ascitis	1.69	0.03	1.71	0.02
Presence vs absence	[1.06; 2.69]		[1.09; 2.68]	
Residual tumour after first laparotomy	1.15	0.55	1.18	0.46
>2 vs <2 cm	[0.73; 1.83]		[0.76; 1.85]	
Tumour type	0.81	0.57	1.09	0.79
Clear cell component vs others	[0.39; 1.69]		[0.58; 2.07]	
Tumour grade	0.92	0.75	0.90	0.70
3/4 vs 1/2	[0.54; 1.57]		[0.54; 1.52]	
Ki-67 expression (% of nuclei surface)	0.96	0.89	0.81	0.41
⩾30 vs <30	[0.56; 1.65]		[0.48; 1.35]	
BCL-2 (% of tumour cells)	1.37	0.20	1.19	0.09
⩾10 vs <10%	[0.85; 2.21]		[0.94; 2.37]	
BAX (% of tumour cells)	0.82	0.41	1.08	0.76
⩾10 vs <10%	[0.51; 1.32]		[0.68; 1.71]	
p53 (% of tumour cells)	1.11	0.68	1.18	0.49
⩾10 vs <10%	[0.69; 1.79]		[0.74; 1.87]	
HER-2 (% of tumour cells)	1.99	0.03	2.37	0.01
⩾10 vs <10%	[1.08; 3.69]		[1.26; 4.46]	

HR = hazard ratio, CI = confidence interval.
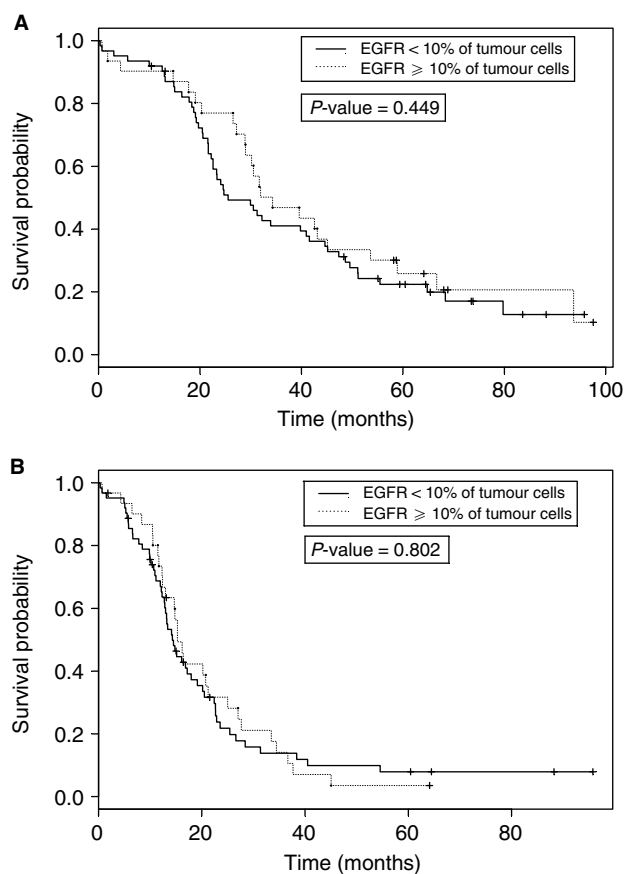
Figure 2 Overall survival (**A**) and progression-free survival (**B**) according to EGFR-1 plasma membrane expression.

Table 3 Multivariate analysis for overall survival and progression-free survival of FIGO stage, ascitis and HER-2 overexpression

Prognostic factor	Overall survival			Progression-free survival		
	HR	95% CI	P-value	HR	95% CI	P-value
FIGO stage						
IIIa/IIIb	1					
IIIc/IIId	2.13	1.02−4.42	0.043	—	—	—
Ascitis						
Absence	1			1		
Presence	1.77	1.06−2.97	0.030	1.96	1.20−3.20	0.008
HER-2						
<10% of tumour cells	1	1		1		
⩾10% of tumour cells	2.66	1.30−5.44	0.008	3.28	1.61−6.70	0.001

HR = hazard ratio, CI = confidence interval.

prognosis impact (Scambia et al, 1992, 1995; Fischer-colbrie et al, 1997; Skirnisdottir et al, 2001), whereas others showed no influence of EGFR-1 overexpression on patient's outcome (Van der Burg et al, 1993; Meden et al, 1995; Bartlett et al, 1996; Baekelandt et al, 1999). Only one study in the literature (Bauknecht et al, 1988) showed a favourable prognostic role of EGFR-1 overexpression, but no multivariate analysis was presented. In this series, we have found no significant prognostic impact on the survival time for EGFR-1 overexpression in a homogeneous series of advanced ovarian cancer patients.

These discrepancies in the prognostic role of EGFR-1 may be related to different technical methods used, different antibodies tested, different cutoff values considered, major differences in the

rate of EGFR-1-positive cases ranging between 13 and 82%. The advantage of the immunohistochemical technique is to select specifically positive plasma membrane expression of receptors by tumour cells. In contrast, ligand-binding assays give a continuous result, in part dependent on the proportion of tumour cells present in the tissue tested, and requires determining a threshold for positivity.

Four main studies have shown a worse prognosis of the EGFR-1-positive ovarian carcinomas (Scambia *et al*, 1992, 1995; Fischer-colbrie *et al*, 1997; Skirnisdottir *et al*, 2001). The first study of Scambia *et al* (1992) concerned 72 subjects with stage III and IV tumours, whereas the second (1995) concerned 117 patients with all clinical stages. In both studies, the EGFR-1 overexpression status, assayed by a radioreceptor method, led to a rate of 54% positive cases. The EGFR-1 overexpression was not linked to clinical and biological characteristics, but was assessed as an independent prognosis factor. The study of Fischer-Colbrie *et al* (1997) included 108 patients of all clinical stages, with a high rate of positive patients for EGFR-1 overexpression (61%), determined with a radioligand-binding assay. The authors found a poor prognosis impact for EGFR-1 overexpression on progression-free and overall survival in univariate analysis, but no adjustment on clinical variables was made, especially as the part of EGFR-1-positive cases increased significantly with clinical stage in this study. However, these papers, showing a poor prognostic impact of EGFR-1 overexpression, had a higher rate of positive cases, suggesting that the studied positive group may be very different from that of our series. Moreover, two of them analysed a heterogeneous series of patients including tumour stages I–IV. Since EGFR-1 overexpression may increase with the FIGO stage (Fischer-Colbrie *et al*, 1997), the exact impact of such correlation on survival results remains to be investigated. The last study (Skirnisdottir *et al*, 2001) included only early stage (FIGO I and II) epithelial ovarian carcinomas, using thus a population very different from ours.

Among the main papers which showed no prognostic influence of EGFR-1 status (Van der Burg *et al*, 1993; Meden *et al*, 1995; Bartlett *et al*, 1996; Baekelandt *et al*, 1999), two included a large number of patients. Baekelandt *et al* (1999), included 185 homogeneous patients with stage III ovarian cancers. Epidermal growth factor receptor 1-positive immunostaining, observed in 22% of the cases, had a tendency towards worse prognosis in univariate analysis, but was not found to be an independent prognosis factor in multivariate analysis. The study published by Meden *et al* included 266 primary ovarian cancer specimens with FIGO stage I–IV. In all, 13% of the cases were scored positive for EGFR-1 with an immunohistochemical method, showing no significant correlation between EGFR-1 status and overall survival. Our series confirms these findings and is the first study to analyse a series of homogeneous patients (FIGO stage III–IV), included in a multicentre clinical trial followed with a long follow-up (median 69 months).

Epidermal growth factor receptor 1 is one of the promising targets for innovative cancer therapies. Among them, the clinical interest of inhibitors targeting the tyrosine kinase domains of EGFR family (Baselga *et al*, 2002) (e.g. ZD1839, Iressa®, Astra-Zeneca) are under clinical investigation. In contrast to HER-2, whose overexpression predicts clinical response to trastuzumab (Herceptin®), it seems that EGFR-1 overexpression is not necessary for using the therapies targeting its activity (Arteaga, 2002). Indeed, in addition to high expression of the receptor, EGFR-1 signalling may be upregulated by increased expression of its ligands, activating receptor mutations, heterodimerisation with other members of the family such as HER-2, and alterations of downstream molecules in the signalling pathway. Only assessment of these different steps may provide a more complete information on the activation of the EGFR pathway and thus on potential response to EGFR-targeted agents.

Some papers suggest that EGFR-1 overexpression could potentialise the antineoplastic activity of other classical drugs of chemotherapy or ionising radiation *in vitro* or *in vivo* in nude mice (Ciardiello *et al*, 1999; Bianco *et al*, 2002; Kari *et al*, 2003). The principle of such cooperation seems to induce apoptosis, following a downregulation of the antiapoptotic BCL-2 and BCL-XL proteins. In our study, we have found no correlation between BCL-2 and EGFR-1 expressions. In a clinical setting, conflicting results have been reported with regard to response to chemotherapy and EGFR-1 overexpression. In contrast to Scambia *et al*. (1995), who found that EGFR-1 overexpression was of borderline significance in response to chemotherapy, we did not observe such a correlation in our series, similar to that observed by other papers (Baekelandt *et al*, 1999; Ferrandina *et al*, 2002). However, further studies are needed to analyse its potential impact on patients treated with other regimens, including paclitaxel.

In our series, a positive correlation of limited significance ($P = 0.06$) was found between EGFR-1 and HER-2 overexpressions, similar to that found by Ferrandina *et al*. (2002) and Skirnisdottir *et al*. (2001). Heterodimerisation of EGFR-1 and HER-2 is functionally active. We have not observed prognostic impact for EGFR-1 and HER-2 co-expressing tumours as compared to the HER-2-positive/EGFR-1-negative group, but this comparison was made with a limited number of patients (data not shown). A recent study from Christensen *et al*. (2001) showed *in vitro* and *in vivo* in nude mice that it is more difficult to inhibit EGFR-1 phosphorylation in cells that express high levels of HER-2 expression. However, in clinical practice, no relationship between HER-2 overexpression and response to ZD1839 (Iressa®) was shown in a series of patients suffering from non-small-cell lung cancer (Cappuzzo *et al*, 2003). Further studies are needed to answer this question in ovarian carcinomas.

In conclusion, our study showed the lack of prognostic impact of EGFR-1 overexpression in a large homogeneous population of stage III and IV ovarian cancer, included in a multicentre clinical trial. However, the absence of prognostic impact eliminates neither a possible role of EGFR-1 in oncogenesis nor a potential benefit of EGFR-targeted agent. Moreover, since a positive correlation of limited significance between EGFR-1 and HER-2 expressions was found, further studies are needed to investigate the clinical effect of such co-expression in a population treated by other regimens, including paclitaxel or targeted therapies.

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