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The Prognostic Value of Epidermal Growth Factor Receptors, Determined by Both Immunohistochemistry and Ligand Binding Assays, in Primary Epithelial Ovarian Cancer: a Pilot Study

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After our previous studies on the incidence of epidermal growth factor receptors (EGF-R) and its relationships with other tumour characteristics in more than 100 ovarian tumours, in the present study we investigated the prognostic value of EGF-R with respect to progression-free survival in 50 patients with primary ovarian cancer and sufficient follow-up (median 26 months, range 10–33 months). EGF-R was measured by both biochemical and two immunohistochemical methods, using two monoclonal antibodies (MAb), in addition to oestrogen receptors (ER) and progesterone receptors (PgR). EGF-R by ligand binding assay and Scatchard analysis were detectable in 63% of the tumours, by immunohistochemistry with MAb 2E9 in 82% and with MAb EGF-R1 in 78% of the tumours. ER-positivity was found in 58% and PgR-positivity in 38% of the patients. The results of the three measurements of EGF-R showed only weak to moderate associations with Spearman rank correlations (Rs) between 0.13 and 0.46. ER and PgR were only weakly correlated (Rs = 0.20) and they showed no significant association with EGF-R status. There was no clear evidence of the existence of correlations between receptor values and FIGO stage and tumour rest. Univariate Cox regression analyses showed that a higher FIGO stage and larger tumour rest were associated with shorter progression-free survival ($P = 0.001$), while PgR positivity was associated with a longer progression-free survival ($P = 0.02$). The level of EGF-R (irrespective of the method of determination used) showed a positive correlation with the risk of progression, but this correlation was not statistically significant.
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INTRODUCTION

EPIDERMAL GROWTH factor (EGF) is a 53 amino acid polypeptide that can influence proliferation and differentiation of a wide variety of cells [1, 2]. EGF and transforming growth factor-α (TGF-α), both of which can activate EGF receptor (EGF-R), are produced locally in many tissues as local growth factors rather than as systemic hormones. There is evidence that EGF plays a role in carcinogenesis and that the EGF-stimulated growth regulatory system is also involved in proliferation of malignant cells [3]. Cellular events are induced by EGF via its cell membrane receptor (EGF-R). The EGF-R is a 170 K glycoprotein that can be divided into an extracellular domain binding EGF or TGF-α, a short transmembrane domain, and an intracellular domain carrying tyrosine kinase activity [4].

EGF and TGF-α also play a role in the function of the normal ovary and have been detected in ovarian follicular fluid [5–10]. Also, EGF and its receptor are involved in the regulation of growth and function in malignant ovarian tissues [10]. EGF-R has been shown to have biological importance in the growth regulation of human ovarian carcinoma-derived cells *in vitro* [11, 12] and *in vivo* in transplanted xenografts in nude mice [11]. Elevated levels of TGF-α have been reported to be present in the urine [13] and in the ascites [14] from patients with disseminated ovarian cancers. The results of several studies [10, 15–23], including ours [24–27], indicate the presence of significant EGF-R levels, as determined by ligand binding assays (LBA) or immunohistochemistry, in 40–75% of ovarian carcinomas. In our series concerning 100 patients, 66% of the primary ovarian cancers were EGF-R$^+$ as measured by LBA and 75% were EGF-R$^+$ by immunohistochemical methods [27]. Interestingly, ovarian carcinomas contain higher median EGF-R$^+$ levels than normal ovaries [26, 28]. However, data on the prognostic significance of EGF-R in ovarian cancer are scarce, concern relatively small series of patients, and are conflicting [18, 20, 23]. In the present study we report on the prognostic value of EGF-R as determined not only biochemically (LBA) but also immunohistochemically using two monoclonal antibodies (Mab 2E9 and EGF-R1), and on the relationship with other prognostic factors such as the oestrogen (ER) and progesterone receptor (PgR) levels.

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PATIENTS AND METHODS

Patients

50 evaluable patients with a primary ovarian cancer and sufficient follow-up were included in the study. Patients' characteristics are presented in Table 1. Tumours were staged according to the International Federation of Gynecology and Obstetrics (FIGO, 1976) [29]. An early stage ovarian cancer was diagnosed in 13 patients and advanced disease in 37 patients. Patients with an early stage ovarian cancer underwent a total abdominal hysterectomy, bilateral salpingo-oophorectomy, omentectomy and staging biopsies. Peritoneal washing for cytology was performed if ascites was absent. Postoperative therapy in patients with early stage ovarian cancer consisted of combination chemotherapy with cyclophosphamide and cisplatin [30] in 5 patients, and with cyclophosphamide monochemotherapy in 1 patient. 7 patients received no adjuvant therapy, 6 patients with FIGO stage IA grade 1, and 1 patient with FIGO stage IC grade 3.

Patients with advanced disease underwent a maximal tumour reduction. Postoperative chemotherapy consisted of cyclophosphamide and cisplatin in 33 patients and cyclophosphamide monotherapy or radiotherapy in 1 patient each. 2 patients with advanced disease FIGO stage III refused postoperative therapy. Tumour response to treatment was defined according to the WHO response criteria [31].

Tissues

Tissue samples were obtained at staging surgery. Specimens were transported to the laboratory immediately after excision, snap frozen in liquid nitrogen and stored at −70°C. Tumour histology was assessed on paraffin-embedded tissue specimens using standard procedures. All tumour samples were reviewed by a single pathologist. The histological type and grade were determined according to the criteria of the World Health Organization [32].

Table 1. Patients' characteristics

No. of evaluable patients	50
Mean age (range)	56 (26–78) years
FIGO stage	
Stage I	13
Stage II	2
Stage III	32
Stage IV	3
Residual tumour	
None	15
≤ 1 cm	13
> 1 cm	22
Ascites	32
Histology	
Serous cystadenocarcinoma	27
Mucinous cystadenocarcinoma	5
Endometrioid carcinoma	6
Clear cell carcinoma	4
Adenocarcinoma	3
Mixed adenocarcinoma	5
Grade	
Grade 1	10
Grade 2	18
Grade 3	19
Not able to classify	3
Treatment	
None	9
Cisplatin/cyclophosphamide	38
Other treatment	3
Median follow-up (range)	26 (10–33) months

Receptor assays

Quantitative assays. For measurement of cytosolic ER and PgR by enzyme immunoassays (ER-EIA and PgR-EIA kits; 26) and of membrane-associated EGF-R by LBA, tumour tissue (0.4–0.8 g) was pulverised and homogenised as recommended by the EORTC for processing of breast tumour biopsies for steroid receptor determinations [33]. Details of the procedure for the preparation of membranes and for assay of EGF-R by Scatchard analysis, based on the separation of bound and free ligand by hydroxylapatite [34] have been extensively described before [27].

Immunohistochemistry. Immunohistochemistry was performed as described before [27]. In summary, serial sections were cut at a thickness of 5 μm. These were air dried and fixed in acetone for 10 min, after which the indirect immunoperoxidase technique was used for visualisation with either the low affinity binding sites with mouse IgG1 MAb 2E9 (50 μg/ml, kindly provided by Dr L.H.K. Defize [35], Hubrecht Laboratory, Utrecht, The Netherlands) or the total (high + low affinity) binding sites with mouse IgG2 MAb EGF-R1 (Amersham, Buckinghamshire, U.K.; 50 μg/ml) as previously described [25, 27]. Briefly, for the scoring of the immunohistochemical staining we counted and scored a maximum of 300 epithelial tumour cells (randomly selected) per sample as described before [27]. A positive or negative mark was given for epithelial tumour cells only. According to the intensity of staining (S score), results were evaluated in grades 0 to 3. Weak but recognisable staining was classified as grade 1, moderate as grade 2, and strong as grade 3. When different intensities within the specimen were noticed the highest grade was recorded. Furthermore, the percentage of reactive cells was recorded (PP score). The average staining intensity (E score) was defined by $\Sigma i.P(i)$ with summation over i = 0–3.

Statistics

The analysis of the data of this group of patients was mainly exploratory and directed at the description and detection of correlations between clinical, biochemical and immunohistochemical parameters, and the relation between these parameters and prognosis, measured by progression-free survival (PFS). It must be emphasised that due to the limited number of patients in this study, a non-significant P value does not exclude a moderate difference. Spearman rank correlations were used to determine the strength of the association between different receptor parameters, together with scatterplots.

The association of each of the receptors with FIGO stage and postoperative residual tumour was studied with cross tabulations and rank correlations. For this purpose, tumours were classified on the basis of the FIGO stage and the postoperative tumour rest on an ordinal scale with 1 = FIGO I, 2 = FIGO > I with residual tumour ≤ 1 cm and 3 = FIGO > I with residual tumour > 1 cm. Univariate Cox regression analyses were used for the determination of the strength of the association of the different factors with PFS. Multivariate analysis was not performed because of the small number of patients. In these analyses receptor values were trichotomised with scores

Table 2. Receptor levels measured by biochemical assay and immunohistochemistry

			fmol/mg protein		
	n	Positive	Median	Mean (± S.D.)	Range
Biochemical					
ER	50	58%*	16	37 ± 46	0–162
PgR	50	38%*	8	38 ± 93	0–546
EGF-R (LBA)	46	63%†	14	18 ± 24	0–104
Immunohistochemistry PP score‡					
Anti-EGF-R					
MAb EGF-R1	49	78%§		39 ± 31%	0–95
MAb 2E9	50	82%§		42 ± 33%	0–100

*> 10 fmol/mg, †> 0 fmol/mg, ‡PP, percentage of positive cells, §PP score > 0.

0 = negative, 1 = medium and 2 = high. Negative corresponds to the samples where no receptor was detectable (i.e. 0 fmol/mg protein or no cells with staining). The cut-off point between medium and high was chosen for each receptor in such a way that sizes of both groups were approximately equal.

RESULTS

Of the 50 patients included in the study, 13 had an early stage ovarian carcinoma and 37 had advanced disease. The response to chemotherapy in the 37 patients with advanced disease was: complete response in 9 patients, partial response in 3, stable disease in 3 and progressive disease in 7 patients, while 15 patients were not evaluable for response (postoperative residual tumour ≤ 1 cm before start of chemotherapy). The actuarial PFS and overall survival for patients with advanced disease (FIGO ≥ II) at 2 years was 25 and 39%, respectively. During the observation period 26 patients (70%) with advanced disease developed tumour progression and 23 (62%) died of disease. The median time to progression of these 26 patients was 8 months (range 1–23 months); the median overall survival of these patients was 12 months (range 1–25 months). All patients with an early stage ovarian cancer were alive without recurrence at the end of the observation period.

Incidence of receptor positivity and receptor levels

Positivities for EGF-R, ER and PgR in these 50 ovarian tumours were 63, 58 and 38%, respectively. Median and mean values are indicated in Table 2. EGF-R immunohistochemistry on cryostat sections was positive with MAb 2E9 in 82% of the tumours and with MAb EGF-R1 in 78% of the tumours. Staining was cytoplasmic in all tumours. The staining intensity varied from absent to strong. The distribution of stained cells was heterogeneous for both Mab, from focal or mosaic to diffuse. A strong staining intensity (3+) was observed in 12% for both MAb EGF-R1 and MAb 2E9. Apart from cytoplasmic EGF-R expression in epithelial tumour cells, low immunoreactivity of EGF-R was also observed in stromal and endothelial cells with both antibodies. Moreover, EGF-R staining was also observed in necrotic areas of eight out of the nine tumours with necrosis.

Correlation between receptor measurements and clinicopathological parameters

Receptor measurements. For both EGF-R antibodies a strong correlation between the three measures for the staining intensity of the samples (S score, PP score and E score) was observed with the largest Spearman rank correlation (Rs) between the PP and E score (Rs = 0.93) (Fig 1a,b). The PP score is easier to

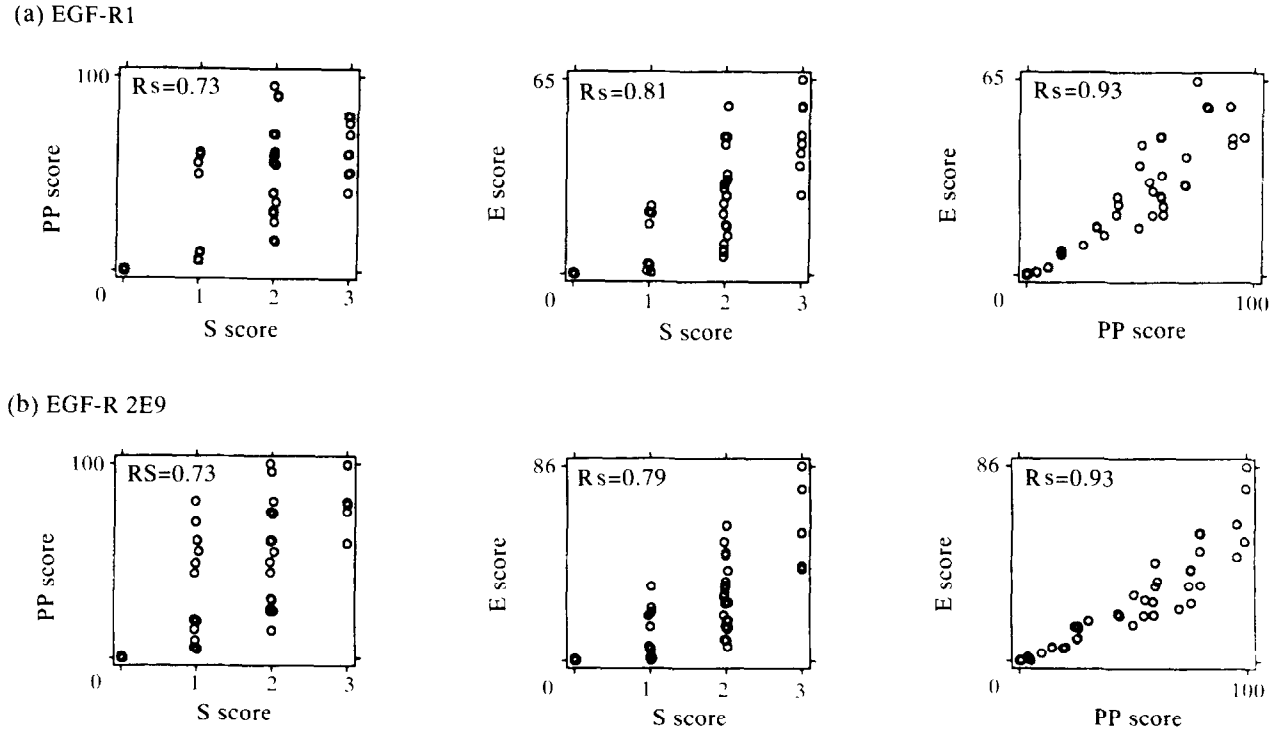

Fig. 1. Relationships between the three different immunohistochemical scoring methods (S score, PP score and E score) for staining of ovarian epithelial tumour cells by the monoclonal antibodies EGF-R1 and EGF-R 2E9. S score = staining intensity, PP score = percentage of positive cells, E score = average staining intensity (see Patients and Methods), Rs = Spearman rank correlation.

determine than the E score, as it does not require a further classification of the positive cells. The PP score seems also to be a more objective measure than the S score, which is based on an overall impression and which may also be influenced by the pattern of staining. Therefore, the PP score was used for further analysis.

Although the percentage of tumour EGF-R positivity was more or less the same for the three methods used (LBA 63%; MAb EGF-R1 78%; MAb 2E9 82%) there were only weak to moderate associations between the individual levels measured by these three methods with Spearman rank correlations between 0.13 and 0.46 (Fig. 2). Also, ER and PgR were only weakly correlated (Rs = 0.20) and they showed no clear association with the EGF-R values (not shown).

Clinicopathological parameters. A positive correlation was observed between FIGO stage/postoperative residual tumour and differentiation grade ($P < 0.05$) and the presence of ascites ($P = 0.01$); patients with residual tumour of larger than 1 cm had more frequent positive ascites and grade 3 tumours (Table 3). None of the rank correlations between receptors and FIGO stage/residual tumour were significant at the 5% level. However, the data suggest that tumours with FIGO stage IIb–IV, especially those with residual tumour > 1 cm, have a higher incidence of ER+, PgR− and EGF-R+ (Table 3).

Table 3. Relation between cell biological parameters and FIGO stage/tumour rest

Parameter	Total	FIGO I	FIGO IIb–IV ≤ 1 cm	FIGO IIb–IV > 1 cm	*P* value*
Total	50	13	15	22	
Grade					
Grade 1	10	5	2	3	< 0.05
Grade 2	18	5	7	6	
Grade 3	19	3	5	11	
Ascites					
No	18	9	5	4	0.01†
Yes	32	4	10	18	
ER					
≤ 10 fmol/mg protein	21	8	6	7	NS‡
> 10 fmol/mg protein	29	5	9	15	
PgR					
≤ 10 fmol/mg protein	31	7	10	14	< 0.10
> 10 fmol/mg protein	19	6	5	8	
EGF-R (fmol/mg protein)					
Negative	17	5	5	7	NS
Median	14	4	6	4	
High	15	3	3	9	
MAb (PP score)§					
Negative	11	2	5	4	< 0.10
Medium	18	8	4	6	
High	20	3	5	12	
MAb 2E9 (PP score)					
Negative	9	3	5	1	< 0.10
Medium	20	6	4	10	
High	21	4	6	11	

*Spearman rank correlation test, †χ^2 test, ‡non-significant, i.e. $P > 0.10$, §PP score as determined by the percentage of positive cells (see Patients and Methods).

Receptor status and clinicopathological parameters in relation to patient prognosis

The clinical relevance of the different clinicopathological parameters and receptor status for PFS were evaluated by separate univariate analyses. For the analysis of the ER, PgR and biochemically and immunohistochemically determined EGF-R status, patients were divided in three equal groups with negative, medium and high values. In the univariate analysis FIGO stage/residual tumour ($P = 0.001$) and PgR ($P = 0.02$) were significant prognostic factors for PFS (Table 4, Fig. 3a,b). Ascites ($P = 0.10$) and a high tumour grade ($P = 0.06$) were related with a higher failure rate but this was not of statistical significance (Table 4). ER showed no prognostic value, neither did EGF-R although patients with high EGF-R levels, biochemically as well as immunohistochemically determined, showed higher failure rates (Fig. 4a–d). If the patients with FIGO stage I were excluded from the analysis (all these patients are still alive with no evidence of disease), none of the parameters showed a statistically significant association with PFS (Table 4). This implies that the correlations of grade, ascites and PgR observed in the total group of 50 patients are partly due to their association with FIGO stage. The limited number of patients does not permit a more detailed analysis.

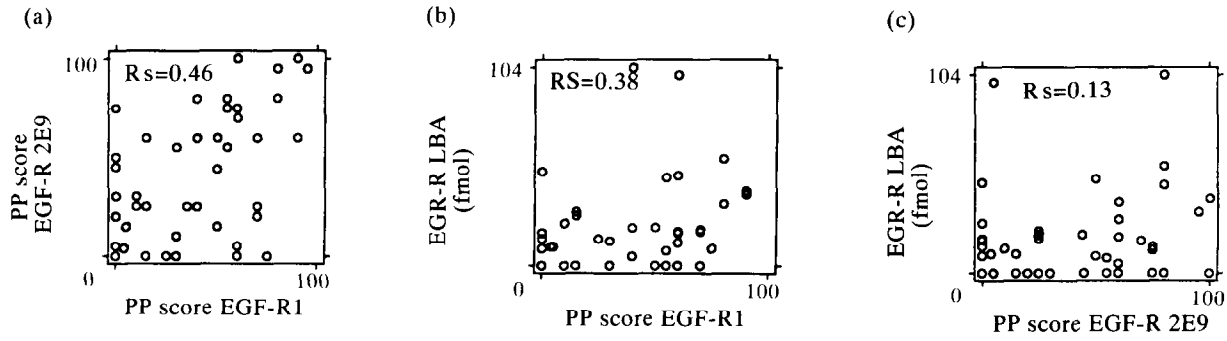

Fig. 2. Relationships between EGF-R measurements by Scatchard analysis (EGF-R LBA) and by immunohistochemistry. (a) Relationship between percentage of positive cells (PP score) measured by Mab EGF-R1 and EGF-R 2E9. (b) Relationship between PP score for EGF-R1 and results of Scatchard analysis. (c) Relationship between PP score for EGF-R 2E9 and results of Scatchard analysis. Rs = Spearman rank correlation.

Table 4. Univariate Cox regression analyses of PFS

Parameter	β	S.E.	*P* value
FIGO/tumour rest	1.16	0.32	0.001
Ascites	0.79	0.47	0.10 (0.98)*
Grade	0.58	0.30	0.06 (0.36)
ER	0.00	0.24	0.99 (0.30)
PgR	−0.66	0.28	0.02 (0.11)
EGF-R (LBA)	0.24	0.25	0.34 (0.43)
EGF-R (MAb 2E9)	0.26	0.29	0.35 (0.74)
EGF-R (MAb EGF-R1)	0.25	0.26	0.34 (0.40)

*P value of analysis restricted to 37 patients with high FIGO stage.
Variable scores: FIGO/tumour rest 0 = FIGO I, 1 = FIGO > I and rest ≤ 1 cm, 2 = FIGO > I and rest > 1 cm; ascites 0 = no, 1 = yes, grades 1, 2, 3; all receptor values trichotomized as 0 = 0, 1 = medium and 2 = high (see Patients and Methods) for the Mab the PP score.

DISCUSSION

Different clinical prognostic factors have been described in primary ovarian cancer [36–38]. Important factors are stage, residual tumour after surgical resection, grade, age and patient performance status. The group of long-term survivors includes patients with a good performance status, a well-differentiated tumour and small tumour residuals after the initial laparotomy [39]. With respect to steroid hormone receptors, both ER and PgR have been described to be related with prognosis, but PgR seems to be the most important one in multivariate analysis [38]. Also in our study, PgR was significantly correlated with survival in contrast to ER.

The rapid developments in cell biology have revealed the importance of a series of new cell biological factors (ploidy, oncogenes/suppressor genes, growth factors) with respect to the growth regulation and prognostic value for several tumour types including ovarian cancer. The role of EGF and its receptor is under extensive investigation in breast cancer [40]. (Over)expression of EGF-R in human breast cancer has been shown to be an indicator of poor prognosis [41] and bad response to endocrine therapy [42]. However, no consensus exists regarding the prognostic significance [40, 43]. With respect to ovarian cancer, seven different groups of investigators including ours showed the presence of EGF-R in 40–75% of primary epithelial ovarian cancers by biochemical or immunohistochemical methods [15–27]. In general, a positive correlation has been found between the results obtained in studies using both methods [18, 27], but discordance exists in 28–45% of the tumours [27]. No correlation was found between EGF-R status and age [23], stage [20, 23] or residual tumour [23]. In contrast to Bauknecht *et al.* [16, 18], who showed a negative relationship between EGF-R and PgR, we found no correlation between EGF-R and steroid receptor status [26] as generally demonstrated in primary breast cancer [40]. With respect to histology, as in our experience [26, 27], no significant association has been demonstrated between tumour EGF-R status and subtypes of ovarian cancer [16, 18, 23] or grade [16, 18, 20, 21, 23]. Bauknecht *et al.* also found no relationship of EGF-R with c-*myc* expression [18]. In contrast, these authors demonstrated a negative correlation between EGF-R and EGF/TGF-α content of the tumours [17, 18]. While Bauknecht *et al.* [16] found no difference in tumour EGF-R levels between primary tumours and metastases or recurrences, both Battaglia *et al.* [19] and our group [27] demonstrated higher EGF-R levels in metastases compared to primary tumours.

Thus far, only three groups have reported on the prognostic value of EGF-R with respect to PFS of patients with ovarian cancer [18, 20, 23]. Initially, Bauknecht *et al.* reported that patients with EGF-R+ tumours had a better survival [16], but later on in a larger series of patients this appeared not to be the case [18]. Although patients with EGF-R+ positive tumours showed a higher response rate to chemotherapy, the duration of response in this subgroup of patients was relatively short resulting in an absence of a significant difference in survival between patients with EGF-R+ and EGF-R− tumours [18]. In contrast, Berchuck *et al.* [20] and Scambia *et al.* [23] reported a negative association between EGF-R positivity and survival. Using the MAb 528, specifically reactive with the extracellular domain of EGF-R, Berchuck *et al.* [20] found in a series of 87 patients that the median length of survival of patients with EGF-R+ tumours (77%) was 26 months compared to 40 months for patients with EGF-R− tumours ($P < 0.05$). If only the 73 patients with stage III and IV disease were considered, the survival advantage for EGF-R− cases was at the limit of statistical significance ($P = 0.06$). Scambia *et al.* [23], using a radioreceptor assay for EGF-R in the tumours of 72 previously untreated patients with FIGO stage III–IV, reported a highly significant ($P = 0.0004$) shorter PFS in patients (54%) with EGF-R+

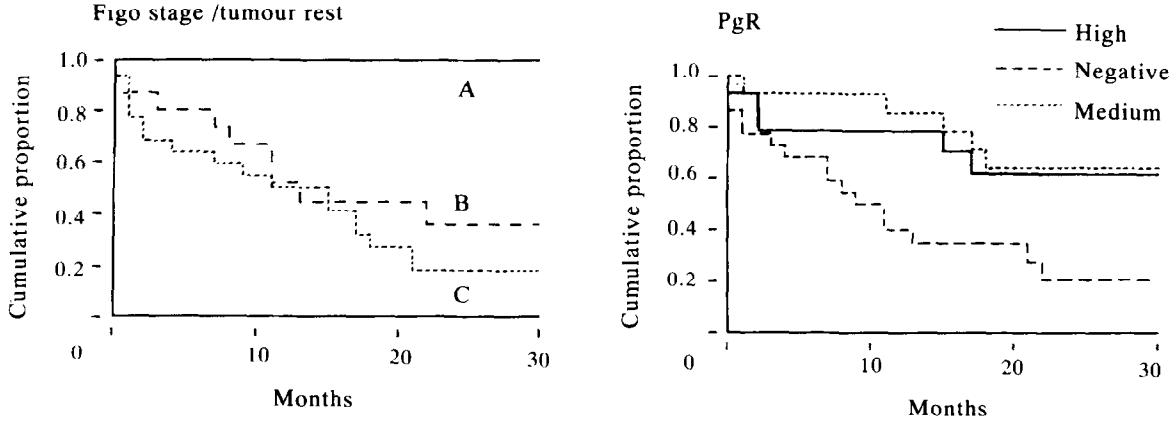

Fig. 3. Progression-free survival according FIGO stage/tumour rest (left) and progresterone receptor levels (right). (A) early stage, (B) advanced stage, tumour rest ≤ 1 cm, (C) advanced stage, tumour rest > 1 cm. PgR negative = 0 fmol/mg protein, PgR medium = 1–22 fmol/mg protein, PgR high = > 22 fmol/mg protein.

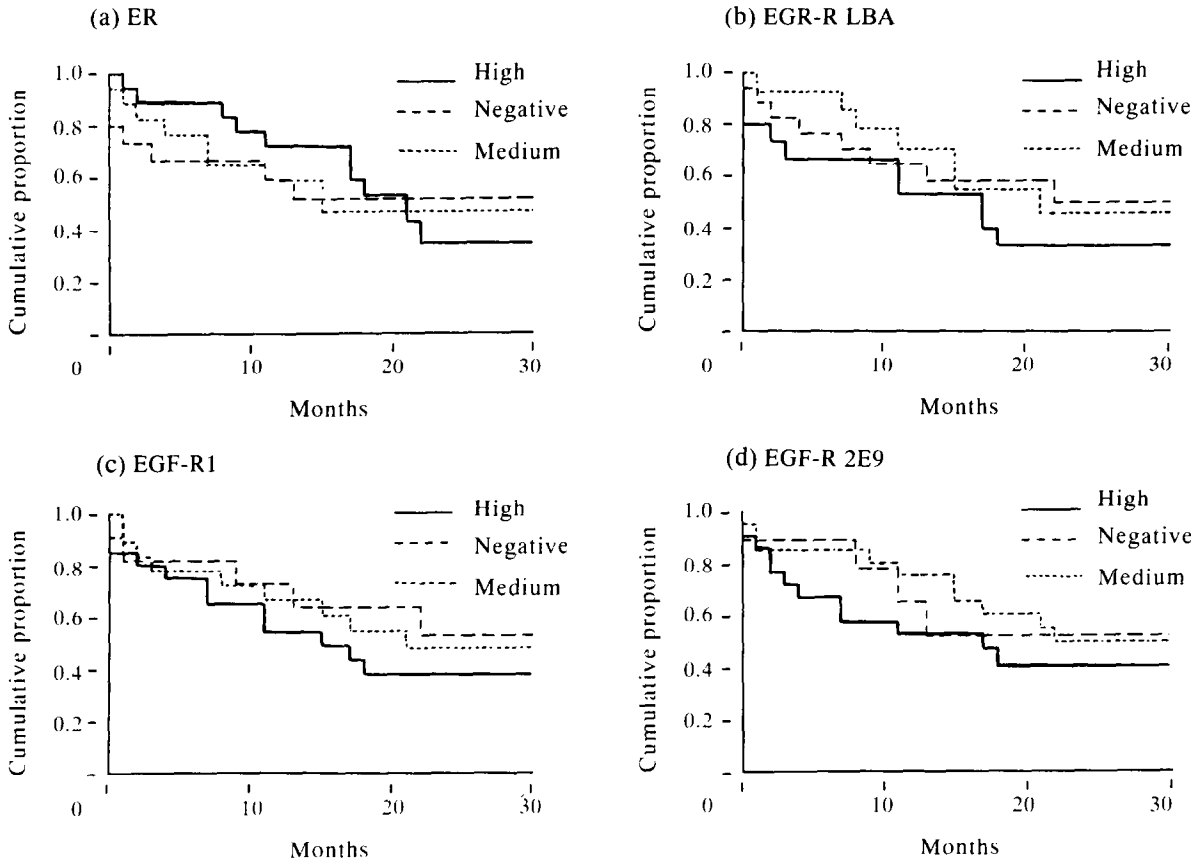

Fig. 4. Progression-free survival as determined by ER and EGF-R status. ER negative = 0 fmol/mg protein, ER medium = 1–32 fmol/mg protein, ER high = >32 fmol/mg protein. EGF-R LBA negative = 0 fmol/mg protein, EGF-R LBA medium = 1–18 fmol/mg protein, EGF-R LBA high = >18 fmol/mg protein. EGF-R1 negative = 0% positive cells, EGF-R1 medium = 4–50% positive cells, EGF-R1 high = >50% positive cells. EGF-R 2E9 negative = 0% positive cells, EGF-R 2E9 medium = 4–50% positive cells, EGF-R 2E9 high = >50% positive cells.

tumours. In a multivariate analysis (including stage, postoperative residual tumour diameter and the presence of ascites), only the postoperative residual tumour ($P = 0.004$) and the EGF-R expression ($P = 0.0005$) remained significantly associated with a high risk of progression [23]. In our study, we have looked for the prognostic significance of EGF-R both for the LBA and for the immunohistochemical methods using two different Mab. In view of the observed discordance in 28–45% of patients [27], such comparison is important. For all three types of determination used, we found only a tendency to a poorer PFS in patients with high EGF-R expression (Fig. 4), but the differences were not statistically significant. However, this does not exclude a possible significant negative association between EGF-R positivity and survival in a larger series of patients. Therefore, we are presently extending the number of patients in a subsequent study. Nevertheless, in this preliminary study, stage, grade and PgR showed a greater prognostic power than EGF-R.

Until now very few clinical data have been available on the relationship between EGF-R status and response to therapy in ovarian cancer. Because cisplatin is the most effective drug for the treatment of human ovarian cancer, the data of Christen *et al.* [44] are interesting, showing that EGF regulates the *in vitro* sensitivity of human ovarian carcinoma cells to cisplatin. Indeed, Bauknecht *et al.* [18] observed that patients with EGF-R+ tumours showed a higher response rate to cisplatin-containing chemotherapy, but no improved survival. Apart from predictability of response to therapy, new treatment modalities directed on growth factors might be increasingly important. In this respect, it has been shown that MAb against TGF-α and EGF-R can inhibit ovarian tumour cell proliferation *in vitro* [22] and in nude mice [11].

It may be concluded from the few studies published thus far that EGF-R might have some therapeutic significance in ovarian cancer, but more and especially larger studies are required to determine the true prognostic value and predictive significance of EGF-R with respect to treatment response.

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