Expression of c-erbB-2, c-myc, and c-ras oncoproteins, insulin-like growth factor receptor I, and epidermal growth factor receptor in ovarian carcinoma

P A van Dam, I B Vergote, D G Lowe, J V Watson, P Van Damme, J-C Van der Auwera, J H Shepherd

Abstract

Aims—To assess whether the overexpression of five dominant oncogene-encoded proteins is crucial to the pathogenesis of ovarian carcinoma and whether this provides any useful prognostic information.

Methods—The expression of the insulin-like growth factor 1 receptor (ILGFR 1), epidermal growth factor receptor (EGFR), and the c-erbB-2, c-ras, and c-myc products was studied by multiparameter flow cytometry in 80 patients with epithelial ovarian cancer for whom long-term follow up was available.

Results—Overexpression of ILGFR 1, EGFR, c-erbB-2, c-ras and c-myc was found in, respectively, nine of 80 (11%), 10 of 80 (12%), 19 of 80 (24%), 16 of 80 (20%) and 28 of 80 (35%) ovarian carcinomas. The levels of expression of ILGFR 1, EGFR, c-erbB-2 and c-ras were significantly higher in the tumours of patients with recurrent or persistent disease after chemotherapy than in the tumours of patients at initial presentation (p < 0.02). Multivariate analysis showed that residual tumour (p < 0.001), FIGO stage (p = 0.002), EGFR overexpression (p = 0.030) and previous chemotherapy (p = 0.034) were independent variables for predicting survival.

Conclusions—Overexpression of these oncoproteins only occurs in a small proportion of ovarian carcinomas but may have an important role in the progression of the disease.

In this study the expression of five dominant oncogene-encoded proteins—insulin-like growth factor 1 receptor (ILGFR 1), epidermal growth factor receptor (EGFR), and the c-erbB-2, c-ras and c-myc products—was measured by multiparameter flow cytometry in 80 patients with ovarian carcinoma. The investigation aimed to assess whether overexpression of these oncoproteins has a crucial role in the pathogenesis of the disease and whether it provides any useful prognostic information. Epidermal growth factor receptor, ILGFR 1, and the c-erbB-2 gene product are transmembrane cell surface glycoproteins which have intrinsic tyrosine kinase activity. They function as signal receptors: ligand binding leads to pleiotropic effects on cells, including stimulation of mitogenesis.2,3 The products of the c-ras gene family are involved with signal transmission in the cytoplasm: they seem to link the effects of growth factor stimulation of receptors with inositol phospholipid metabolism.4 C-myc is a nuclear oncoprotein involved in regulating cell proliferation and possibly in cell differentiation.8

Methods

Eighty patients with epithelial ovarian cancer were included in this study. Each patient had an exploratory laparotomy as part of treatment for epithelial ovarian carcinoma at the Norwegian Radium Hospital between 1982 and 1989. The stage of the disease was assigned according to the International Federation of Gynaecologists and Obstetricians' (FIGO) staging criteria.9 In 40 patients tumour was obtained at the initial operation before any adjuvant treatment was given. Of these 40 patients, five also had tumour samples frozen at second-look or subsequent laparotomy. All of the remaining 40 patients had persistent disease after chemotherapy; tumour samples were collected only at second-look or subsequent laparotomy.

Samples of normal ovary were collected during the study period from 30 age matched patients undergoing hysterectomy and salpingo-oophorectomy for benign uterine disease. Histological material from each case was reviewed by one pathologist. The histological type and grade were assigned with the use of World Health Organization criteria.10 All of the patients with advanced disease in this study received platinum based chemotherapy. The patients' details are given
Expression of oncoprotein encoded proteins in ovarian carcinoma

Table 1. Clinical and histological characteristics of 80 patients with epithelial ovarian cancer

<table>
<thead>
<tr>
<th>Sampling at</th>
<th>Initial laparotomy</th>
<th>Subsequent laparotomy</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age *</td>
<td>57.0 (13.2)</td>
<td>52.0 (12.5)</td>
<td>NS *</td>
</tr>
<tr>
<td>Stage:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>24</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>9</td>
<td>6</td>
<td>NS †</td>
</tr>
<tr>
<td>Residual tumour after debulking:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>7</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>0-2 cm</td>
<td>14</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>&gt;2 cm</td>
<td>19</td>
<td>24</td>
<td>NS †</td>
</tr>
<tr>
<td>Histological type:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>23</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Mucinous</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Endometrioid</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Clear cell</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>8</td>
<td>4</td>
<td>NS †</td>
</tr>
<tr>
<td>Histological grade:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade I</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Grade II</td>
<td>15</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Grade III</td>
<td>17</td>
<td>23</td>
<td>NS †</td>
</tr>
<tr>
<td>Mean survival (months) after sampling:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>17.5</td>
<td>16</td>
<td>NS †</td>
</tr>
</tbody>
</table>

* Student's t test
† ‡ test
§ Mean (standard deviation)
NS = not significant

in table 1. The follow up of the patients was assessed in March 1991. No patients were lost to follow up.

Representative samples of the primary ovarian tumours were obtained from surgical specimens in the operating theatre. They were immediately dissected free from necrotic tissue and divided into two adjacent parts. The first block, of about 0.5 cm³, was snap frozen in liquid nitrogen and stored at −70°C.11 The second part was fixed in formalin and processed routinely for haematoxylin and eosin staining.

The monoclonal antibody NEU3 (provided as ascites) was used to label the internal domain of the human c-erbB-2 oncoprotein (Cambridge Research Biochemicals Ltd, Harston, England).12 The EGFR was probed with the well characterised R1 monoclonal antibody provided by Drs B Guillick and M Waterfield (ICRF, Laboratories, London) (1982).13 The 6E10 monoclonal antibody (Cambridge Research Biochemicals, England) was used to label the c-myc gene product.14 The F132-G2 and a R3 antibodies, recognising p21 pan-ras and ILGFR I receptor, respectively, were purchased from Oncogene Science (USA). All primary monoclonal antibodies were mouse IgG and were stained with fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse IgG (Dako, Denmark).

Samples were analysed as a batch. Cryopreserved tissue blocks were thawed rapidly and disaggregated mechanically, as described before.11 12 The resulting cell suspension was filtered through a 35 μm nylon mesh and divided into seven samples containing about 2-5×10⁶ cells each, which were spun down. A cytospin preparation was made from the first sample, which was used as a cytological control. Only tumour samples that contained more than 50% of tumour cells were included for further analysis; this resulted in the exclusion of six of the original 86 patients. The five other aliquots were incubated for one hour with the primary antibody raised against EGFR (50 μg/ml), c-erbB-2 (1:250), c-myc (1:20), pan-ras (20 μg/ml) and IGF-1 (20 μl/ml), respectively.16 The pellet of the seventh aliquot was resuspended in phosphate buffered saline and served as a background fluorescence control.15 The optimal staining technique was identified by performing a checkerboard titration of the primary and secondary antibody, optimising the incubation times and volumes and the number of washes.17 Labelling the primary antibody with FITC conjugated rabbit anti-mouse IgG and the staining the DNA was performed, as described before.16

The cells were analysed simultaneously for DNA and oncoprotein in the Cambridge MRC custom-built dual laser flow cytometer. Excitation, optical filtration, calibration, setup of the flow cytometer, data collection, data processing and data analysis have been given in detail elsewhere.8 16 17

Cross-blocking assays were carried out using the NEU3 peptide used as the immuno- gen. Cultured cells known to express high oncoprotein concentrations were used as positive controls: A431 cells (EGFR), SKBR3 (c-erbB-2), NIH3T3 EC816 (Ha-ras), and COLO320 HSR (c-myc). Cultured cells and tumour samples incubated with monoclonal antibodies against lymphocyte markers (B1, T015, T05 all Dako Ltd, Denmark) gave no substantial signal above background for the gated cells.

Results

Oncoprotein and receptor expression in normal and carcinomatous ovary is shown in table 2. The median level of expression of all measured oncoproteins was significantly higher in the ovarian cancers. When oncoprotein and receptor concentrations in normal and neoplastic tissue sampled in 1982–1985 were compared with the samples obtained during the period 1986–1989, no significant differences were found. This suggests that there was no major time dependent deterioration of the measured proteins during storage of the biopsy specimens at −70°C.

Overexpression (defined as protein concentrations exceeding the 97th centile of the oncoprotein expression in the normal ovary) of c-erbB-2, c-ras, c-myc, EGFR and ILGFR I

Table 2. Tumour DNA aneuploidy, S-phase fraction, and oncoprotein expression in normal and carcinomatous ovary

<table>
<thead>
<tr>
<th>Normal ovary n = 30</th>
<th>Ovarian carcinoma n = 80</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA aneuploidy*</td>
<td>0</td>
<td>47</td>
</tr>
<tr>
<td>SPhase</td>
<td>9.4 (±3.2)</td>
<td>18.1 (±7.6)</td>
</tr>
<tr>
<td>EGFR†</td>
<td>3 (0–36)</td>
<td>12 (0–45)</td>
</tr>
<tr>
<td>ILGFR†</td>
<td>7 (0–45)</td>
<td>26 (0–90)</td>
</tr>
<tr>
<td>c-erbB-2‡</td>
<td>8 (0–35)</td>
<td>50 (12–111)</td>
</tr>
<tr>
<td>c-ras‡</td>
<td>8 (0–49)</td>
<td>45 (1–108)</td>
</tr>
<tr>
<td>c-myc‡</td>
<td>16 (0–30)</td>
<td>121 (12–198)</td>
</tr>
</tbody>
</table>

* Z test
† Mean (standard deviation), Student’s t test
‡ Median (upper-lower quartile), Mann-Whitney U test
was found in, respectively, 19 of 80 (24%), 16 of 80 (20%), 28 of 80 (35%), 10 of 80 (12%) and nine of 80 (11%) of ovarian carcinomas (table 3). There was no correlation between the degree of expression and stage, histology, grade or DNA ploidy of the tumour. S phase fraction was correlated with tumour ploidy (p < 0.001) and EGFR expression (p < 0.02). No correlation could be found among the expression of any of the studied oncoproteins.

Expression of EGFR, ILGFR1, c-erbB-2 and c-ras oncoproteins in tumours of patients with recurrent or persistent disease after chemotherapy was significantly higher than in tumours of patients at initial presentation (table 4). In five cases tissue was available for evaluation before and after platinum based chemotherapy. Two of these patients had a similar pattern of oncoprotein expression in tumour retrieved at initial presentation and at second look laparotomy. The other three patients had normal pretreatment tumour oncoprotein concentrations, but expression of one or more oncoproteins was found in the persistent tumours.

Oncoprotein expression in relation to actuarial overall survival is given in the figure. Life table analysis (log rank analysis) showed there was a tendency for patients with primary or recurrent tumours overexpressing EGFR (p < 0.13) and c-ras (p < 0.17) to have a worse prognosis than patients with normal expression of these oncoproteins. Patients with DNA diploid tumours survived significantly longer than patients with DNA aneuploid tumours (p < 0.013). Normal expression of all of the oncoproteins studied was more likely in patients who survived more than 30 months than in those who survived 30 months or less (table 4).

Survival after surgery was calculated using a Cox proportional hazard analysis to determine which variables had an independent prognostic influence and to evaluate their prognostic effect. Initial FIGO stage, tumour histology, tumour grade, residual tumour after surgery, previous platinum based chemotherapy, age, DNA ploidy, S phase fraction, and the expression of EGFR, c-erbB-2, ILGFR1, c-myc and c-ras proteins were analysed. Multivariate analysis revealed that residual tumour, FIGO stage, EGFR overexpression and previous chemotherapy were the only independent variables predicting survival time (table 5).

Discussion

Several reports have shown that EGFR, ILGFR1, c-myc, c-ras and c-erbB-2 oncoproteins can be detected in the normal ovary by immunohistochemistry, radioimmunoassay, or blotting techniques. By measuring messenger RNA, Slamon et al demonstrated an increased expression of the c-fos, c-myc, Ha-ras and Ki-ras oncogenes in ovarian carcinomas compared with normal tissues. Press et al demonstrated that c-erbB-2 expression concentrations in normal tissues were similar to those found in non-ovulated, non-ovulated breast cancers, and breast cancer cell lines. Median oncoprotein concentrations in the neoplastic samples were significantly higher than in the normal ovaries in the present investigation, though there was a considerable overlap between both groups. These findings support the idea that these oncoproteins have a role in growth control and differentiation of the normal ovary.

In agreement with other investigators, overexpression of one or more of the studied oncoprotein products could be detected in only a minority of the ovarian cancers. Just over one third of the tumours we studied had normal expression of ILGFR1, EGFR, c-erbB-2, c-ras and c-myc oncoproteins, which means that overexpression of one or more of these oncoproteins seems not to be the common molecular biological event leading to ovarian cancer. Alternatively, a small subset of malignant neoplasms might use this type of abnormal growth control. The molecular biological heterogeneity of epithelial ovarian cancer is illustrated in the present study.

It has been proposed that the hallmarks of tumour progression can probably be attributed to changes in the genes that regulate these functions, allowing sublines of the origi-
Expression of oncogene encoded proteins in ovarian carcinoma

Relation between oncogene expression and actuarial survival for 80 patients with epithelial carcinoma of the ovary. The number of patients at risk of death at 0, 10, 20, 30, 40, 50 and 60 months, respectively, is shown.

- Normal c-erb B-2 expression (n = 61)
- c-erb B-2 overexpression (n = 19)

- Normal c-ras expression (n = 64)
- c-ras overexpression (n = 16)

- Normal EGFR expression (n = 70)
- EGFR overexpression (n = 10)

- No oncogene overexpression (n = 41)
- Overcompression of one oncogene (n = 14)
- Overcompression of two or more oncogenes (n = 25)

- DNA - diploid (n = 33)
- DNA - aneuploid (n = 47)

Higher incidence of c-erbB-2 overexpression in the former group and a similar trend for the other oncogenes.

Further prospective studies are needed to determine whether these alterations in oncogene or receptor expression were already present in the tumour before treatment or were the result of clonal adaptation under chemotherapy. Our observations in patients in whom oncogene expression was measured before and after treatment suggests that in some tumours clonal evolution may be responsible.

Bercshuck et al and Kacinski et al found that advanced ovarian tumours with high c-erbB-2 expression were significantly less likely to have a complete response to primary therapy. This trend was also described in patients with neuroblastoma and leukaemia. Preisl er et al found that high levels of c-myc expression in patients with acute non-lymphocytic leukaemia are associated with a high probabil-
ity that the patients will not respond to remis-

sion induction treatment or will have only short remissions.\textsuperscript{10,11} The ras oncogene may have a role in tumour progression and also influence the development of radiation resis-
tance.\textsuperscript{12,13} In vitro and in vivo experiments indicate that exposure of c-myc and c-ras expressing cells to hypomethylating agents can lead to tumour progression.\textsuperscript{14} This indi-
cates that pre-existing alterations in oncogene expression may facilitate the malignant evolution in some tumours treated with certain types of chemotherapy.

It was found in the present study that EGFR overexpression is an independent prognostic factor in patients with epithelial ovarian cancer. Studies by Bauknecht \textit{et al.} and Berchuck \textit{et al.} have suggested that high levels of EGFR expression are a marker of bad prognosis in ovarian cancer patients.\textsuperscript{1,2,15} Although our data and a recently published study by Henzen-Logmans \textit{et al.} show that EGFR overexpression occurs only in about 12% of ovarian carcinomas, the present findings suggest that it is an important molecular biological event in the progression of ovarian cancer.\textsuperscript{16} The investigation by Henzen-

Logmans \textit{et al.} indicates that EGFR overexpression is rare due to amplification of the EGFR gene and that altered transcription seems important.\textsuperscript{17}

Both c-erbB-2 amplification and over-

expression are associated with decreased sur-
vival in ovarian carcinoma according to some authors.\textsuperscript{18,22} Haldane \textit{et al.}, on the other hand, could not confirm that there was an adverse prognostic effect of c-erbB-2 expression in a series of 104 immunostained epithelial ovarian malignancies.\textsuperscript{19} Kacinski \textit{et al.} could not identify a correlation between the intensity of c-erbB-2 in situ hybridisation staining and either relapse free or overall survival in 72 patients with stage I to IV ovarian carcino-

ma.\textsuperscript{20} This agrees with the findings of this study.

Amplification and overexpression of the Ki-

ras oncogene has been reported in only a small proportion of ovarian cancers.\textsuperscript{37,38} In immunohistochemical studies of patients with advanced ovarian cancer, Rodenburg \textit{et al.} and Yaginuma \textit{et al.} found no correlation between staining intensity of the ras gene product p21 and the histological type, grade, the ploidy class, and the clinical outcome.\textsuperscript{40} The relevance of ras gene product p21 to the diagnosis and prognosis of gynaecological malignancies remains to be determined.

Polacarz \textit{et al.} detected strong c-myc immunostaining of the nucleus and entire cytoplasm in all 22 cases of mucinous cystade-

nocarcinoma of the ovary.\textsuperscript{41} Although only small numbers of cases have been studied, c-

myc amplification and overexpression seem to be a common finding (25–50%) in advanced stage ovarian cancer.\textsuperscript{42} In an immunohisto-

chemical study Sasano \textit{et al.} could find no signif-

icant correlation in cases of ovarian cancer between intracellular localisation patterns of the c-myc oncprotein and histological and nuclear grades or mitotic activity.\textsuperscript{43}

We thank Dr Bill Gillick (London) for providing the RI mono-

clonal antibody. Dr P van Dam is supported by a Research Werk tegen Kanker, a British Council and a St Bartholomew's Cancer Research Campaign grant.


15. van Dam PA, Watson JV, Lowe DG, Chard T, Shepherd JH. Comparative evaluation of fresh, fixed and cryopre-


19. Slamon DJ, DeKernion JB, Verma MI, Clince MJ. Expression of cellular oncoproteins in human malignan-


23. Bauknecht T, Janz J, Kohler M, Pfleiderer A. Human ovarian carcinomas: correlation of malignancy and sur-


27. Comberet V, Wang Q, Favor MC, Thiese P, Philip I,