

# 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

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## 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.

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Recent insight into the molecular biology of cancer raises the hypothesis that carcinogenesis can be explained by a complex multistep process of activation of oncogenes and inactivation of tumour suppressor genes, these events acting synergistically to produce the malignant phenotype.<sup>1</sup> Alterations in the expression of oncogenes and antioncogenes, caused by mutation, rearrangement or gene amplification, or altered gene transcription, can occur spontaneously or be induced by carcinogens.<sup>2,3</sup> Although for some tumour types (such as colonic carcinoma) the cascade of tumourigenic events is on the verge of being revealed, the molecular biology of epithelial ovarian cancer remains an enigma.<sup>4</sup>

In this study the expression of five dominant oncogene-encoded proteins—insulin-like growth factor I receptor (ILGFR I), 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 I, 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.<sup>2,5,6</sup> 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.<sup>7</sup> *C-myc* is a nuclear oncoprotein involved in regulating cell proliferation and possibly in cell differentiation.<sup>8</sup>

## 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.<sup>9</sup> 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 Organisation criteria.<sup>10</sup> All of the patients with advanced disease in this study received platinum based chemotherapy. The patients' details are given

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

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

\* Student's t test

†  $\chi^2$  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 cm<sup>3</sup>, was snap frozen in liquid nitrogen and stored at -70°C.<sup>11</sup> 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).<sup>12</sup> The EGFR was probed with the well characterised R1 monoclonal antibody provided by Drs B Gullick and M Waterfield (ICRF, Laboratories, London) (1982).<sup>13</sup> The 6E10 monoclonal antibody (Cambridge Research Biochemicals, England) was used to label the *c-myc* gene product.<sup>14</sup> 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.<sup>11 15 16</sup> The resulting cell suspension was filtered through a 35  $\mu$ m nylon mesh and divided into seven samples containing about 2.5-5  $10^5$  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  $\mu$ g/ml), *c-erbB-2* (1/250), *c-myc* (1/20), pan-*ras* (20  $\mu$ g/ml) and IGF-1 (20  $\mu$ l/ml), respectively.<sup>16</sup> The pellet of the seventh aliquot was resuspended in phosphate buffered saline and served as a background fluorescence control.<sup>15</sup> 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.<sup>17</sup> Labelling the primary antibody with FITC conjugated rabbit anti-mouse IgG and the staining the DNA was performed, as described before.<sup>16</sup>

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.<sup>8 16 17</sup>

Cross-blocking assays were carried out using the NEU3 peptide used as the immunogen. Cultured cells known to express high oncoprotein concentrations were used as positive controls: A431 cells (EGFR), SKBRIII (*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

	Normal ovary n = 30	Ovarian carcinoma n = 80	Significance
DNA aneuploidy*	0	47	< 0.001
SPF†	9.4 ( $\pm$ 3.2)	18.1 ( $\pm$ 7.6)	< 0.001
EGFR††	3 (0-36)	12 (0-45)	0.031
ILGFR††	7 (0-45)	26 (0-90)	0.018
<i>c-erbB-2</i> ††	8 (0-35)	50 (12-111)	< 0.001
<i>c-ras</i> ††	8 (0-49)	45 (1-108)	0.016
<i>c-myc</i> ††	16 (0-30)	121 (12-198)	< 0.001

\*  $\chi^2$  test

† Mean (standard deviation), Student's t test

†† Median (upper-lower quartile), Mann-Whitney U test

**Table 3** Tumour aneuploidy, S phase fraction, and oncoprotein expression in samples obtained at initial presentation of primary carcinoma of the ovary and in patients with recurrent or progressive ovarian carcinoma after chemotherapy

	Initial carcinoma (n = 40)	Recurrent carcinoma (n = 40)	p value
Tumour aneuploidy†	24/40	23/40	NS
SPF *	18.4 (7.6)	17.6 (6.9)	NS
EGFR expression †	3 (0-36)	23 (5-48)	0.020
EGFR overexpression‡	3/40	7/40	NS
ILGFR1 expression †	7 (0-45)	69 (6-106)	0.001
ILGFR1 overexpression †	2/40	7/40	NS
c-erbB2 expression †	35 (0-55)	91 (31-177)	< 0.001
c-erbB-2 overexpression †	4/40	15/40	< 0.005
c-ras expression †	19 (0-76)	61 (12-147)	0.014
c-ras overexpression‡	5/40	11/40	NS
c-myc expression †	95 (0-189)	130 (73-199)	0.100
c-myc overexpression‡	12/40	16/40	NS

\* Mean (SD), Student's t test

† Median (lower-upper quartile), Mann-Whitney U test

‡ Number of cases/total,  $\chi^2$  test

NS = not significant

**Table 4** Oncoprotein overexpression in poor and good responders

	Survival†		p value*
	≤ 30 months	> 30 months	
EGFR	10/67 (15)	0/13 (0)	NS
ILGFR 1	8/67 (12)	1/13 (8)	NS
c-erbB-2	17/67 (25)	2/13 (15)	NS
c-ras	16/67 (24)	0/13 (0)	< 0.07
c-myc	26/67 (39)	2/13 (15)	NS
Normal expression of all oncoproteins	30/67 (45)	10/13 (77)	< 0.04
Overexpression of two or more oncoproteins	23/67	2/13	NS

\*  $\chi^2$  test

† number of cases/total (percentage)

Patients with a survival of > 30 months were defined as good responders and patients with a survival of ≤ 30 months as poor responders.

NS = not significant

**Table 5** Actuarial survival after surgery: multivariate analysis (Cox regression)

Prognostic factor	p value	Coefficient	Standard error	Hazard ratio
Residual tumour	< 0.001	-1.838	(0.541)	0.1591
FIGO stage	0.002	1.320	(0.420)	3.745
EGFR overexpression	0.030	1.769	(0.816)	5.864
Previous chemotherapy	0.034	1.111	(0.532)	3.037

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 overexpression 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).

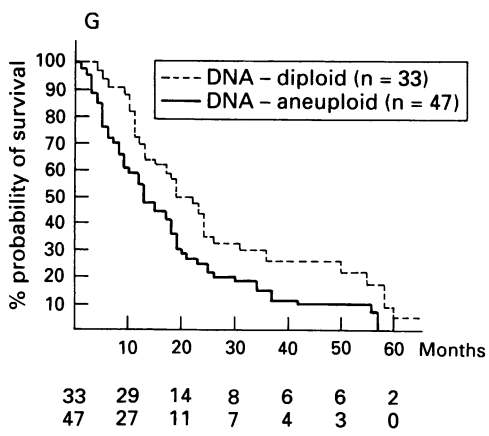
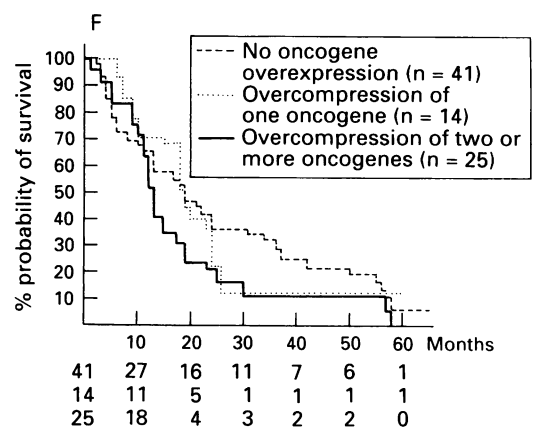
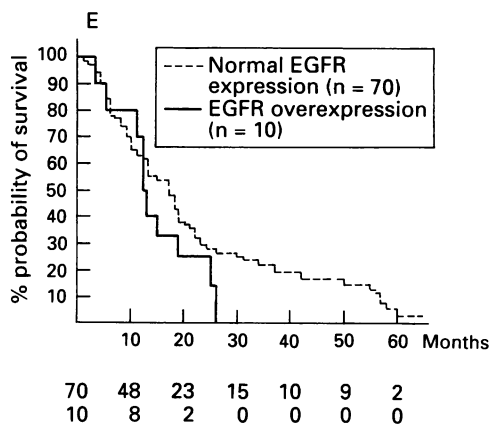
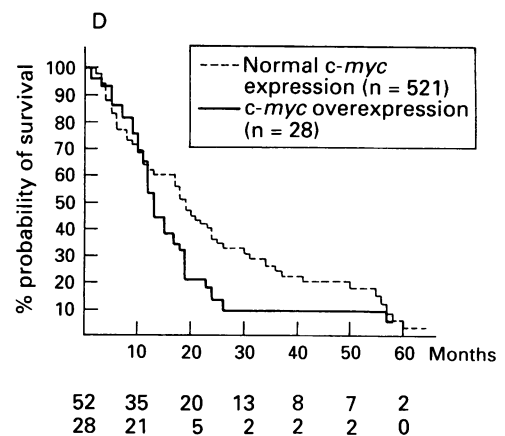
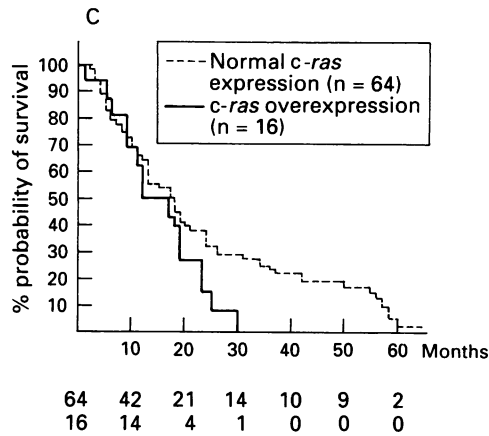
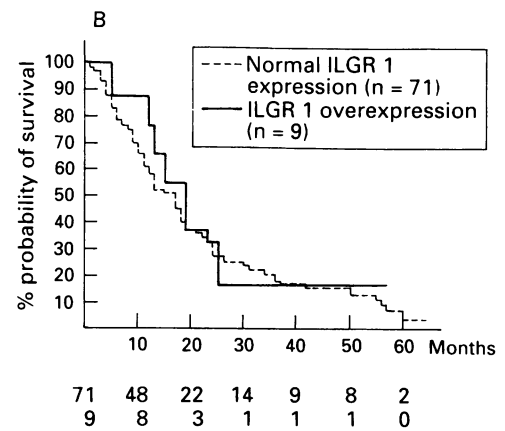
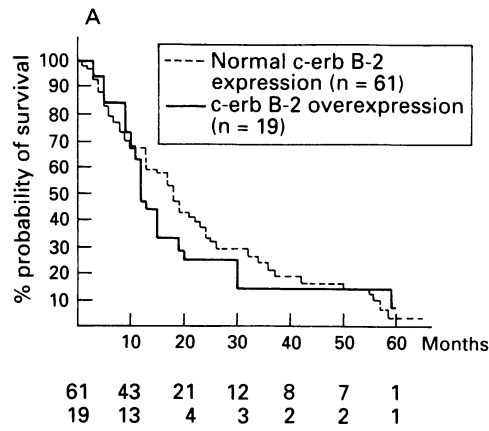
## Discussion

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.<sup>7,8,16,18</sup> 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.<sup>19</sup> Press *et al* demonstrated that c-erbB-2 expression concentrations in normal tissues were similar to those found in non-amplified, non-overexpressed breast cancers, and breast cancer cell lines.<sup>20</sup> 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 oncogene products could be detected in only a minority of the ovarian cancers.<sup>21-24</sup> Just over half 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-

Relation between oncoprotein overexpression 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.



higher incidence of *c-erbB-2* overexpression in the former group and a similar trend for the other oncoproteins.

Further prospective studies are needed to determine whether these alterations in oncoprotein 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.

Berchuck *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.<sup>21, 26</sup> This trend was also described in patients with neuroblastoma and leukaemia.<sup>27-29</sup> Preisler *et al* found that high levels of *c-myc* expression in patients with acute non-lymphocytic leukaemia are associated with a high probab-

nal neoplastic clone to emerge as predominant.<sup>25</sup> The present study shows for the first time that there are higher levels of oncoprotein expression in progressive or recurrent tumours than in similar tumours at initial presentation. There also was a significantly

ity that the patients will not respond to remission induction treatment or will have only short remissions.<sup>30-31</sup> The *ras* oncogene may have a role in tumour progression and also influence the development of radiation resistance.<sup>32-33</sup> 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.<sup>34</sup> This indicates 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 *et al* and Berchuck *et al* have suggested that high levels of EGFR expression are a marker of bad prognosis in ovarian cancer patients.<sup>3-23-24</sup> Although our data and a recently published study by Henzen-Logmans *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.<sup>35</sup> The investigation by Henzen-Logmans *et al* indicates that EGFR overexpression is rarely due to amplification of the EGFR gene and that altered transcription seems important.<sup>35</sup>

Both *c-erbB-2* amplification and overexpression are associated with decreased survival in ovarian carcinoma according to some authors.<sup>18-22</sup> Haldane *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.<sup>36</sup> Kacinski *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 carcinoma.<sup>26</sup> 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.<sup>37-39</sup> In immunohistochemical studies of patients with advanced ovarian cancer, Rodenburg *et al* and Yaginuma *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.<sup>7-40</sup> The relevance of *ras* gene product p21 to the diagnosis and prognosis of gynaecological malignancies remains to be determined.

Polaczar *et al* detected strong *c-myc* immunostaining of the nucleus and entire cytoplasm in all 22 cases of mucinous cystadenocarcinoma of the ovary.<sup>41</sup> 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.<sup>42-43</sup> In an immunohistochemical study Sasano *et al* could find no significant correlation in cases of ovarian cancer between intracellular localisation patterns of the *c-myc* oncoprotein and histological and nuclear grades or mitotic activity.<sup>43</sup>

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