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Ki-67 STAINING IN BENIGN, BORDERLINE, MALIGNANT PRIMARY AND METASTATIC OVARIAN TUMORS: CORRELATION WITH STEROID RECEPTORS, EPIDERMAL-GROWTH-FACTOR RECEPTOR AND CATHEPSIN D

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Ki-67 immunoreactivity was studied in relation to immunohistochemically assessed expression of epidermal-growth-factor receptor (EGFR), estrogen receptor (ER) progesterone receptor (PgR) and cytosolic levels of cathepsin D in advanced human ovarian adenocarcinomas, borderline and benign cystadenomas and normal ovaries. A significantly higher number of Ki-67-positive cells were found in metastatic tumors *vs.* primary adenocarcinomas and in the total group of adenocarcinomas *vs.* benign/borderline cystadenomas. Cathepsin-D levels were also significantly higher in metastatic tumors than in primary adenocarcinomas, which in turn presented higher levels than were found in normal ovaries. However, no significant difference was observed between cathepsin-D levels in malignant adenocarcinomas and borderline/benign cystadenomas. Immunohistochemically assessed expression of ER and PgR was detected in variable percentages of epithelial tumor cells, and stromal cells were occasionally positive as well. In the group of primary adenocarcinomas, 46% were ER-positive and 34% were PgR-positive, although there was no significant difference between primary and metastatic lesions with respect to ER or PgR expression. Concordance between immunohistochemically assessed ER or PgR data and cytosolic ER and PgR levels measured with enzyme immunoassay was relatively low. EGFR, immunohistochemically assessed with MAb-EGFR1, was positive in 76% of the primary and in 78% of the metastatic adenocarcinomas. A strong positive association was detected between ER and PgR, and EGFR was observed to present a weak positive correlation with Ki-67 and ER. Cathepsin-D levels were not found to be significantly correlated with the expression of ER, PgR, EGFR or Ki-67.

Although the precise pathogenesis of ovarian cancer remains unknown, it appears that hormones and growth factors involved in menstrual activity contribute to it. Attention has been drawn to estradiol, progesterone, epidermal growth factor (EGF) and, more recently, cathepsin D. With regard to their function in breast cancer, it has been shown that binding of estradiol to its receptor activates both PgR synthesis and cell proliferation. Binding of EGF to its receptor activates a variety of biochemical and physiological changes in the target cell, normally leading to enhanced DNA synthesis and cell division. Cathepsin D, which is over-expressed and induced by estrogen and growth factors in hormone-dependent breast-cancer cells (Westley and Rochefort, 1980), is an aspartic lysosomal proteinase and is synthesized as a 52-kDa precursor (reviewed by Rochefort *et al.,* 1987). This cathepsin-D (52-kDa) protein shows an autocrine mitogenic effect in breast-cancer cells through binding to the insulin-like growth-factor-II receptor (Mathieu *et al.,* 1990) and a proteolytic effect on extracellular matrix after its autoactivation at acidic pH (Briozzo *et al.,* 1988). It may therefore play a role in controlling the growth and spread of human breast cancer (Rochefort *et al.,* 1987).

The potential function of steroid receptors, growth-factor receptors and cathepsin D is complicated by the fact that estradiol can stimulate the production of both EGF and cathepsin D, meaning that the observed mitogenic effect of estradiol can be mediated by local action of EGF or cathepsin D.

Furthermore, studies of ovarian tumor cell lines have shown that PgR synthesis and the proliferation of cells regulated through the binding of estradiol to its receptor can become uncoupled (reviewed by Rao and Slotman, 1991). Translating the findings in human breast cancer and ovarian tumor cell lines to the situation *in vivo* several studies have reported the incidence of both ER and PgR in ovarian cancer, with percentages ranging from 38% ER and 14% PgR positivity to 100% ER and 100% PgR positivity (Rao and Slotman, 1991). With regard to EGFR, the reported incidence varies from 40% (Owens *et al.,* 1991) to 77% (Berchuck *et al.,* 1991). We expanded these data by studying ER, PgR and EGFR status in advanced human ovarian cancer using ELISA or ligand-binding assay techniques and found a positive incidence of 60% for ER, 35% for PgR and 65% for EGFR (Berns *et al.,* 1992). While both ER and PgR have been described as favorable prognostic factors in ovarian cancer, PgR appears to be the most important (Rao and Slotman, 1991) and our own experience concurs (van der Burg *et al.,* 1993). Data on the prognostic significance of EGFR in ovarian cancer are contradictory and concern relatively small series of patients (Bauknecht *et al.,* 1988, 1990; Berchuck *et al.,* 1991; Owens *et al.,* 1991; van der Burg *et al.,* 1993). With respect to cathepsin-D levels, published data are scarce. Scambia *et al.* (1991) found detectable levels of cathepsin D in all 68 normal and neoplastic ovarian tissues examined. Interestingly, cathepsin-D levels were significantly higher in malignant tissues than in normal ones.

Although the presence of ER, PgR, EGFR and cathepsin D suggests that ovarian cancers are endocrinologically regulated tumors, their function and biological and clinical significance for patients with ovarian cancer remain to be clarified in large series of patients.

Rutgers *et al.* (1987) have shown the importance of classical prognosticators such as ovarian cancer growth kinetics, including S-phase values assessed by flow cytometry. Immunostaining with MAb Ki-67 gives reliable data on the growth fraction (McGurrin *et al.,* 1987; Kuenen-Boumeester *et al.,* 1991). In ovarian cancer samples (Wong and Tattersall, 1989; Isola *et al.,* 1990) high expression is associated with poor prognosis (Isola *et al.,* 1990). We expanded these data by studying ER, PgR and EGFR expression in relation to Ki-67 expression in human ovarian cancer (Berns *et al.,* 1992; Henzen-Logmans *et al.,* 1992; van der Burg *et al.,* 1993). Results obtained for 103 carcinoma specimens from 99 patients were compared with those obtained for benign and borderline malignant epithelial

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ovarian tumor tissues and normal ovarian tissues. In addition, the incidence and levels of cathepsin D were assessed since this protease may play a role in tumor spread and may also assist in determining evolution.

MATERIAL AND METHODS

Patients

A total of 121 epithelial tumor specimens were classified according to the WHO system, and analyzed. Tumors were classified as primary when they were taken from the ovarian tumor mass and as metastatic when the biopsy was taken from metastases in the omentum, peritoneum or diaphragm. Forty-seven patients (mean age, 58 years) had serous adenocarcinoma (38 primary, 9 metastatic); 20 patients (mean age, 59 years) had mucinous adenocarcinoma (19 primary, 4 metastatic, including 3 patients with both); 7 (mean age, 57) had endometrioid adenocarcinoma (6 primary, 1 metastatic); 10 (mean age, 56) had clear-cell carcinoma (8 primary, 2 metastatic); 8 (mean age, 54) had mixed-type adenocarcinoma (7 primary, 2 metastatic, 1 patient with both); and 7 (mean age, 57 years) had poorly differentiated carcinoma (5 primary, 2 metastatic). Overall, 103 specimens (83 primaries and 20 metastases) from 99 patients were analyzed. Apart from these carcinoma patients, 8 patients (mean age, 59 years) had benign cystadenomas (5 serous, 3 mucinous) and 10 (mean age, 56 years) had borderline malignant cystadenomas (5 serous, 5 mucinous). Furthermore, 24 non-tumorous ovaries (9 from patients between 43 and 55 years of age, and 15 from patients over 55) were analyzed.

Immunohistochemistry

Representative tissue samples were snap-frozen in liquid nitrogen and stored at −70°C until use. Serial sections were cut at 5 μm. These were air-dried and fixed in acetone for 10 min, after which the indirect immunoperoxidase technique was used for visualizing Ki-67 MAb (DAKO, Glostrup, Denmark) and EGFR1 MAb (Amersham, Aylesbury, UK) as described by us in detail (Kuenen-Boumeester *et al.*, 1991; Henzen-Logmans *et al.*, 1992). For immunostaining of ER and PgR commercially available kits were used (ER-ICA and PgR-ICA kits, Abbott, Chicago, IL). After the final washing procedure, nuclear counterstaining was achieved for all antibodies by incubation in Mayer's hematoxylin for 1 min. Control samples consisted of known positive and negative specimens identified by the ligand-binding assay (for ER, PgR, EGFR). In addition, for negative control sections, the first layer antibody was changed to PBS or non-immune ascites fluid.

Quantification

The percentage of immunohistochemically assessed ER-, PgR-, EGFR- and Ki-67-positive tumor cells was calculated by counting the number of positive cells in a total of 3 × 100 cells in 3 different areas of the tumor section. For cytosolic ER, PgR and cathepsin-D assays, tumor tissue (0.4–0.8 g) was pulverized and homogenized as recommended by the EORTC for processing breast tumor tissue for cytosolic ER and PgR determinations. The homogenate was centrifuged for 30 min at 100,000 *g* at 4°C and the supernatant fraction (cytosolic extract) was used for ER and PgR determination by enzyme immunoassays (ER-EIA and PgR-EIA kits, Abbott) as described before (Berns *et al.*, 1992). Cathepsin-D levels were measured in the same cytosols with a commercially available radiometric immunoassay kit (ELSA-CATH-D, CIS bio international, Gif-sur-Yvette, France) as described before for human breast cancer samples (Foekens *et al.*, 1993).

Statistics

Associations between ER, PgR, EGFR, cathepsin D and Ki-67 were analyzed by Spearman rank correlation. Differences between groups were tested non-parametrically by means of the Wilcoxon 2-sample test (Mann-Whitney U-test).

RESULTS

Ki-67 and cathepsin D in histological subgroups

Ki-67. In tumor cells, various levels of immunoreactivity for Ki-67 were observed exclusively in the nuclei. Cells expressing Ki-67 were randomly distributed over the tissue sections. Stromal elements such as fibroblasts occasionally stained with the antibody. Figure 1 (top) shows the percentage of cells stained with the Ki-67 MAb according to the histological classification of the tumors. For the total group of 83 primary adenocarcinomas, the median value of Ki-67-positive cells was 18%. Metastatic tumors contained a significantly higher ($2p < 0.03$) number of Ki-67-positive cells (median, 28%; n = 20) than did primary adenocarcinomas, and in the total group of adenocarcinomas the number of Ki-67-positive cells was higher than in 18 benign/borderline cystadenomas ($2p < 0.01$). The median percentage of the number of Ki-67-positive cells in benign tumors was 4.5% and in borderline serous and mucinous tumors 4% and 23% respectively. Normal ovarian tissues (n = 24) did not express Ki-67 (Fig. 1, top).

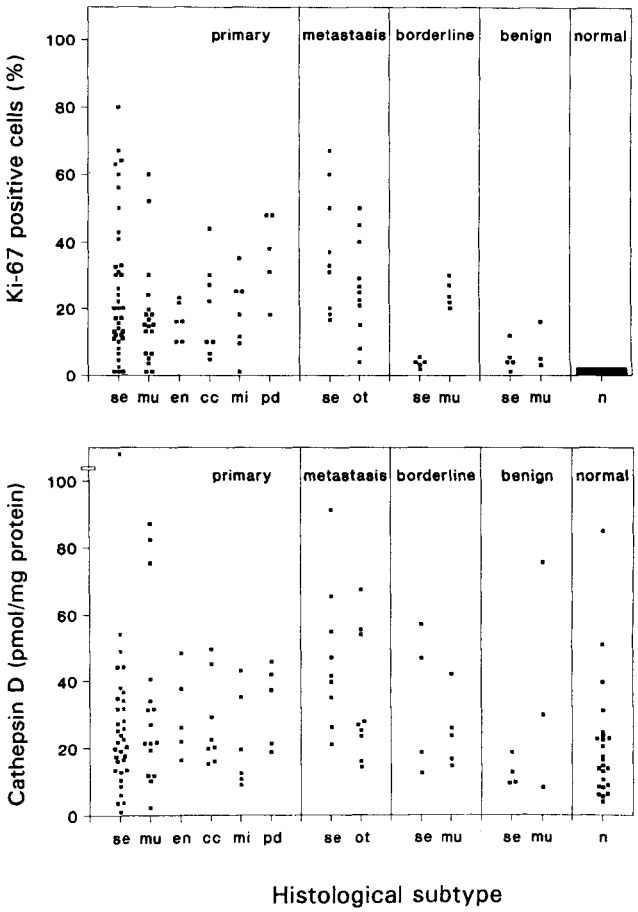

FIGURE 1 – Ki-67 and cathepsin D in various histological subtypes of ovarian tissues. The percentage of Ki-67-positive epithelial cells (top panel) was determined by immunohistochemistry; cytosolic cathepsin-D content (bottom panel) was determined with an enzyme immunoassay. Abbreviations: se = serous, mu = mucinous, en = endometrioid, cc = clear-cell, mi = mixed, pd = poorly differentiated, ot = other (metastatic) adenocarcinomas and n = normal ovarian tissues.

Cathepsin D. Cathepsin-D levels were measured in cytosols by quantitative radiometric immunoassay. The distribution of cytosolic cathepsin-D levels according to histological classification of the tumors is shown in Figure 1 (bottom). For the total group of 74 primary adenocarcinomas analyzed for cathepsin-D content, the median value was 22 pmol cathepsin D/mg cytosolic protein (range, 1–255). The 18 metastatic tumors analyzed contained significantly higher ($2p < 0.01$) levels of cathepsin D (median, 37 pmol/mg cytosolic protein) than did primary tumors. As in the case of Ki-67 expression, there was an overlap in the ranges of cathepsin-D values in all subgroups of primary and metastatic adenocarcinomas. Both metastatic and primary adenocarcinomas contained significantly higher ($2p < 0.03$) levels of cathepsin D than were present in normal ovarian tissues (median, 15 pmol/mg cytosolic protein; n = 24). The ranges of cathepsin-D values observed in borderline and benign tumors did not differ from those in either adenocarcinomas or normal tissues (Fig. 1, bottom).

ER, PgR and EGF-R

Adenocarcinomas. Immunohistochemically detectable nuclear ER and PgR were present in a variable percentage of tumor cells. Tumor stromal cells were occasionally positive, also in tumors lacking ER and PgR expression in epithelial tumor cells (ER: 6 cases, 2 with negative tumor cells; PgR: 6 cases, 5 with negative tumor cells). The occasional presence of ER and/or PgR in stromal tissue may partly explain the relatively weak correlation between data obtained by immunohistochemistry (IHC, scoring epithelial cells only) and data obtained by enzyme immunoassay (EIA) on cytosolic extracts (epithelial cells + stromal components). By defining ≥ 10 fmol/mg cytosolic protein as positive for the EIAs and by arbitrarily describing a tumor as being positive by IHC in those cases when any epithelial tumor cell reacted with the antibody, the observed concordances between IHC and EIA were 82% for ER and 70% for PgR (Table I). The Spearman correlation coefficients between the number of epithelial tumor cells stained in the IHC assay and the amount of cytosolic ER and PgR expressed by EIAs were Rs = 0.65 for both ER and PgR (n = 80, $2p < 0.0001$). Immunohistochemically assessed ER and PgR data were included in further analyses.

In 47% of 102 adenocarcinomas studied (primary and metastatic tumors), ER was detectable by IHC; a histoscore (percentage of positive epithelial cells) of 15 ± 22 (mean ± SD) was observed for primary tumors (46% positive, n = 83) and of 20 ± 26 for metastatic tumors (53% positive, n = 19). PgR was detectable in 32% of the adenocarcimonas, with a mean percentage of PgR-positive cells of 14 ± 26% (± SD) for the primary tumors (34% positive), and of 6 ± 15% for the metastatic tumors (26% positive). Neither for ER nor for PgR were these differences between primary and metastatic tumors statistically significant.

With MAb EGFR1, a heterogeneous level of expression was seen and staining was predominantly cytoplasmic. In all, 76% of 80 primary tumors and 78% of 18 metastatic adenocarcinomas analyzed for EGFR by IHC were positive for EGFR1 staining. In accordance with previous reports, this difference was not statistically significant (Henzen-Logmans *et al.,* 1992).

Benign and borderline tumors and normal ovarian tissues. Among borderline tumors, all 5 mucinous tumors were negative for ER and only a small number of the cells of 1 mucinous tumor were positive for PgR. By contrast, in all 5 borderline serous tumors, ER (mean percentage of positive cells ± SD: 25 ± 25%) and PgR (53 ± 12%) were detectable. The reverse was observed with respect to Ki-67 expression in mucinous *vs.* serous borderline tumors (Fig. 1, top). All 5 benign serous tumors studied were positive for ER (63 ± 25% positive cells) and PgR (54 ± 34%), whereas ER (4 ± 3% positive cells) and PgR (2 ± 2%) were either absent from or barely detectable in the 3 mucinous benign tumors studied. In non-tumoral ovarian tissues, stromal cells were occasionally positive for ER and/or PgR (ER, 6 out of 32 cases; PgR, 23 out of 32 cases). This high incidence of stromal expression of PgR probably accounts for the high (median, 152 fmol/mg protein) levels measured by EIA in cytosols prepared from normal ovarian tissues as described before (Berns *et al.,* 1992).

All 5 borderline serous (median, 60%; range, 30–90% positive cells) and 3 out of 5 mucinous tumors (60%, 60%, and 100% positive cells) studied were positive for EGFR1 expression. Of the benign neoplasms studied, 3 out of 5 serous tumors (40%, 60%, and 100% positive cells) and all 3 mucinous tumors (10%, 70%, and 100% positive cells) were positive for EGFR1 expression. When surface epithelial cells were present in normal ovarian tissues, EGFR1 immunoreactivity was localized on these cells. In addition, as reported before (Henzen-Logmans *et al.,* 1992), non-tumorous spindle cells and vessel walls sometimes stained as well.

Correlations between Ki-67, ER, PgR, cathepsin D and EGFR

Correlations between the percentage of Ki-67-positive cells and immunohistochemically assessed ER, PgR, and EGFR and cytosolic levels of cathepsin D were studied in primary adenocarcinoma tissues. Tumors containing high levels (above the median) of cathepsin D were found to be equally distributed among ER-, PgR- and EGFR-positive and -negative tumors and among tumors expressing high or low numbers of Ki-67-positive cells (Table II). Similarly, primary adenocarcinomas expressing high or low numbers of Ki-67-positive cells were found to be equally distributed among ER- and PgR-positive and -negative tumors. However, a trend towards a positive correlation between EGFR and Ki-67 was observed: 54% of EGFR-positive tumors *vs.* 37% of EGFR-negative tumors contained a high number of Ki-67-positive cells (Table II). The respective Spearman correlation coefficients between the various cell biological parameters listed in Table III show weak positive associations of EGFR with Ki-67 and ER ($2p < 0.05$) and a strong positive correlation between immunohistochemically assessed ER and PgR ($2p < 0.0001$). None of the other associations were statistically significant.

TABLE I – ER AND PgR STATUS IN PRIMARY OVARIAN ADENOCARCINOMAS: IMMUNOHISTOCHEMISTRY *VS.* ENZYME IMMUNOASSAY

		ER-EIA[1]			PgR-EIA[1]		
		+	–	Total	+	–	Total
IHC[2]	+	33	3	36	21	6	27
	–	11	33	44	18	35	53
	Total	44	36	80	39	41	80

[1]EIA: enzyme immunoassay; positive defined as ≥ 10 fmol/mg cytosolic protein.–[2]IHC: immunohistochemistry; positive defined as > 0 stained epithelial tumor cells.

TABLE II – Ki-67 AND CATHEPSIN-D STATUS ACCORDING TO OTHER VARIABLES IN PRIMARY OVARIAN ADENOCARCINOMAS

Variable	Ki-67 (≥ 18% positive cells)[1]		Cathepsin D (≥ 22.0 pmol/mg protein)[1]	
	number/total	(%)	number/total	(%)
Total	42/83[2]	(51)	37/74	(50)
ER-positive[3]	20/38	(53)	16/32	(50)
ER-negative	22/45	(49)	22/42	(52)
PgR-positive[3]	14/28	(50)	12/24	(50)
PgR-negative	28/55	(51)	25/50	(50)
EGFR-positive[3]	33/61	(54)	30/55	(54)
EGFR-negative	7/19	(37)	7/16	(46)
Cathepsin D-high[4]	18/37	(49)		
Cathepsin D-low[4]	20/37	(54)		

[1]18% and 22.0 pmol/mg protein represent the median values of the percentage of Ki-67-positive cells and cytosolic cathepsin-D levels respectively.–[2]Note that, due to missing values, total numbers do not all add up to 83.–[3]Positive by immunohistochemistry (> 0 positive epithelial tumor cells).–[4]High and low indicate above and below the median value of 22.0 pmol/mg cytosolic protein respectively.

TABLE III – SPEARMAN CORRELATION COEFFICIENTS

	ER[1]	PgR[1]	EGFR[1]	Ki-67[1]
PgR	0.73[2]	—	—	—
EGFR	0.23[2]	0.18	—	—
Ki-67	0.14	0.09	0.23[3]	—
Cathepsin D[4]	0.10	0.18	0.02	−0.02

[1]Immunohistochemistry: percentage positive cells, n = 79 to 83.–[2]$p < 0.0001$.–[3]$p < 0.05$.–[4]Cytosol assay, n = 71 to 74.

DISCUSSION

Our study was undertaken to determine whether a relationship exists between the levels of EGFR, steroid hormone receptors, cathepsin D and proliferative activity as assessed by Ki-67 reactivity in human ovarian cancer. ER and PgR were detected by IHC in a variable percentage of tumor cells and their expression was found to be strongly correlated. ER was detectable in 46% of the primary and in 53% of the metastatic specimens examined. PgR expression was found in 34% of the primary and in 26% of the metastatic adenocarcinomas studied. These differences in expression between primary and metastatic tumors were not statistically significant. EIAs showed ER to be positive in 55% and PgR to be positive in 49% of the primary adenocarcinomas examined in our series. Correlating data obtained from the same specimens by IHC and EIA, we found the observed concordances to be moderate (82% for ER and 70% for PgR). The discrepancies can be partially explained by expression of both receptors demonstrated immunohistochemically in stromal cells. Using IHC, only positive epithelial tumor cells are scored and the possible presence of one or both receptors in stromal cells may therefore give positive results when assays are performed on cytosolic extracts of tumor tissues. Expression of one or both receptors on ovarian stromal cells, as assessed by IHC, has also been reported by Isola *et al.* (1990), who likewise observed a relatively low concordance between IHC and the ligand-binding assay. Other explanations could be related to low receptor levels and loss of antigenicity due to tissue-storage and processing procedures.

In our series, ER and PgR positivity, when assessed by IHC, was not related to tumor type. However, Sutton *et al.* (1986) reported higher expression of PgR in endometrioid carcinomas, and in our previous study using EIA (Berns *et al.*, 1992) higher levels of ER were measured in serous *vs.* mucinous adenocarcinomas. Differences in detection methods and/or in the number of tumors examined may be responsible for this discrepancy. In ovarian cancer patients positivity for both ER and PgR is associated with better prognosis, PgR having the stronger prognostic power (Rao and Slotman, 1991). We have made the same observation in a limited series of 50 patients with primary ovarian cancer who had a sufficiently long follow-up period (van der Burg *et al.*, 1993).

The presence of EGFR and the mitogenic response of some ovarian (cancer) cell lines to EGF *in vitro* (Berchuck *et al.*, 1990) suggest that EGF and its receptor may play an important role in the regulation of ovarian cancer growth *in vivo*. The relatively high incidence of EGFR expression detected in the present study (76% of the primary tumors using IHC) and in previous ones (66% using ligand-binding assay; Henzen-Logmans *et al.*, 1992) also suggests that EGF may have a growth-regulatory role in ovarian carcinomas. In the total group of ovarian adenocarcinomas, no inverse relation was found between the levels of EGFR, ER and PgR such as is generally observed in breast cancer (reviewed by Klijn *et al.*, 1992). On the contrary, we observed a weak positive correlation between EGFR and ER. This finding is puzzling since both tumors are supposed to be endocrinologically regulated. One explanation may be related to the fact that the pathways of ER and/or EGF regulation are not necessarily the same in breast and ovarian cancers, with a possibly reduced functionality of ER in the latter (Rao and Slotman, 1991). As to clinical outcome, data on the prognostic value of EGFR are conflicting with respect to both ovarian cancer (Bauknecht *et al.*, 1988, 1990; Berchuck *et al.*, 1991; Owens *et al.*, 1991; van der Burg *et al.*, 1993) and breast cancer (Klijn *et al.*, 1992).

When comparing our EGFR data with proliferative activity by counting Ki-67 reactivity in adenocarcinomas, we noted a trend towards a positive correlation between EGFR and Ki-67, *i.e.*, 54% of EGFR-positive tumors *vs.* 37% of EGFR-negative tumors contained ≥ 18% (median) Ki-67-positive cells. Such a positive relationship between EGFR and Ki-67 has also been reported in a few studies on breast cancer tissues (Klijn *et al.*, 1992).

Concerning cathepsin D, significantly higher levels were measured in adenocarcinomas *vs.* normal ovarian tissues and in metastatic lesions *vs.* primary tumors. Higher levels of cathepsin D in malignant tumors *vs.* normal tissues have also been reported by Scambia *et al.* (1991). Since cathepsin D is a proteolytic enzyme that may facilitate tumor invasion when it is secreted by cancer cells (Rochefort *et al.*, 1987), differences in levels of expression might represent a biochemical characteristic reflecting biological tumor aggressiveness. In our series, however, no significant differences in levels of expression were noticed between benign/borderline adenomas and adenocarcinomas. Cathepsin D may have mitogenic activity through an autocrine mechanism (Mathieu *et al.*, 1990). However, no relationship between Ki-67 activity and cathepsin-D levels was found in our study. In ER-positive breast-cancer cells, estradiol stimulates the production of cathepsin D (Westley and Rochefort, 1980). In our study however, which concords with data reported by Scambia *et al.* (1991), no positive correlation was found between the expression of cathepsin D and ER in ovarian carcinoma tissues. This lack of association between cathepsin D and ER is also observed for breast tumors (Rochefort, 1990), although we have found a very weak, but significant ($p < 0.05$), association between ER and cathepsin D in a series of 710 breast tumors (Foekens *et al.*, 1993).

We have shown that the percentage of Ki-67-positive cells in adenocarcinomas varies without any correlation to tumor type but with a significant trend to higher expression in metastatic lesions. We also observed that Ki-67 expression in the total group of adenocarcinomas was significantly higher than in

adenomas. Wong and Tattersall (1989) have also reported a positive correlation between high Ki-67 activity and distant metastases. Correlating Ki-67 reactivity with the level of expression of ER, PgR, EGFR and cathepsin D as markers of possible mitogenic functionality, we have shown that the levels of expression of these factors vary in adenocarcinomas without relation to tumor type. Apart from a weak positive association of EGFR with ER and Ki-67, and a strong positive correlation between ER and PgR, no statistically significant correlations were found.

Further studies are in progress to assess the prognostic significance of Ki-67 and cathepsin D in ovarian carcinomas in relation to progression-free survival and death. The lack of correlation between Ki-67 and ER, PgR and cathepsin D suggests that hormone- and cathepsin-D -mediated pathways and cell proliferation are not directly linked in ovarian carcinomas.

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REFERENCES

BAUKNECHT, T., BIRMELIN, G. and KOMMOSS, F., Clinical significance of oncogenes and growth factors in ovarian carcinomas. *J. steroid Biochem. mol. Biol.*, **37**, 855–862 (1990).

BAUKNECHT, T., RUNGE, M., SCHWALL, M. and PFLEIDERER, A., Occurrence of epidermal growth-factor receptors in human adnexal tumours and their prognostic value in advanced ovarian carcinomas. *Gynecol. Oncol.*, **29**, 147–157 (1988).

BERCHUCK, A., OLT, G., EVERITT, L., SOISSON, A.P., BAST, R.C. and BOYER, C.M., The role of peptide growth factors in epithelial ovarian cancer. *Obstet. Gynecol.*, **75**, 255–262 (1990).

BERCHUCK, A., RODRIGUEZ, G.C., KAMEL, A., DODGE, R.K., SOPER, J.T., CLARKE-PEARSE, D.L. and BAST, R.C., JR., EGF-R expression in normal ovarian epithelium and ovarian cancer. 1. Correlation of receptor expression with prognostic factors in patients with ovarian cancer. *Amer. J. Obstet. Gynecol.*, **164**, 669–674 (1991).

BERNS, E.M.J.J., KLIJN, J.G.M., HENZEN-LOGMANS, S.C., RODENBURG, C.J., VAN DER BURG, M.E.L. and FOEKENS, J.A., Receptors for hormones and growth factors and (onco)-gene amplification in human ovarian cancer. *Int. J. Cancer*, **52**, 218–224 (1992).

BRIOZZO, P., MORISSET, M., CAPONY, F., ROUGEOT, C. and ROCHEFORT, H., *In-vitro* degradation of extracellular matrix with Mr 52,000 cathepsin D secreted by breast-cancer cells. *Cancer Res.*, **48**, 3688–3692 (1988).

FOEKENS, J.A., VAN PUTTEN, W.L.J., PORTENGEN, H., DE KONING, Y.W.C.M., THIRION, B., ALEXIEVA-FIGUSCH, J. and KLIJN, J.G.M., Prognostic value of PS2 and cathepsin D in 710 human primary breast tumors: multivariate analysis. *J. clin. Oncol.*, **11**, 899–908 (1993).

HENZEN-LOGMANS, S.C., BERNS, E.M.J.J., KLIJN, J.G.M., VAN DER BURG, M.E.L. and FOEKENS, J.A., Epidermal growth-factor receptor in ovarian tumours: correlation of immunohistochemistry with ligand binding assay. *Brit. J. Cancer*, **66**, 1015–1021 (1992).

ISOLA, J., KALLIONIEMI, O.-P., KORTE, J.-M., WAHLSTRÖM, T., AINE, R., HELLE, M. and HELIN, H., Steroid receptors and Ki-67 reactivity in ovarian cancer and in normal ovary: correlation with DNA flow cytometry, biochemical receptor assay and patient survival. *J. Pathol.*, **162**, 295–301 (1990).

KLIJN, J.G.M., BERNS, P.M.J.J., SCHMITZ, P.I.M. and FOEKENS, J.A., The clinical significance of epidermal growth-factor receptor (EGFR) in human breast cancer: a review on 5232 patients. *Endocrine Rev.*, **13**, 3–17 (1992).

KUENEN-BOUMEESTER, V., VAN DER KWAST, TH., VAN LAARHOVEN, H.A.J. and HENZEN-LOGMANS, S.C., Ki-67 staining in histological sub-types of breast carcinoma and fine-needle aspiration smears. *J. clin. Pathol.*, **44**, 208–210 (1991).

MATHIEU, M., ROCHEFORT, H., BARENTSON, B., PRÉBOIS, C. and VIGNON, F., Interactions of cathepsin D and IGF-II on the IGF-II/M-6-P receptor in human-breast-cancer cells and possible consequences on mitogenic activity of IGF-II. *Mol. Endocrinol.*, **4**, 1327–1335 (1990).

MCGURRIN, J.F., DORIA, M.I., DAWSON, P.J., KARRISON, T., STEIN, H.O. and FRANKLIN, M.O., Assessment of tumor cell kinetics by immunohistochemistry in carcinoma of the breast. *Cancer*, **59**, 1744–1750 (1987).

OWENS, O.J., STEWART, C., BROWN, I. and LEAKE, R.E., Epidermal growth-factor receptors (EGFR) in human ovarian cancer. *Brit. J. Cancer*, **64**, 907–910 (1991).

RAO, B.R. and SLOTMAN, B.J., Endocrine factors in common epithelial ovarian cancer. *Endocrine Rev.*, **12**, 14–26 (1991).

ROCHEFORT, H., Cathepsin D in breast cancer. *Breast Cancer Res. Treat.*, **16**, 3–13 (1990).

ROCHEFORT, H., CAPONY, F., GARCIA, M., CAVAILLÈS, V., FREISS, G., CHAMBON, M., MORISSET, M. and VIGNON, F., Estrogen-induced lysosomal protease secreted by breast-cancer cells. A role in carcinogenesis? *J. cell. Biochem.*, **35**, 17–29 (1987).

RUTGERS, D.H., WILS, I., SCHAAP, A. and VAN LINDERT, A., DNA flow cytometry, histological grade, stage and age as prognostic factors in human epithelial ovarian carcinomas. *Pathol. Res. Pract.*, **182**, 207–213 (1987).

SCAMBIA, G., BENEDETTI, P., FERRANDINA, G., BATTAGLIA, F., BAIOCCHI, G. and MANCUSO, S., Cathepsin-D assay in ovarian cancer: correlation with pathological features and receptors for oestrogen, progesterone and epidermal growth factor. *Brit. J. Cancer*, **64**, 182–184 (1991).

SUTTON, G.P., SENIOR, M.B., STRAUSS, J.F. and MIKUTA, J.J., Estrogen and progesterone receptors in epithelial ovarian malignancies. *Gynecol. Oncol.*, **23**, 176–182 (1986).

VAN DER BURG, M.E.L., HENZEN-LOGMANS, S.C., FOEKENS, J.A., BERNS, E.M.J.J., RODENBURG, C.J., VAN PUTTEN, W.L.J. and KLIJN, J.G.M., The prognostic value of epidermal growth-factor receptors, determined by both immunohistochemistry and ligand binding assay, in primary epithelial ovarian cancer: a pilot study. *Europ. J. Cancer*, **29**, 1951–1957 (1993).

WESTLEY, B. and ROCHEFORT, H., A secreted glycoprotein induced by estrogen in human-breast-cancer cell lines. *Cell*, **20**, 352–362 (1980).

WONG, W.S.F. and TATTERSALL, M.H.N., Immunohistochemical determination of tumour growth fraction in human ovarian carcinoma. *Brit. J. Obstet. Gynaecol.*, **96**, 720–724 (1989).