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Immunocytochemical Determination of the Estrogen-regulated Protein M_r 24,000 in Primary Breast Cancer and Response to Endocrine Therapy

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Abstract—*Monoclonal antibodies against an estrogen-regulated* M_r 24,000 *cytosol protein (p24) were used for immunocytochemical localization of p24 in formalin-fixed paraffin-embedded specimens from the primary tumor in* 103 *patients who received endocrine therapy for advanced breast cancer. Sixty-one per cent of the tumors showed p24-positive staining, and this correlated well with the presence of estrogen receptor (ER)* (P = 0.00017, *chi-square test). Response to endocrine therapy was obtained in* 43% *of the patients. A statistically significant association between p24 status and response could not be established, while response and ER status were highly correlated* (P = 0.0000029, *chi-square* = 21.6). *Discrimination between ER-positive responders and ER-positive non-responders was not possible using p24 staining.*

INTRODUCTION

THE PROBABILITY of response to hormonal treatment in human breast cancer is in general predicted from the level of estrogen (ER) and progesterone receptors (PgR) in the tumor tissue [1, 2]. The predictive value of receptor determinations is, however, limited because a substantial fraction of patients with receptor-positive tumors do not respond to endocrine therapy.

In search of a potential marker of estrogen responsiveness in breast cancer, Edwards *et al.* have identified and characterized an estrogen-regulated protein, M_r 24,000 (p24) in a human breast cancer cell line (MCF-7) [3, 4]. Using a quantitative enzyme-linked immunosorbent assay, p24 was found in human breast cancer cytosols and was found to correlate with the content of ER and PgR [5]. Monoclonal antibodies raised against p24 have been used for immunohistochemical detection of p24 in formalin-fixed paraffin-embedded breast tumors grown in nude mice and in human breast cancer biopsies [6].

The aim of this study was to evaluate clinically immunohistochemical analysis of p24 in the primary tumor from patients with advanced breast cancer as a predictor of response to hormonal treatment.

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PATIENTS AND METHODS

Patients

We studied 103 consecutive patients admitted to the Department of Oncology, Aarhus, Denmark who met the following inclusion criteria: (1) recurrent or advanced breast cancer with evaluable disease parameters, (2) no previous systemic treatment, (3) evaluable disease parameters treated with hormones only. At the time of recurrence, 93 (90%) of the patients were postmenopausal and 10 (10%) were premenopausal. The dominant metastatic sites were defined as viscera, bone or soft tissue in decreasing order of priority, and were distributed as follows: viscera 38 (37%), bone 25 (24%) and soft tissue 40 (39%). Endocrine therapy was as follows: tamoxifen 79 (76.7%), oophorectomy five (4.9%), oophorectomy and tamoxifen five (4.9%), fluoxymesterone two (1.9%), other endocrine treatment 12 (11.6%). All local recurrences were confirmed histologically and dated from the time first observed. Relapses in bone were dated from the time X-rays became abnormal. Pleural effusions were accepted as a sign of relapse only when proved malignant by cytology. Characteristic changes on isotope or ultrasound liver scan were accepted as recurrence. Criteria of response followed UICC recommendations [7] and were reviewed without knowledge of the result of the p24 analysis.

Antibodies

Monoclonal antibodies against p24 were kindly provided by Dr. W.L. McGuire. The production

and characterization of p24 has previously been described [8, 9].

Immunocytochemistry

p24 analyses were carried out on archive formalin-fixed paraffin-embedded biopsy specimens from the primary tumor. The immunohistochemical staining technique was as follows: after deparaffinization and rehydration, slides were washed in Tris/PBS [0.5 M Tris–HCl in PBS (NaCl, 8.5 g; KH_2PO4, 0.25 g; $K_2HPO_4 \times 3H_2O$, 1.43 g in 1000 ml H_2O), pH 7.6] for 2 × 5 min and incubated with trypsin for 15 min at 37°C (0.1% trypsin in 0.1% $CaCl_2$, pH 7.8). The reaction was stopped by washing in 4°C Tris/PBS for 5 min followed by Tris/PBS for 5 min and incubation with normal goat serum for 20 min (1:10 dilution in Tris/PBS). After pouring off excess material, the slides were covered with the monoclonal p24 antibody for 24 h at 4°C (5 μg/ml in Coons' buffer), washed in Tris/PBS 2 × 5 min and incubated with biotinylated goat anti-mouse for 60 min (69 μg/ml in Tris/PBS). After washing in Tris/PBS 2 × 5 min, the slides were incubated with AB complex for 30 min (according to the manufacturer's description), washed in Tris/PBS 3 × 10 min and stained with carbazole for 10 min (0.04% 3-amino-9-ethylcarbazole with 0.01% H_2O_2 in 50 mM acetate buffer pH 5.0). After washing in H_2O for 2 min, the slides were slightly counterstained with hematoxylin, washed and mounted with glycerol/gelatine. All procedures were performed at room temperature unless otherwise indicated. A control specimen was processed for each biopsy specimen by replacing the primary antibody with normal mouse IgG in the same dilution. With every run, a known p24-positive and p24-negative specimen were incubated in parallel with the unknown sections. p24-positive staining was defined as a red-brown reaction product in the cytoplasm of malignant epithelial cells after incubation with the specific antibody but absent in parallel incubation with the non-immune antibody.

Immunohistochemical ER analysis was performed on the same sections using a method previously described in detail [10, 11]. A tumor was designated as ER-positive if any tumor cell presented specific staining.

RESULTS

In all p24-positive tumors, the specific staining was confined to the cytoplasm of malignant epithelial cells and appeared uniformly throughout the cytoplasm. Almost all tumors contained a mixture of stained and unstained epithelial cells both in terms of cell to cell differences and area to area differences. A tumor was designated p24-positive if any degree of specific staining was seen, and the average number of p24-positive cells was subjectively classified as none, rare (<10% positive), moderate (10–50% positive) or numerous (>50% positive). Sixty-three patients (61%) had p24-positive tumors and 40 (39%) patients had p24-negative tumors. The relationship to receptor status is shown in Table 1, where a significant correlation is established (receptor data only available in 100 patients).

Response to endocrine therapy (CR + PR) was obtained in 44 patients (43%), while 59 patients (57%) were non-responders (NC + PD). The relationship between response and p24 status is shown in Table 2. A statistically significant association between response and p24 status could not be found. This was true even when correlating the number of p24-positive cells and response (data not shown). In contrast, 38 (56%) of the patients with ER-positive tumors obtained a response vs. two (6%) of the patients with ER-negative tumors ($P = 0.0000029$, chi-square = 21.6). Table 3 shows the association between response and p24 status in the 68 patients who harbored ER-positive tumors. As can be seen, p24 staining does not allow discrimination between responders and non-responders in these patients. There was no correlation between p24 status and the dominant metastatic site.

Table 1. Correlation of p24 with ER status

ER status	p24 status	
	Positive	Negative
Positive	50	17
Negative	11	21

Chi square = 14.16, $P = 0.00017$.

Table 2. Correlation of p24 with response to endocrine therapy

	Response			
	CR	PR	NC	PD
p24-positive	15	14	7	27
p24-negative	9	6	0	19

Chi square = 1.09, $P = 0.78$.

Table 3. Correlation of p24 with response in patients with ER-positive tumors

	p24 status	
	Positive	Negative
+ response	27	11
− response	23	7

Chi square = 0.06, $P = 0.81$.

DISCUSSION

The lack of response to hormonal treatment in a substantial number of the ER-positive tumors could, among other reasons, be caused by an inoperative ER system. It has therefore been postulated that demonstration of an end product of estrogen stimulation may improve the predictive value of ER as a sign of hormone-responsive tumor cell growth [12]. Several estrogen-regulated proteins have been described, among which the p24 protein was identified and isolated by Edwards *et al.* [3]. In this study we found 61% of the breast tumors to be p24-positive and p24 status was highly correlated with ER status. This is in accordance with the data published by Adams and McGuire who compared p24 analyzed by an ELISA technique with the steroid receptor status in 102 breast tumors [5]. Ciocca *et al.* found p24 expression in 3/6 breast tumors using an immunohistochemical technique [6]. We were unable to demonstrate any association between p24 status and the response to endocrine therapy of metastatic breast cancer, while, as expected, the association between ER status and response was highly significant. As in most other studies, the value of an ER-negative status as a predictor of non-response is high, while the prediction of response by an ER-positive status is less powerful. If one accepts that the production of p24 in the cell is an indicator of an intact receptor mechanism, as suggested [4], it might be hoped that determination of the p24 status would allow discrimination between ER-positive responders and ER-positive non-responders.

Disappointingly, the fraction of p24-positive tumors was generally equal in these two groups of patients, indicating that there were no differences between responders and non-responders regarding the ER-mediated response pathway. This assumption is supported by a recent study on endocrine therapy of recurrent breast cancer showing equal response rates in patients with ER-positive/PgR-positive tumors and in patients with ER-positive/PgR-negative tumors [13]. This is an analog because like p24, PgR is synthesized in tissues with normally functioning ER [14].

The above study together with our own results imply that the problem of ER-positive non-responders is caused by factors other than a non-functioning receptor.

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