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Differences in oestrogen and progesterone receptors, HER-2 and p53 expression and proliferation in ductal breast cancers in relation to histopathological grade

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In case of breast cancer the grade of differentiation and expression of oestrogen and progesterone receptors falls within the first category of prognostic factors according to the College of American Pathologists. HER-2, p53 and Ki67 belong to the second category and their significance still awaits confirmation. The aim of the present study was to examine the relationship between the intensity of expression of oestrogen receptors (ER), progesterone receptors (PgR), HER-2, p53 and Ki67 in cells of ductal breast cancer of G1, G2 or G3 differentiation grade. In paraffin sections of 60 ductal breast cancers (20 cases in G1, 20 in G2 and 20 in G3), immunocytochemical reactions were performed to detect the expression of ER, PgR, HER-2, p53 and Ki67. Following a semi-quantitative appraisal of the preparations under examination, appropriate statistical tests were used to document significant relationships. We noted significant positive correlations between ER and PgR (the entire group studied, G1–3, and the G1 group), HER-2 and p53 (G2) and between p53 and Ki67 expression (G2). Significant negative correlations were found between ER and p53 (G1–3), PgR and p53 (G1–3, G1, G3) and between PgR and Ki67 (G1–3, G2). The studies performed demonstrated distinct relationships between the expression intensity of various proteins in tumour cells in relation to the grade of differentiation of the tumour. We also showed that a parallel determination of ER, PgR and p53 expression may carry high predictive value as to response to tamoxifen treatment.

Key words: breast cancer, prognostic factors, grade, immunocytochemistry

INTRODUCTION

Breast cancer is the most common malignant tumour among females in the western world. The incidence of breast cancer remains high and clinical courses are highly variable. It is of general importance to predict the biology of the tumour and thus the course of the disease in the individual patient in order to ensure adequate therapy and patient sur-

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veillance. In 1999, under the auspices of the College of American Pathologists, a multi-disciplinary group of clinicians, pathologists and statisticians examined prognostic and predictive factors related to breast cancers and categorised them according to their value and the number of respective publications [4]. Three categories of prognostic factors were distinguished. Factors of the first category carried an established clinical value and continue to be used in daily clinical practice (tumour size, metastases to the lymph nodes, grade, histological type, mitotic index and receptors for oestrogen and progesterone). The second category grouped factors whose significance required confirmation in subsequent clinical studies (HER-2, p53, Ki67, PCNA). Factors of the third category are at present at the stage of testing and their prognostic value has not yet been established (DNA ploidy, neoplastic angiogenesis, EGFR, TGF alpha, Bcl-2, pS2 and cathepsin D).

Breast cancer grade is appraised on the basis of its histological structure and the extent of atypia of the cell nuclei. In cases of an infiltrating ductal cancer, grade is established according to the modified grading of Bloom and Richardson [3]. Studies on the relationship between the grading of the tumours and the survival of patients demonstrated an extensive correlation between the variables, despite the fact that the data were accumulated by several pathologists and various scales of evaluation were employed [12].

In around 60% of all breast cancer cases tumour cells carry oestrogen receptors (ER) and progesterone receptors (PgR). Around 20% of cases manifest no receptors for the hormones. The cases demonstrating expression of both receptors are known to be the ones to respond most frequently by remission to treatment with tamoxifen. The presence of ER by itself has been accepted as an independent prognostic and predictive factor. As compared to ER, the significance of PgR expression has proved to be much less unequivocal [15].

In 1987 Slamon et al. [14] reported that cases of advanced breast cancer with amplification of the HER-2/neu gene manifested shorter remission and shorter survival. Later, overexpression of the HER-2 receptor was also noted as being linked to a less favourable response to treatment in schemes based on cyclophosphamide, methotrexate and 5-fluorouracil but to a better clinical response to administration of anthracyclines [9].

The P53 gene was first described in 1979. Its mutations are detected in approximately one third of breast cancer cases. Mutations of the P53 gene extend the half-life of p53 protein. Immunocytochemical detection of overexpression of the protein, therefore, is in most cases equivalent to detection of mutation of the gene. Breast cancer cases with P53 mutation are less differentiated and exhibit a more aggressive course and shorter survival [5, 10].

Ki67 protein is characteristic for cells in the mitotic cycle and represents an unfavourable prognostic index [13]. The intensity of expression of the protein correlates directly with the grade of tumour differentiation [2].

The aim of the present study was to examine the relationships between the intensity of expression of oestrogen and progesterone receptors, HER-2, p53 and the intensity of proliferation (Ki67) in cells of ductal breast cancer of individual grades of differentiation (G1, G2, G3). Such an analysis of relationships between prognostic factors of the first category with those of the second category may enable the significance of the latter to be defined more precisely.

MATERIAL AND METHODS

Immunocytochemical analysis was performed retrospectively on tissue samples that were taken for routine diagnostic purposes. Based on histology (invasive ductal breast cancer) and grade (equal groups for each grade), 60 patients with primary invasive breast cancer who were diagnosed in the years 1999 to 2000 in the Lower Silesian Centre of Oncology (Wroclaw, Poland) were analysed. The mean age of the patients amounted to 55.62 years ± 9.86 SD (age range: 43 to 72 years). Each of the grades G1, G2 and G3 were represented by 20 cases. Samples isolated from the tumours were fixed in 10% buffered formalin and embedded in paraffin. In all cases haematoxylin and eosin stained preparations were prepared and subjected to histopathological evaluation, including independent assessment of the grade by two independent pathologists, according to a modified version of the Bloom-Richardson scale.

Formalin-fixed paraffin-embedded tissue was freshly cut (4 μm). The sections were mounted on Superfrost slides (Menzel Glaeser, Germany), dewaxed with xylene, and gradually rehydrated. The activity of endogenous peroxidase was blocked by 30-minute incubation in 1% H_2O_2. The sections under examination were boiled for 15 minutes in Target Retrieval Solution in a microwave oven. Immunocytochemical reactions were performed using the antibodies to oestrogen receptor (clone 1D5, optimally prediluted), progesterone receptor (clone 1A6, optimally prediluted), HER-2 (polyclonal antibodies, dilution 1:250),

p53 (clone DO-7, dilution 1:100) and Ki67 (clone MIB-1, dilution 1:100). The antibodies were diluted in the Antibody Diluent, Background Reducing. The sections tested were incubated with the antibodies for 1 hour at room temperature. Subsequently, incubations were performed with biotinylated antibodies (15 minutes at room temperature) and with streptavidin-biotinylated peroxidase complex (15 minutes at room temperature) (LSAB+, HRP). DAB was used as a chromogen (7 minutes at room temperature). All the sections were counterstained with Meyer's haematoxylin. In each case controls were included in which a specific antibody was substituted by the Primary Mouse Negative Control. All the reagents originated from DakoCytomation, Denmark.

The intensity of the immunocytochemical reactions was quantitated by two pathologists using the following scales:

— in cases of ER and PgR the scales applied took into account the percentage of cells yielding a positive reaction (0: negative, 1: < 10%, 2: 10–30% and 3: >30% of positive cells);
— for evaluation of HER-2 reactivity the DakoCytomation scoring system was used (0: negative; + partially membranous; ++ completely membranous, weak; +++ completely membranous, strong);
— p53 was evaluated using the semi-quantitative IRS scale, which paid attention to the proportion of positive cells as well as the intensity of the reaction. The final result was obtained by multiplying the scores given for each of the traits and the product ranged between 0 and 12 [11];
— expression of Ki67 was quantitated by scoring cells with a positive reaction.

Statistical analysis of the results obtained was conducted using Statistica 98 PL software (Statsoft, Poland). The expression intensities of individual antigens in groups G1, G2 and G3 were compared using the Kruskal-Wallis ANOVA rank test. Since the distribution of the ER, PgR, HER-2 and p53 variables could not be considered normal (as shown by the Lilliefors test), gamma correlation was employed to examine relations between individual variables.

RESULTS

The studies performed documented the following pattern of immunocytochemical reactions:

— in cases of oestrogen receptor, progesterone receptor, p53 and Ki67 the colour reaction was localised in the cell nuclei (Fig. 1–4). The intensity of the reaction varied in individual cases (Table 1);

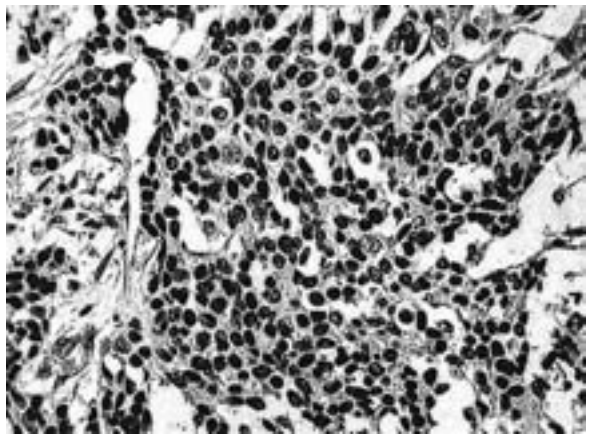

Figure 1. Immunocytochemical localisation of oestrogen receptor expression in the cells of invasive ductal breast cancer (haematoxylin, × 200).

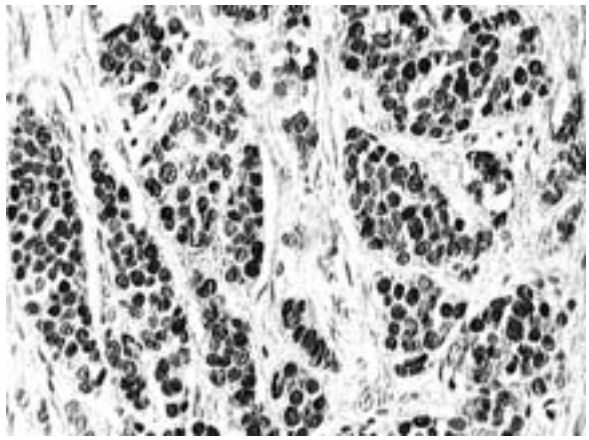

Figure 2. Immunocytochemical localisation of progesterone receptor expression in the cells of invasive ductal breast cancer (haematoxylin, × 200).

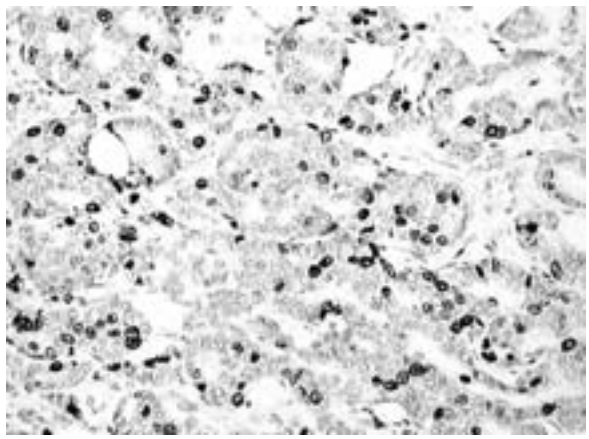

Figure 3. Immunocytochemical localisation of p53 expression in the cells of invasive ductal breast cancer (haematoxylin, × 200).

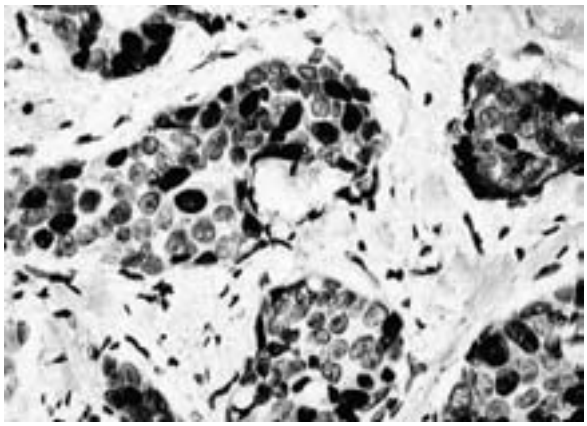

Figure 4. Immunocytochemical localisation of Ki67 expression in the cells of invasive ductal breast cancer (haematoxylin, × 400).

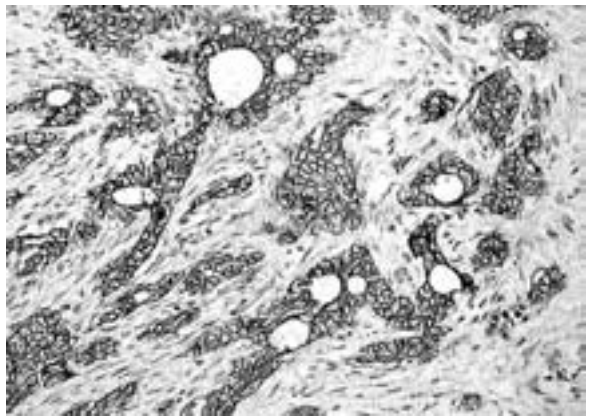

Figure 5. Immunocytochemical localisation of HER-2 expression in the cells of invasive ductal breast cancer (haematoxylin, × 100).

Table 1. Intensity of expression of the antigens studied in the examined material in relation to grade of differentiation (ANOVA Kruskal-Wallis test)

Antigen	Grade	Mean ± SD	Min	Max	p
ER	G1	2.27 ± 0.66	1	3	0.18
	G2	1.57 ± 0.97	0	3	
	G3	1.6 ± 1.24	0	3	
	G1–3	1.73 ± 1.02	0	3	
PgR	G1	2.27 ± 0.66	0	3	**0.02**
	G2	1.52 ± 1.07	0	3	
	G3	0.95 ± 1.14	0	3	
	G1–3	1.46 ± 1.15	0	3	
HER-2	G1	2.27 ± 0.79	1	3	0.45
	G2	1.47 ± 0.88	0	3	
	G3	1.8 ± 0.86	0	3	
	G1–3	1.77 ± 0.89	0	3	
p53	G1	2.18 ± 1.4	0	6	0.09
	G2	3.85 ± 2.57	0	12	
	G3	4.7 ± 2.77	0	12	
	G1–3	3.82 ± 2.51	0	12	
Ki67	G1	11.91 ± 5.34	5	25	**0.0001**
	G2	34.14 ± 14.62	2	80	
	G3	51.25 ± 13.75	5	75	
	G1–3	36.02 ± 18.9	2	80	

— in the case of the HER-2 receptor the colour reaction showed membrane localisation (Fig. 5). In this case the intensity of the reaction also varied between individual cases (Table 1).

Comparison of the expression intensity of the markers studied between the individual grades of differentiation demonstrated the following significant differences:

— the intensity of PgR expression proved to be the highest in the G1 group and the lowest in the G3 group of cases (Table 1);
— Ki67 was detected in the lowest fraction of cells in the G1 group and the highest proportion of cells in the G3 group (Table 1).

In cases of ER, HER-2 and p53 no significant differences were disclosed between groups G1, G2 and G3 (Table 1).

At the second stage of statistical analysis the relationships of all the variables were examined in pairs in patients of all 3 groups taken together (G1, G2 and G3) and in each group individually (G1, G2 or G3).

In the entire material (groups G1 to G3) significant correlations were detected between:

— ER and PgR (positive correlation),
— p53 and Ki67 (positive correlation),
— ER and p53 (negative correlation),
— PgR and Ki67 (negative correlation),
— PgR and p53 (negative correlation).

In the G1 group significant correlations were detected between:

— ER and PgR (positive correlation),
— PgR and p53 (negative correlation).

In the G2 group significant correlations were detected between:

— HER-2 and p53 (positive correlation),
— PgR and Ki67 (negative correlation).

In the G3 group only a negative correlation was observed between PgR and p53.

In the remaining pairs of variables no correlations were detected. The results of the analysis are presented in Table 2.

DISCUSSION

The aim of the study was to analyse the manifestation of receptors for female sex steroids (ER and

Table 2. Correlations between the intensity of expression of the antigens studied in the group as a whole (G1–3) and in the G1, G2 an d G3 groups (Gamma correlation)

Pair of variables studied	Grade	Gamma	p
ER and PgR	G1–3	0.35	**0.005**
	G1	0.93	**0.003**
	G2	0.23	0.33
	G3	0.29	0.20
ER and HER-2	G1–3	–0.20	0.28
	G1	–0.21	0.28
	G2	–0.24	0.25
	G3	0.22	0.34
ER and p53	G1–3	–0.28	**0.02**
	G1	–0.19	0.56
	G2	–0.24	0.57
	G3	–0.16	0.44
ER and Ki67	G1–3	–0.38	0.29
	G1	–0.21	0.34
	G2	–0.14	0.57
	G3	0.26	0.19
PgR and HER-2	G1–3	–0.22	0.94
	G1	–0.24	0.32
	G2	–0.08	0.64
	G3	0.05	0.85
PgR and p53	G1–3	–0.31	**0.006**
	G1	–0.66	**0.04**
	G2	–0.06	0.75
	G3	–0.45	**0.04**
PgR and Ki67	G1–3	–0.31	**0.01**
	G1	–0.02	0.93
	G2	–0.41	**0.03**
	G3	0.26	0.23
HER-2 and p53	G1–3	0.31	0.14
	G1	0.09	0.65
	G2	0.38	**0.05**
	G3	0.12	0.55
HER-2 and Ki67	G1–3	0.21	0.28
	G1	0.11	0.60
	G2	0.02	0.91
	G3	–0.24	0.22
p53 and Ki67	G1–3	0.29	**0.01**
	G1	0.33	0.40
	G2	0.23	0.50
	G3	0.00	1.00

PgR), HER-2, p53 and of proliferation-related antigen Ki67, in relation to grade in primary invasive ductal breast cancers in women. The parameters studied represented the first or second category of prognostic factors in breast cancer [4].

In the case of ER no significant differences were detected in the intensity of expression of the receptor between groups G1, G2 and G3. The result did not confirm the observations of other authors, who found a lower intensity of ER expression in less differentiated cases [1]. The absence of differences in ER expression between groups in our material does not necessarily indicate that the less differentiated cases, associated with less favourable prognosis, are equally sensitive to the action of oestrogens or anti-oestrogen drugs. The possibility exists that in less differentiated cases ER may fail in its function as a transcription factor. This might reflect a defect or the prevailing effect on the cells of signals from receptors of the HER group.

When differences in PgR expression were examined between groups G1, G2 and G3, the first group demonstrated the highest and the last group the lowest intensity of expression of the receptor. This observation confirmed the hypothesis advanced in the discussion of differences in ER expression between individual groups. In the G3 group, PgR was noted in the lowest percentage of cells. PgR is known to represent an oestrogen-dependent protein, appearing in the cell as a result of interaction between the oestrogen-ER complex and DNA [17]. The less pronounced expression of PgR in the G3 group demonstrated that the presence of ER in cancer cells is not necessarily linked to the preserved activity of the receptor. Moreover, this work supplies evidence that a more complete evaluation of the sensitivity of breast cancer cells to the action of oestrogen and thus their potential sensitivity to anti-oestrogen treatment requires an evaluation of both receptors for female sex steroids.

No differences were detected between individual groups in the expression of HER-2 and p53.

In the analysis of Ki67 antigen expression, its intensity proved to be lowest in cells of cancers of the G1 grade and highest in those of the G3 grade. The result is consistent with reports of other investigators [16] and confirms the high value of Ki67 as an exponent of proliferation.

Analysis of relations between variables within the entire material (Groups 1 to 3) demonstrated a positive correlation between ER and PgR and a negative one between PgR and Ki67. The former correlation corroborates the corresponding data given in the literature [6, 7] and may confirm the oestrogen--dependent character of PgR. It is surprising that a negative correlation was detected between the intensity of expression of PgR and Ki67 but no reverse correlation could have been detected between ER expression and proliferation intensity measured by Ki67 expression. This seems to confirm the view cited above that ER expression does not necessarily indicate a preserved function of the receptor and that

PgR may represent an index of the preserved activity of ER. At this point the problem should be posed in relation to the ER(–) and PgR(+) cases. Such cases have been analysed in the past. In 1996, Keshgegian and Cnaan [8] described studies performed on 300 breast cancer patients, among whom the ER(–) and PgR(+) cases proved to carry the worst prognosis. Thus it seems that PgR represents a favourable prognostic and predictive factor only when it is co-expressed with ER.

In groups G1–3 we also demonstrated a positive correlation between p53 and Ki67 as well as negative correlations between p53 on the one hand and ER and PgR on the other. The information indicates that cases with P53 mutation exhibit a higher degree of proliferation intensity and a lower degree of sensitivity to oestrogens and, linked to this, a lower degree of sensitivity to tamoxifen treatment [15].

Studies on the relationships between variables in the G1 group detected a positive correlation between ER and PgR and a negative correlation between PgR and p53. In the G2 group a positive correlation was detected between HER-2 and p53 and a negative correlation between PgR and Ki67. In the G3 group we detected a negative correlation between PgR and p53. The results corroborate the data obtained for the entire material (groups G1 to G3). PgR expression has been found to be negatively related to proliferation intensity and has demonstrated a relation to ER. The negative correlation between PgR and p53 expression, detected in all groups except G2, deserves attention. The absence of a relationship between the expression of PgR and p53 in the G2 group may have reflected a decrease in PgR expression and a parallel increase in p53 expression in the course of transition of the tumour from G1 to G3. The intensity of HER-2 expression was higher in cases with mutation of the p53 gene.

The studies performed indicate that the value of individual markers and relations between individual variables vary depending upon the grade of differentiation of cancerous cells. The conclusion can be drawn that the value and function of the individual proteins tested can be recognised only after examining them in various groups of tumours. As a starting point, the parameters analysed should include the grade of tumour differentiation, tumour size, the presence of metastases and histological type. If in our studies expression of ER, PgR, HER-2, p53 and Ki67 had been examined in only one of the groups, the results obtained would have been different. It is worth noting that we have disclosed most of the relations in the entire group studied (consisting of an identical number of cases of each of the individual grades of differentiation) and the least frequent relations have been observed in the G3 group with cases of low differentiation. The extensive differences between the results of various authors probably reflect groups for study that were too narrow or inappropriately selected.

The present study indicates that the value of ER as an independent prognostic and predictive factor can depend upon the grade of tumour differentiation. The study also indicates that a parallel evaluation of ER, PgR and p53 expression may prove to be of high predictive value in prognosis concerning response to tamoxifen treatment.

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