Article is available online at http://www.webio.hu/por/2004/10/4/0207

ARTICLE

Steroid Receptor Status, Proliferation and Metallothionein Expression in Primary Invasive Ductal Breast Cancers

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The most important immunocytochemical prognostic and predictive factors in cases of breast cancer include estrogen receptor alpha (ER) and progesterone receptor (PgR). The present study aimed at examining the relationship between the manifestation intensity of proliferation markers (Ki-67 and nucleolar organizer regions - AgNORs) on one hand, and expression of ER and PgR on the other in a uniform group of invasive ductal breast cancers of G2 grade. Moreover, the study aimed at examining the relationship between the above mentioned markers and expression of metallothionein (MT). The studies were performed on samples of invasive ductal breast cancers of G2 grade, originating from 60 females. In paraffin sections originating from the studied cases immunocytochemical reactions were performed using monoclonal antibodies to ER, PgR, Ki-67 and MT, and silver staining was conducted to localize AgNORs. The obtained results were subjected to statistical analysis using Statistica software. Results indicate that manifestation of AgNORs does not correlate with any of the studied antigens (ER, PgR, Ki-67, MT) (p>0.05). Moreover, no relationship could be demonstrated between the intensity of MT expression and proliferation markers or steroid receptor status (p>0.05). A negative correlation was shown between the expression of ER and Ki-67 (p=0.0009). The most intense proliferative activity was demonstrated in cases of breast cancer showing PgR expression but no ER expression (p=0.015), while the lowest proliferative activity was detected in breast cancers with expression of both ER and PgR (p<0.05). (Pathology Oncology Research Vol 10, No 4, 207–211)

Keywords: Breast cancer, hormone receptor status, metallothionein, Ki-67

Introduction

According to the College of American Pathologists, expression of estrogen receptor alpha (ER) and progesterone receptor (PgR) belongs to the first category of prognostic factors. The category includes only factors the value of which has been verified and which are used in daily clinical practice. The group includes in addition the size of primary tumor, axillary lymph nodes metastases, histological type and grade.[4] Expression of ER and PgR points to lower grade and, therefore, to lower proliferative activity and to potential for tumor growth control by sex steroids. A reciprocal correlation has also been demonstrated between ER expression and proliferation intensity, measured by the expression of Ki-67 protein.[2] A significance of PgR expression has been less recognized. The receptor is known to represent an estrogen-dependent protein, i.e. it is synthesized following stimulation of target cells with estrogens.[18,19] Breast cancer cases that exhibit expression of both receptors for female sex steroids carry the best prognosis. Also, ER(–) and PgR(+) cases are associated with the worst prognosis.[13]

Some indicators of cell proliferative activity (Ki-67, PCNA) have been included in the second category of prognostic factors in cases of breast cancer. The prognos-

Received: Oct 5, 2004; *accepted:* Nov 26, 2004

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Abbreviations: ER: estrogen receptor alpha; PgR: progesterone receptor; AgNOR: argyrophilic nucleolar organizer region; MT: metallothionein

tic significance of these factors has not been fully clarified, but is widely used to monitor the fraction of proliferating cells. Ki-67 antigen has been found to be the most significant marker of proliferation, detected using monoclonal antibody produced by the MIB-1 clone.[4]

Much less clear seems the status of nucleolar organizers. They represent DNA segments which carry genes responsible for synthesis of rRNA. Transcriptionally active NORs are associated with specific non-histone acidic and argentophilic proteins.[14] Therefore, impregnation with silver permits to localize transcriptionally active NORs. Positive correlation has been described between the number of AgNORs and cell proliferative activity.[17]

The group of less recognized factors includes metallothioneins (MT), the group of low molecular weight proteins (6-7 kDa), containing 61 to 62 amino acids each. These proteins are characterized by high content of cystein residues (23 to 33%) and low content of aromatic and hydrophobic amino acids. Due to their unique structure, MT play a number of important functions in the cell. They are important for heavy metal homeostasis, participate in processes of cell growth and differentiation and are involved in multidrug resistance to cytostatic drugs.[12] Cells of ductal mammary carcinoma may express two isoforms of metallothionein, MT-1 and MT-2.[16] Jin et al. (2001) have demonstrated that MT-1F expression positively correlates with grade in infiltrating breast cancers.[11] Other studies have described correlation between MT expression and proliferative activity of tumor cells.[7] In 1998, Friedline et al. found that in some cell lines MT-1E gene might be down-regulated by ER.[5] In 2001, Harris et al. noted the estradiol-induced up-regulation of MT expression in cells which carried estrogen receptor beta.[6]

The present study aimed at examining the relationship between ER and PgR expression, and proliferation measured by detecting Ki-67 and AgNORs, in a uniform group of invasive ductal breast carcinomas of G2 grade. Moreover, the study aimed at examining relationship between steroid receptors and proliferation on one hand and, on the other hand, intensity of MT expression.

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 (G2), 60 patients with primary invasive breast cancer, diagnosed in the years 1999 to 2000 in the Lower Silesia Centre of Oncology (Wroclaw, Poland) were defined. Mean age of the patients was 53.94±8.16 years (range: 41 to 69 years).

From the studied tumors samples were taken, fixed in 10% buffered formalin, and embedded in paraffin. In all cases hematoxylin and eosin stained sections were prepared, which were histopathologically evaluated by two pathologists. Tumors were graded according to the Bloom-Richardson grading modified by Elston and Ellis.

Table 1. **Evaluation of the intensity of immunocytochemical reactions using ImmunoReactive Score (IRS) according to Remmele.[15] The final result represents the product of scores given for individual traits.**

Percentage of positive cells	*Score*	*Intensity of reaction*	*Score*
None	0	No reaction	0
<10%	1	Weak color reaction	1
10-50%	2	Moderate intensity	2
51-80%	3	Intense reaction	3
>80%	4		

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 hydrated. Immunohistochemical reactions were performed using the following mouse monoclonal antibodies applied for 1 h at room temperature (all from DakoCytomation, Denmark): clone 1D5 to ER (optimally prediluted), clone 1A6 to PgR (optimally prediluted), clone MIB-1 to Ki-67 (1:100), clone E9 to MT (1:100).

Each of the reactions was accompanied by a negative control using primary negative control (DakoCytomation). The antigens were visualized using biotinylated antibodies, streptavidin-peroxidase complex (LSAB2, DakoCytomation) and 3,3'-diaminobenzidine (DAB) (DakoCytomation). Detection of ER, PgR and Ki-67 expression was preceded by 15-min exposure of the sections to boiling in antigen retrieval solution (DakoCytomation) in a microwave oven.

Material of the same cases was also subjected to reactions of impregnation with silver in order to demonstrate location of nucleolar organizer regions (NORs). For this purpose AgNOR kit (Bio-Optica, Italy) was used.

In the case of ER, PgR and MT, intensity of the immunohistochemical reactions was evaluated using the semi-quantitative scale of the ImmunoReactive Score (IRS),[15] which took into account the intensity of the color reaction as well as the proportion of positive cells (*Table 1*). Intensity of Ki-67 expression was expressed by the proportion of cells showing positive reaction.

Manifestation of AgNORs was evaluated calculating an average number of silver grains per cell nucleus. In each preparation, the mean was calculated for 48 cell nuclei (the number of AgNOR grains was scored in four microscopic fields, in 12 cell nuclei each).

The obtained results were subjected to statistical analysis using the Statistica 98 PL software (Statsoft, Poland).

Results

In the case of ER, a color reaction of nuclear localization was obtained, which was of various intensity in individual cases (*Figure 1*). Mean intensity of the reaction was 3.05±3.03 (SD). In 17 cases no ER was detected (IRS=0). The highest intensity of ER expression (IRS=12) was noted in two cases.

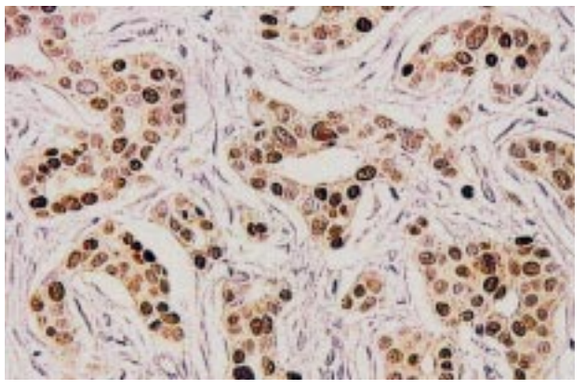

Figure 1. *Immunocytochemical localization of estrogen receptor in cells of ductal breast cancer of G2 grade (x200)*

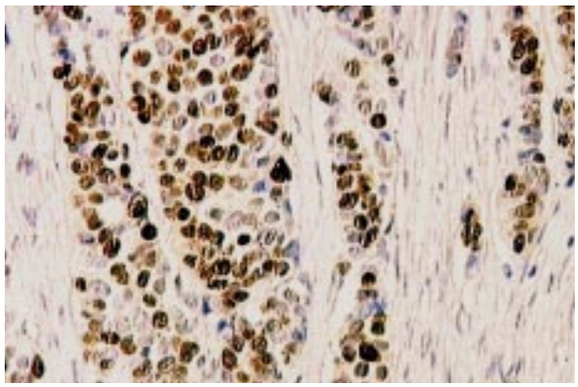

Figure 2. *Immunocytochemical localization of progesterone receptor in cells of ductal breast cancer of G2 grade (x200)*

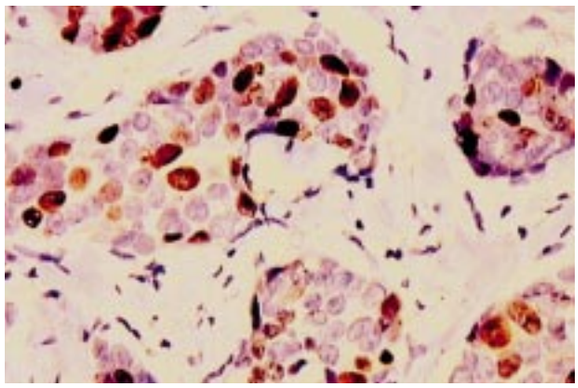

Figure 3. *Immunocytochemical localization of Ki-67 antigen in cells of ductal breast cancer of G2 grade (x200)*

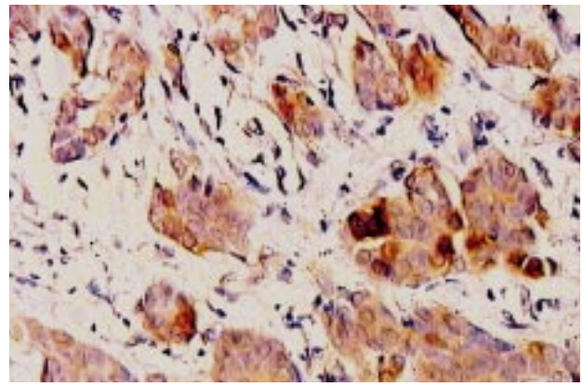

Figure 4. *Immunocytochemical localization of metallothionein in cells of ductal breast cancer of G2 grade (x200)*

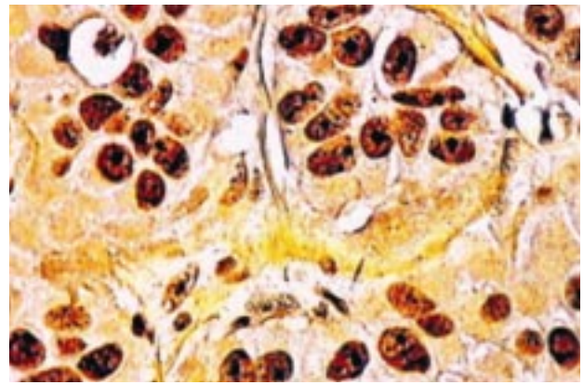

Figure 5. *Silver impregnation for localization of AgNORs in cells of ductal breast cancer of G2 grade (x400)*

PgR labeling also demonstrated nuclear localization of the reaction. Its intensity differed between individual cases (*Figure 2*). In 25 cases no PgR presence could be demonstrated (IRS=0). The highest intensity of the reaction (IRS=12) was noted in 6 cases. Mean intensity of the reaction on the IRS scale was 3.35±4.11.

Reactions using antibody against Ki-67 also exhibited nuclear localization of various intensity in individual cases (*Figure 3*). In one case no Ki-67 could be demonstrated, in two cases the antigen was present in 90% of cell nuclei, while on the average it was present in 32.3%±26.78% of cells.

In the case of metallothionein the color reaction showed both nuclear and cytoplasmic localization of various intensity in individual cases (*Figure 4*). On the IRS scale its mean intensity was 5.1±3.52, and the highest IRS value of 12 was detected in 5 cases. In 7 cases no MT could be disclosed in the cells.

Table 2. **Relationship within pairs of individual variables (ER, PgR, Ki-67, MT, AgNORs) analyzed using Spearman's rank correlation.**

Examined pair of variables	*r*	*p*
ER and PgR	0.076599	0.560762
ER and Ki-67	–0.417084	0.000916
ER and AgNORs	–0.029568	0.822551
ER and MT	0.067514	0.608269
PgR and Ki-67	–0.018953	0.885713
PgR and AgNORs	0.044514	0.735574
PgR and MT	0.100393	0.445335
Ki-67 and AgNORs	0.111585	0.395989
Ki-67 and MT	0.103659	0.430596
AgNORs and MT	0.156368	0.232832

Table 3. **Comparison of PgR, Ki-67, AgNOR and MT manifestation between ER(–) (IRS 0 to 2) and ER(+) (IRS 3 to 12) groups performed using Mann-Whitney U-test; p = probability, n = number of cases.**

Compared marker	*Sum of ranks – ER(–)*	*Sum of ranks – ER(+)*	*p*
PGR	780.5	1049.5	0.51
Ki-67	1033	797	0.001
AgNORs	858.5	971.5	0.60
MT	818	1012	0.93

Silver impregnation of nucleolar organizer regions yielded a granular, dark-brown reaction in cell nuclei of studied tumors (*Figure 5*). The number of AgNOR grains was different in individual cases. On the average 4.19±1.39 AgNOR grains were noted per cell nucleus. The lowest observed mean number of AgNOR grains per cell nucleus was 1.79, while the highest mean number was 7.23.

The performed statistical analyses yielded the following relationships:

• Relations in the group of all variables (i.e. relations between the presence of individual markers) studied using Spearman's rank correlation were weak and far from statistical significance, except the correlation between expression of Ki-67 and ER, which was highly significant ($p<0.001$) (*Table 2*).

• At the next stage intensity of manifestation of individual markers was compared in two groups of patients: ER(–) (IRS 0 to 2), and ER(+) (IRS=3 to 12). The comparison was conducted using Mann-Whitney U-test. In general, weak relations were disclosed between ER expression and the remaining variables. A significant difference was demonstrated only in the case of Ki-67, i.e. higher expression in the ER(–) than in the ER(+) group (*Table 3*).

• At the last stage of statistical analysis differences in Ki-67, AgNOR and MT manifestation were examined in groups of patients with tumors of distinct expression of ER and PgR: ER(+) / PgR(+), ER(+) / PgR(–), ER(–) / PgR(+), and ER(–) / PgR(–). The comparisons were performed using Mann-Whitney U-test, and demonstrated differences only in the case of Ki-67. It was significantly higher in the ER(–)/PgR(–) group as compared to the ER(+)/PgR(+) one ($p=0.03$). On the other hand, Ki-67 expression was significantly higher in the ER(–)/PgR(+) group as compared to the ER(+)/PgR(–) ($p=0.01$) or the ER (+)/PgR(+) one ($p=0.003$) (*Table 4*).

Discussion

These studies have shown that although the examined cases represented the same grade (G2), all the other examined variables manifested various intensity in individual cases.

Our calculations have demonstrated no relationship between the manifestation of AgNORs and that of Ki-67. Each of the markers is regarded to represent an index of cell proliferative activity. Such a lack of relationship between Ki-67 and AgNORs has been reported in the past. In studies on samples of breast cancer no relation could be demonstrated between the presence of Ki-67, estimated using antibody of the MIB-1 clone, and AgNORs.[1] Increase in the number of AgNORs in cells may indicate not only their proliferative activity but also their augmented translation activity (e.g., due to stimulated secretory activity).

In none of the performed statistical analyses could a significant relationship be disclosed between the expression of metallothionein and the other studied markers. In multiple investigations augmented expression of MT has been linked to higher proliferative potential of tumor cells,[8,10] less favorable clinical course[20] and lower expression of ER.[9] Our studies have failed to confirm the relationship between MT and ER or Ki-67. The absence of statistically significant relationships between MT expression and the remaining studied variables might have resulted from selection of a uniform group of patients in respect to their grade (G2). Jin et al. have reported that MT manifestation in breast cancer cells is positively correlated with grade.[11] This might also explain why a relationship has been described between metallothionein expression and proliferative activity of breast cancer cells. The higher is the grade, the higher is proliferative activity of the tumor cells. Thus, selection of patients with G2 tumors might have obliterated the reported relationship between metallothionein expression and proliferative activity of breast cancer cells.

PgR is known to represent an estrogen-dependent protein.[3] Our studies have not detected relationships between intensities of expression of PgR and ER. Obviously, the result does not suggest that no relations exist between ER function and synthesis of PgR. The current receptor status of a cell reflects not only the potential capacity to synthesize PgR but also the presence of the appropriate ligands. The synthesis of

Table 4. **Statistical significance level (p) of differences in Ki-67 expression in ER(–) / PgR(–), ER(–) / PgR(+), ER(+) / PgR(–) and ER(+) / PgR(+) groups, performed using Mann-Whitney U-test.**

	ER(–) / PgR(–)	*ER(–) / PgR(+)*	*ER(+) / PgR(–)*	*ER(+) / PgR(+)*
ER(–) / PgR(–)	X	0.35	0.10	0.03
ER(–) / PgR(+)		X	0.01	0.003
ER(+) / PgR(–)			X	0.31

the proteins is controlled by feedback mechanisms and depends also on sex steroid levels in the patient's serum.

Analysis of the relationships between all the studied variables using Spearman's rank correlation has shown a statistically significant, negative correlation only between the expression of ER and Ki-67. The negative correlation between ER and Ki-67 is consistent with the literature data.[9]

Analysis of the expression of the studied markers in ER(+) and ER(–) groups has demonstrated a relationship only between ER and Ki-67. Manifestation of Ki-67 in ER(–) patients was significantly higher as compared to that of ER(+) patients. The result verifies the clinically applied IRS scale for evaluation of ER expression. ER(+) cases have been found to exhibit a lower proliferative activity.

At the final stage of the study we have considered the complete receptor status of the cells as a starting point. This reflected the routine diagnostics in which expression not only of ER but also of PgR is estimated. Again, significant differences have been noted only in the case of Ki-67. The calculations have demonstrated that expression of Ki-67 was significantly higher in the ER(–)/PgR(–) group as compared to the ER(+)/PgR(+) one, as well as in the ER(–)/PgR(+) group as compared to the ER(+)/PgR(–) or the ER(+)/PgR(+) group.

The performed studies indicate that in every case both ER expression and PgR expression should be examined in parallel. We have demonstrated that ER(+) and PgR(+) breast cancer cells exhibit the lowest, while ER(–) and PgR(+) cells demonstrate the highest proliferative activity.

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