Immunohistochemical evaluation of human epidermal growth factor receptor 2 and estrogen and progesterone receptors in invasive breast cancer in women

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Abstract

Introduction: Estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER-2) expression are crucial in the biology of breast carcinoma. *HER-2/neu* gene is amplified and overexpressed in 15-30% of invasive breast cancers. HER-2-positive breast cancers have worse prognosis than HER-2 negative tumors and possess distinctive clinical features. The aim of this study was to assess the expression of HER2 in cancer tissue of patients with invasive breast cancer in correlation with tumor type, histological grade, tumor size, lymph node status, and expression of estrogen receptor and progesterone receptor.

Material and methods: Formalin-fixed, paraffin-embedded tissues from 40 patients with invasive HER-2-positive breast cancer and from 191 patients with HER-2-negative breast cancer were used in this study. HER2 expression was determined using the test HerceptTest™ DAKO.

Results: Among 231 cases of breast cancer, 18 invasive lobular carcinomas and 213 invasive ductal carcinomas were diagnosed. Sixty percent of HER-2-positive breast cancers were ER-positive compared with 77% in the HER-2-negative group ($p = 0.002$). The expression of PR was observed in 43% of HER-2-positive breast cancers and in 72% of HER2-negative tumors ($p = 0.003$). Excessive expression of HER2 protein was detected in 60% of patients positive for estrogen receptors, which may worsen prognosis in these patients.

Conclusions: Determination of HER2 overexpression in breast cancer patients, allows for a determination of a group of patients with a worse prognosis.

Key words: invasive ductal carcinoma, invasive lobular carcinoma, human epidermal growth factor receptor 2, estrogen receptor, progesterone receptor.

Introduction

Breast cancers are characterized by high production of growth factors and their receptors. The gene for type 2 receptor for human epidermal growth factor (HER2, HER2/neu, c-erbB-2) is a proto-oncogene located on chromosome 17q21 [1]. It encodes a transmembrane glycoprotein, a receptor, called HER2. HER2 overexpression plays a significant role in the process of malignant transformation and growth of many cancers, especially breast cancer, in which it occurs in 15-30% [2] of cases. Determination of HER2 overexpression in breast cancer patients indicates a more aggressive form of the disease [2]. This is also associated with resistance to hormonal

therapy despite the presence of receptors for estrogen and progesterone [3]. Patients with tumors exhibiting HER2 overexpression live shorter and distant metastases occur earlier [2-5]. Several reports indicate that overexpression of HER2 in breast cancer is an indicator of more aggressive disease. Changes in HER2 coexist with other prognostic factors such as histological grade, histological type, the absence of estrogen receptors (ER) and progesterone receptors (PR), and lymph node involvement [6]. It is also suggested that HER2 status in breast cancer may be useful in selecting an appropriate treatment regimen.

The aim of this study was to assess the expression of HER2 in cancer tissues of patients with invasive breast cancer in correlation with tumor type, histological grade of malignancy, tumor size, the status of lymph nodes, the expression of estrogen receptor (ER) and progesterone receptor (PR).

Material and methods

Material consisted of histological preparations derived from patients treated for invasive breast cancer. Histological and immunohistochemical studies were performed in the Department of Pathology, Military Medical Institute in Warsaw. Samples of tumors were fixed in 10% buffered formalin phosphate. Paraffin blocks were cut into sections with a thickness of 4 μm. The resulting sections were stained with different methods for diagnostic purposes. Preparations stained with H&E were used to identify tumor type (WHO classification) and histological grade of malignancy.

Immunohistochemistry was performed using EnVision™ + HRP DakoCytomation (EnVision™ Dual Link System-HRP, DAB+, Code: K4065). In order to determine the expression of steroid receptors, monoclonal antibodies against receptors for estrogen (Monoclonal Mouse Anti-Human Estrogen Receptor alpha, 1 : 50 dilution, Clone: 1D5, Code: IR654, DAKO) and progesterone (Monoclonal Mouse Anti-Human Progesterone Receptor, 1 : 400 dilution, Clone: PgR636, Code: IR068, DAKO) were used. The study was conducted as follows: Sections were incubated in an incubator at 60°C overnight, then preparations were dewaxed. The next step was revealing the epitope by heating slides in buffer for 40 min. Subsequently, preparations were left at room temperature for 20 min. Preparations were rinsed in buffer and then endogenous peroxidase was blocked by washing in 3% H_2O_2 for 10 min. In the next stage, the preparations were incubated with an appropriate antibody for 30 min. After incubation, preparations were rinsed in buffer for 10 minutes, and then incubated with the reagent (Visualization Reagent) for 30 min. After incubation with the reagent, preparations were washed in TBS (Tris-Buffered Saline, Code: S1968) pH 7.6 for 10 min, and

then incubated with 3,3'-diaminobenzidine (DAB) (Substrate – Chromogen Solution) for 10 min to visualize the color of the reaction. At the end of the procedure, preparations were stained with hematoxylin. Nuclear staining in > 10% of tumor cells was considered positive for ER and PgR.

HER2 expression was determined using HerceptTest™ DAKO test (Code: K5204). It enabled the detection of HER2 expression using a polyclonal antibody against this protein (Rb A-Hu HER2 – Rabbit Anti-Human HER2 Protein). Antigen retrieval for HER2 using HerceptTest was performed by immersing and incubating the slides in 10 mmol/l citrate buffer in a calibrated water bath (95-99°C) for 40 ±1 min. After decanting the epitope-retrieval solution, the sections were rinsed in the wash buffer and later soaked in the buffer 5 to 20 min before staining. The slides were loaded onto the autostainer using the HercepTest program as described in the manufacturer's insert. In the autostainer, the slides were rinsed, placed in 200 μl peroxidase-blocking reagent for 5 min, rinsed, placed in 200 μl primary anti-HER2 protein (or negative control reagent) for 30 min, rinsed twice and finally immersed in 200 μl substrate chromogen solution (DAB) for 10 min. The slides were counterstained with hematoxylin and finally were coverslipped. HER2 results were determined based on the maximum area of staining intensity according to the package insert and the ASCO/CAP guidelines as follows. Strong, circumferential membranous staining in > 30% of invasive carcinoma cells was scored as 3+; moderate, circumferential membranous staining in ≥ 10% of invasive tumor cells or 3+ staining in ≤ 30% of cells was scored as 2+; weak and incomplete membranous staining in invasive tumor cells was scored as 1+; and no staining was scored as 0. Tumors with 0 and 1+ staining were considered negative (Figure 1), cases scored as 2+ were considered equivocal, and cases with 3+ staining were considered positive (Table I). As a reference for showing overexpression
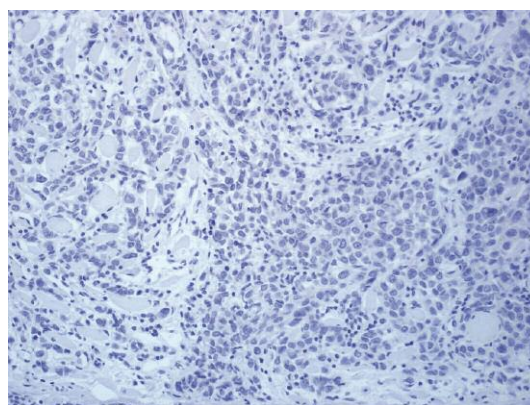
Figure 1. Example of immunohistochemical HER2 staining of invasive ductal carcinoma (IDC), scored 0 (stained cells were less than 10% of total tumor cells). The micrograph was taken with objective 20×

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Table I. Scoring system for HER2

HER2 IHC Scoring	
Score	ASCO/CAP Scoring Interpretation/Staining pattern
0	Negative/no staining Staining in < 10% of tumor cells
1+	Negative/faint/barely perceptible incomplete membrane staining in > 10% of tumor cells
2+	Equivocal/weak to moderate complete membrane staining in > 10% of tumor cells
3+	Positive/strong complete membrane staining in > 30% of tumor cells
HER2 FISH Scoring	ASCO/CAP Scoring Interpretation/ratio (HER2/CEP17)
Equivocal/1.8-2.2	Negative/< 1.8 Positive/> 2.2

All abbreviations are defined in the text of the manuscript

of HER2 we used cancer tissue in which the staining reaction was assessed as 3+. Results identified as HER2 2+ were verified by fluorescence in situ hybridization (FISH). Positive and negative control preparations were previously determined.

Statistical analysis

All statistical analyses were performed with SPSS software version 12.0 for Winows. The frequency of HER2 expression according to joint ER/PR status and the distribution of the hormone receptor status (ER, PR, and joint ER/PR) according to HER2 were also calculated. To assess the relationship between HER2 expression and expression of steroid receptors, histological type of tumor, its degree of histological malignancy and clinical stage of tumor, Mantel-Haenszel test was used (StatsDirect package). Differences were considered statistically significant at $p \leq 0.05$.

Results

Pathological examination was performed in tumors obtained from 231 patients suffering from invasive breast cancer. Ages of patients ranged from 34 to 86 years; mean age was 52 years. Patients were divided into two age groups: < 50 years and $\geq$ 50 years old.

Among 231 cases of breast cancer, 18 invasive lobular carcinomas (ILC) and 213 invasive ductal carcinomas (IDC) were diagnosed. Given the histological grade of malignancy, the largest group of invasive carcinomas comprised second grade (G2) tumors, and the least numerous group comprised first grade (G1) tumors. Among invasive ductal carcinomas (IDC), most of the diagnosed cancers were in the second grade (G2) (65%) (Table II).

During analysis of the pre-operative staging of studied cancers, it was found that the largest group among the IDC tumors comprised those assessed as T1c (38%) and T2 (46%) stages (T1c – larger than 1 cm, up to 2 cm in diameter; T2 – tumor larger than 2 cm but not larger than 5 cm in diameter). The ILC constituted the largest group of tumors staged at T2 (38%). Statistically significant differences were found for cancers at stage T4 (p = 0.0225) (Table II).

Lymph node status was also assessed during the study. It was noted that in all investigated invasive carcinomas (IDC, ILC) women with invasive breast cancer without metastases to regional lymph nodes constituted the largest group (pN0) (59.3%) (Table II).

Table II. Clinicopathological characteristics of studied groups

	Type of tumor		$P \leq 0.05$
	IDC [%]	ILC [%]	
Tumor grade (G1-3)			
G1	5	0	0.7363
G2	65	89	0.1747
G3	30	11	0.151
Tumor size			
T1	1	0	0.3619
T1a	3	0	0.9481
T1b	8	6	0.8691
T1c	38	28	0.4087
T2	46	38	0.5349
T3	1	11	0.0611
T4	3	17	0.0225
Lymph node			
pNx	4	0	0.8685
pN0	59	60	0.8711
pN1	23	17	0.7131
pN2	8	6	0.9289
pN3	6	17	0.1848

All abbreviations are defined in the text of the manuscript
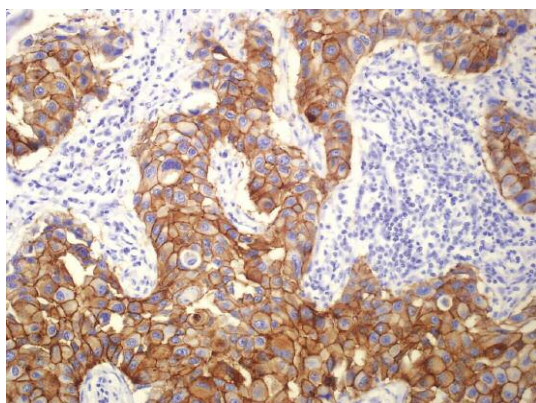
Figure 2. Strong, circumferential staining in > 30% of invasive ductal carcinoma (IDC) cells was scored as 3+; interpretation – positive for HER2 amplification (original magnification, 20×)

Table III. The relationship between histological type of tumor and expression of ER, PR, HER2

Variable	IDC (n = 213)		ILC (n = 18)		$P \leq 0.05$
	n	%	n	%	
ER+	158	74	14	78	0.7367
ER–	55	26	4	22	
PR+	143	67	11	61	0.6026
PR–	70	33	7	39	
HER2+	40	19	0	0	0.0896
HER2–	173	81	18	100	

All abbreviations are defined in the text of the manuscript

Table IV. Correlation between HER2 expression and hormonal receptor status

Variable	HER2(–) (n = 191)		HER2(+) (n = 40)		$P \leq 0.05$
	n	%	n	%	
ER+; n = 172	148	77	24	60	0.0211
ER–; n = 59	43	23	16	40	
PR+; n = 155	138	72	17	43	0.003
PR–; n = 76	53	28	23	57	
ER+/PR+; n = 147	130	69	17	42	0.0022
ER+/PR–; n = 25	18	9	7	18	0.1349
ER–/PR+; n = 8	8	4	0	0	0.3999
ER–/PR–; n = 51	35	18	16	40	0.0027

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In women under 50 years of age, expression of HER2 was found in 17% of cases, while in 83% HER2 expression was not detected. In patients over 50 years of age, the expression of HER2 was demonstrated in 14% of cases and the lack of expression of HER2 was found in 86%. Taking into account the histological type of tumor, HER2-positive reaction was observed in 19% of IDC (Figure 2) and there was no expression of HER2 in ILC (p = 0.0896) (Table III). Considering the grade of histological malignancy, HER2 expression in the largest percentage (9.1%) was observed in second grade invasive carcinomas (G2). There was no expression of HER2 in first grade (G1) cancers. There was no relationship between histological type of tumor, histological grade of malignancy and expression of HER2.

In the group of 137 patients with breast cancer without lymph node metastases (pN0), HER2 overexpression was found in 8.2% of cases. There was no significant association between the status of the lymph nodes, and expression of the receptor. Analysis of the pre-operative staging of cancers and of HER2 expression showed that the largest group to demonstrate the expression of this receptor comprised cancers staged at T1c and T2. Expression of HER2 was found in 7.4% of tumors staged at T1c and 6.5% of cases at T2 stage. No relationship was found between the overproduction of HER2 and tumor size.

During the study, HER2 overexpression among patients with positive estrogen receptors was found in 60% of cases, and 43% of PR-positive patients. In ER-negative patients, HER2 overexpression was found in 40% of cases, and in PR-negative patients HER2 overexpression was demonstrated in 57% of cases (Table IV). Among the patients expressing both estrogen and progesterone receptors (ER+/PR+), tumors that overexpress HER2 accounted for 42% of cases (p = 0.0022) (Table IV). In the group of ER+/PR- patients HER2 overexpression was found

in 18% (p = 0.3999). In the ER-/PR- group, HER2 overexpression was observed in 40% of patients (p = 0.0027) (Table IV). In the ER-/PR+ group there was no overexpression of HER2. Based on the data analysis, there is a statistically significant correlation between the presence or absence of ER expression and HER2 (p = 0.0211). Results concerning the relationship between PR and HER2 expression demonstrated a statistically significant relationship (p = 0.003). In patients with ER+/PR+ hormonal status, there was a statistical correlation between the expression of steroid receptors and HER2 expression (p = 0.0022). Similarly, a statistical correlation between the expression of ER, PR, and HER2 was found in patients with ER-/PR- hormonal status (p = 0.0027) (Table IV).

Outcome was also assessed for HER2 in G1, G2, and G3 tumors, depending on hormonal status. Among the G1 and G2 tumors with hormonal status ER+/PR+, expression of HER2 was found in 9 tumors. As for G3 tumors with the same hormonal status, HER2 expression was found in 8 tumors. There was no statistical correlation (Table V).

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Table V. Frequency of HER2 expression for tumor grade I-II and grade III lesions by joint ER/PR status

Variable	G I-II	G III	$P \leq 0.05$
	HER2-positive ($n = 23$)	HER2-positive ($n = 19$)	
ER–/PR+	0	0	–
ER+/PR–	6	1	0.1656
ER+/PR+	9	8	0.845
ER–/PR–	8	10	0.982

All abbreviations are defined in the text of the manuscript

Discussion

Numerous scientific studies have shown that the expression of HER2 in malignant tumors influences their biological behavior compared to tumors that do not show changes in the HER2 gene. From the literature it is known that breast, ovarian, lung, bladder and pancreatic cancers of HER2-positive phenotype are characterized by less favorable clinical course [7-9]. The largest pool of research on HER2 protein overexpression and its prognostic and predictive significance was performed on breast cancers. Based on the literature, it can be concluded that overexpression of HER2 can be regarded as an independent prognostic factor in advanced breast cancers. In the study, in a group of women aged 50 years, HER2 overexpression was found in 17% of cases, whereas in postmenopausal women, or women over 50, HER2 overexpression was detected in 14% of cases. It is known from the literature that in a group of premenopausal women with breast cancer, worse prognosis of the disease is associated with overexpression of HER2 because it points to a more aggressive form of cancer [4, 10]. Our results are in accordance with the data from the available literature, where HER2 overexpression was found in approximately 15-30% of cases of breast cancer [2, 10, 11]. In our study, among patients with invasive ductal form of cancer (IDC), HER2 overexpression was found in 19% of cases (Table III). This expression was not detected in any of the patients with lobular carcinoma. The absence of HER2 overexpression in a more promising less aggressive form of cancer could be considered an important fact, but the very small number of cases of lobular carcinoma does not allow us to draw such conclusions.

Detection of cancer cells in the presence of estrogen receptors allows for the use of estrogen receptor blocking preparations such as tamoxifen, which significantly improves the prognosis. Many researchers have confirmed that the presence of HER2 overexpression indicates a more aggressive form of the disease, which contributes to resistance to hormonal therapy [2, 10, 11]. During the study, HER2 overexpression was found in 60% of patients positive for estrogen receptors and 43% of PR-positive cases. Similar results were obtained in the study by Almasri and Hamad [12]. Several studies confirm a correlation between overexpression of HER2 protein, the presence of lymph node metastases and poor clinical prognosis [13]. There is a significant correlation between the degree of differentiation of breast cancer cells and HER2 overexpression [14-16]. In poorly differentiated carcinomas (Bloom III), HER2 protein overexpression is found more often than in other groups (Bloom I, II).

There are also works showing that in HER2-positive patients, survival and disease-free time were shorter than in patients lacking HER2 in the tumor regardless of the presence or absence of lymph node metastases [17]. In our study, there was no relationship between tumor size, lymph node status, and HER2 expression. Huang *et al.* presented similar results. Interesting, but still unanswered is the question concerning the relationship between overexpression of HER2 in breast cancer and the type of therapy [18]. Numerous studies have been conducted in women with breast cancer showing overexpression of HER2 in tumors, but no response to treatment with tamoxifen [12, 18, 19]. There are also scientific reports demonstrating a correlation between tamoxifen treatment and survival in patients diagnosed with breast cancer regardless of the presence of HER2 protein in the tumor [20].

Some authors have observed that overexpression of HER2 may be a potential marker which indicates sensitivity to treatment with anthracycline derivatives [21].

In conclusion, it is possible that the unfavorable course of disease in premenopausal women is associated with overexpression of HER2, which was found in 17% of patients in this group. There was no relationship between overproduction of HER2 and histological type of tumor and its size. Excessive expression of HER2 protein was detected in 60% of estrogen receptor-positive patients, which may worsen the prognosis in these patients. Determination of HER2 overexpression in breast cancer patients allows a group of patients with a worse prognosis to be identified.

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