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Estrogen (ER), progesterone (PR) and human epidermal growth factor HER-2 receptors expression status in breast cancers: study on the patients population at the Oncology Centre in Bydgoszcz

Status ekspresji receptorów estrogenowych (ER), progesteronowych (PR) i ludzkiego epidermalnego czynnika wzrostu HER-2 w rakach piersi: badania populacji pacjentek Centrum Onkologii w Bydgoszczy

Summary
In Poland, the number of newly diagnosed cases of breast cancers increases continuously and currently amounts to ca. 16000 cases per annum, with a reduced tendency in a number of deaths caused by the breast cancer observed for last 20 years. According to statistical data for 2010, the breast cancer represented 22.4% of all neoplasms in women and was first in terms of occurrence. Routine prognostic and predictive tests for breast cancer include estrogen (ER) and progesterone (PR) receptors and human epidermal growth factor receptor 2 (HER-2) expression in cancer cells. There is also a group of breast cancer patients in which no expression of any of these receptors is observed. They represent from 10 - 30% of all cases. These are called triple-negative cancers. A significant percentage of patients with triple-negative breast cancers have BRCA1 gene mutations. The triple-negative cancers are characterised by more aggressive clinical course, and quicker and more frequent recurrence. The aim of this study was to analyse estrogen, progesterone and HER-2 receptors expression in the population of patients treated at the Oncology Centre in Bydgoszcz. In the studied group the triple-negative (ER-/PR-/HER-2-) status was found in 68 patients (16.2% of the cases). Estrogen receptor expression was found in 286 cases (68.1% of the patients), progesterone receptor expression in 231 cases (55% of the patients), and HER-2 receptor expression in 91 cases (21.7%). Simultaneous estrogen and progesterone receptors expression was found in 222 (52.9%) patients. One of the breast cancer subtypes associated with the worst prognosis is the triple-negative subtype (ER(-), PR(-), HER-2(-)). Frequency of that subtype is high. The high ratio of these tumours in the population of patients with breast cancer requires a research aiming at finding new predictive and prognostic factors, to characterise their malignant potential and plan effective therapeutic scenarios. Subsequent studies will concern distribution of histological

types, grades and prognoses in that subgroup of breast cancer patients. Moreover, neoplasms occurring in young patients will be studied in detail (histological type, grade, response to hormonal and chemotherapy).

Key words: breast cancer, estrogen receptors, progesterone receptors, HER-2 receptor

Streszczenie
Liczba nowych zachorowań na raka piersi w Polsce stale rośnie i aktualnie wynosi około 16000 na rok, przy czym na przestrzeni minionych 20 lat obserwuje się tendencję do obniżenia liczby zgonów spowodowanych rakiem piersi. Według danych statystycznych za rok 2010, rak piersi stanowił 22,4% wszystkich nowotworów u kobiet i znajdował się na pierwszym miejscu w kolejności występowania. Do rutynowych badań prognostycznych i predykcyjnych w raku piersi należy określenie ekspresji receptorów estrogenowych (ER) i progesteronowych (PR) w komórkach nowotworowych oraz ocena ekspresji receptora HER-2/neu (receptora ludzkiego epidermalnego czynnika wzrostu, ang. Human Epidermal Growth Factor Receptor 2). Istnieje grupa pacjentek z rakami piersi, w których nie obserwuje się ekspresji żadnego z tych receptorów. Stanowią one 10 - 30% wszystkich przypadków. Są to tzw. raki potrójnie ujemne (ang. triple negative). Znaczna część pacjentek z potrójnie ujemnymi rakami piersi ma mutacje w genie BRCA1. Raki potrójnie ujemne cechuje bardziej agresywny przebieg kliniczny oraz szybsze i częstsze wznowy. Celem niniejszej pracy była analiza ekspresji receptorów estrogenowych, progesteronowych oraz receptora HER-2 w populacji pacjentek leczonych w Centrum Onkologii w Bydgoszczy. W badanej grupie status potrójnie ujemny (ER-/PR-/HER-2-) stwierdzono u 68 pacjentek (16,2% przypadków). Ekspresję receptorów estrogenowych stwierdzono w 286 przypadkach (68,1% pacjentek), receptorów progesteronowych w 231 przypadkach (55% pacjentek), natomiast ekspresję receptora HER-2 w 91 przypadkach (21,7%). Jednoczesną ekspresję receptorów estrogenowych i progesteronowych stwierdzono u 222 pacjentek (52,9% pacjentek). Jednym z najgorzej rokujących subtypów raka piersi jest subtyp potrójnie ujemny (ER(-), PR(-), HER-2(-)). Częstość występowania tego subtypu jest wysoka. Uzyskane rezultaty są zgodne z obserwacjami innych autorów. Wysoki odsetek tych guzów w populacji chorych na raka piersi wymaga poszukiwania nowych czynników predykcyjnych i prognostycznych, pozwalających charakteryzować ich złośliwy potencjał i planować skuteczne scenariusze terapii. Kolejne badania będą dotyczyły rozkładu typów histologicznych, zaawansowania oraz rokowania w tej subgrupie pacjentek z rakiem piersi. Ponadto zostaną szczegółowo zbadane (typ histologiczny, zaawansowanie, odpowiedź na hormono- i chemioterapię) nowotwory występujące u pacjentek młodych.

Słowa kluczowe: rak piersi, receptory estrogenowe, receptory progesteronowe, receptor HER-2

Introduction

Breast cancer is a malignant neoplasm prevailing in women, although it also occurs rarely in men. In women it is diagnosed ca. 100-150 times more often, and analyses conducted in Poland show it may occur in one out of ten women (Chabner et al. 2009; Pawlicki 2011; Donegan and Spratt 2012). In Poland, the number of newly diagnosed cases increases continuously and currently amounts to ca. 16000 cases per annum, with

a reduced tendency in a number of deaths caused by the breast cancer observed for last 20 years. According to statistical data for 2010, the breast cancer represented 22.4% of all neoplasms in women and was first in terms of occurrence. The highest morbidity ratio (48%) concerned the age interval from 50 to 64 years, while only 2% of all breast cancers were diagnosed in young women (up to 35 years). On a basis of data published for 2010 it can be seen that the number of fatalities resulting from that cancer increases after 40 years of age and reaches its peak at the age between 50 to 59 years. In 2010, the malignant breast cancer, the second cause of death from malignant neoplasms in women after the lung cancer, resulted in death of almost 13% of all patients with malignant neoplasm. With the breast cancer morbidity/mortality ratio being 3.0 this means that over 5200 women received help too late (Zatoński et al. 2012).

It is not possible to distinguish just one factor involved in malignant neoplasm development. Usually, cancer development results from interaction of several agents (Pawlicki 2011). The following factors are listed amongst those predisposing to breast cancer development: age, gender, genetic factors, hormonal influence (including hormone replacement therapy, hormonal contraception), lifestyle, diet, exposure to ionising radiation, as well as the reproduction factor. The high-risk group includes women who had their first child at older age, these whose first period occurred early, as well as with late-onset menopause. An important factor for the breast cancer development are hereditary BRCA1 and BRCA2 gene mutations, with BRCA1 mutation being a stronger risk factor. These autosomal recessive genes act as tumour growth suppressors (Chabner et al. 2009). Mutations in these genes increase a risk of breast and prostate cancers in men and breast, ovarian and uterine cancers in women (Kirchhoff et al. 2004; Ding et al. 2012). A risk for malignant breast cancer developing in mutated gene carriers may reach even 80% throughout their life (Easton et al. 1993).

From early 1990s, a decrease in mortality for malignant breast neoplasms has been observed (Chabner et al. 2009; Donegan and Spratt 2012). The most important condition for effective treatment and, eventually, healing, is quick diagnosis of a neoplasm at an early stage of its development (Pawlicki 2011). A correct diagnosis, implementation of effective therapy and relevant auxiliary treatments allow achieving the intended objective with as minimum intervention as possible in the patient's body. It is possible, amongst the others, due to increasing application of screening imagining, as well as due to extensive modern laboratory diagnostic methods (Chabner et al. 2009; Donegan and Spratt 2012).

Routine prognostic and predictive tests for breast cancer include estrogen (ER) and progesterone (PR) receptors expression in cancer cells, and therefore, confirming or excluding their sensitivity to hormones (Nomura et al. 1992; Pawlicki 2011). Many breast cancers are hormone-dependant tumours, thus exposition to increased estrogen and/or progesterone is related to increased risk of their development (Dunnwald et al. 2007; Chabner et al. 2009).

Estrogen (ER) and progesterone (PR) receptors belong to the family of intracellular steroid receptors. Estrogen and progesterone are hormones that can penetrate into a cell where after binding to receptors in cytoplasm they move to the nucleus. Their interaction with DNA facilitates appropriate control over gene expression (Cui et al. 2005). So far,

a role and functions of estrogen receptors and estrogen in breast cancer biology has been studied in more detail. Properties of progesterone and its receptors, and their role in cancer biology, on the other hand, are less known, although similarity to estrogen and its receptors roles is suspected. Activated estrogen and progesterone receptors influence expression of genes responsible for proliferation, apoptosis and angiogenesis. ER receptors participation in these processes significantly contributes to promoting uncontrolled neoplastic cell growth (http://www.cancer.gov 30.04.2012; Cui et al. 2005; Thomas et al. 2008).

Determining ER and PR receptors expression is a crucial procedure in pathomorphological and clinical assessment of breast cancer and facilitates qualification for an effective supplementary hormonal therapy. As it was mentioned above, expression of these receptors is a predictive marker for response to hormonal treatment blocking steroid receptors activity. A hormonal medication used in breast cancers exhibiting ER or PR receptors expression is tamoxifen, having antimitogenic properties and acting as an estrogen antagonist (Johnston and Dowsett 2003). The hormonal therapy is ineffective in patients with the hormone-receptor-negative breast cancer (Chabner et al. 2009). Currently, determining receptors' presence in neoplastic cells is a sole predictive factor widely used in all patients. According to some authors, expression of these receptors depends on age and they are more often found in elderly patients (Pieńkowski 2000).

Besides assessment of ER and PR receptors expression, being now a routine procedure, HER-2/neu receptor (Human Epidermal Growth Factor Receptor 2) expression is also assessed in patients with invasive breast cancer (Harrison, 2010). HER-2 is a transmembrane glycoprotein with tyrosine kinase activity (Barros et al.; Brennan et al. 2000; Gullick 2009). Activated HER has a very strong oncogenic effect by stimulating cellular division processes, inhibiting apoptosis and increasing migration rate and independence from extracellular signals (Alroy and Yarden 1997). Because of these properties, HER-2 expression is linked to a neoplastic transformation process and/or change in neoplastic cells behaviour (Badache and Goncalves 2006). Disorders in a neoplastic cells are related to HER-2/neu gene amplification and/or overexpression of its product, reflected in the increased number of protein molecules on the cell surface. HER-2 overexpression and/or amplification was found in many neoplasms, including colonic, lung, ovarian and breast cancers (Ramieri et al. 2010). Breast cancers with HER-2 overexpression and/or amplification are characterised by poorer prognosis due to increased risk of metastases and recurrence, and changed sensitivity to chemotherapeutic agents and the hormonal therapy administered. Consequently, overall survival periods in these patients are shorter by half than in patients with a negative HER-2 result (Pawlicki 2011). However, some patients with the invasive cancer and positive expression of that oncogene, respond positively to a therapy with monoclonal antibody anti-HER-2/neu, i.e., trastuzumab, particularly, in combination with specific chemotherapeutic agents. In HER-2-negative patients it is necessary to implement the cytostatic therapy (Chabner et al. 2009). A possibility to determine the HER-2/neu gene amplification degree and its expression allows using that parameter as a prognostic and predictive factor, as well as facilitates selection of the most suitable adjuvant therapy regime (Pawlicki 2011). There is also a group of breast cancer patients in which no expression of any of these

receptors is observed. They represent from 10 - 30% of all cases (Osborne 1998; Althuis et al. 2004; Dunnwald et al. 2007). These are called triple-negative cancers. A significant percentage of patients with triple-negative breast cancers have BRCA1 gene mutations. The triple-negative cancers are characterised by more aggressive clinical course, and quicker and more frequent recurrence (in: (Stevens et al. 2013)).

The aim of this study was to analyse estrogen, progesterone and HER-2 receptors expression in the population of patients treated at the Oncology Centre in Bydgoszcz.

Materials and study design

Patients

Breast cancer patients treated at the Prof Franciszek Łukaszczyk Memorial Oncology Centre in Bydgoszcz in the years 2004-2009 were enrolled in the study. The computer database of the Oncology Centre was used for patients' selection. The patients who had estrogen, progesterone and HER-2 receptors expression determined with immunohistochemical methods were qualified for the study. In patients with the uncertain HER-2 status, gene amplification was assessed by in situ hybridisation (FISH or CISH). Ultimately, 420 patients were qualified for the study. The average patient's age was 58.4 years (ranging from 29 to 89 years; median – 57 years). For the needs of this study the patients were assigned to age groups, with the patients' group of up to 40 years old called young patients.

Immunohistochemical determination of nuclear and membrane receptors expression

ER, PR and HER-2 receptors were determined immunochistochemically in standard 4-5 μm sections fixed in 10% buffered formaldehyde and embedded in paraffin.

To determine the nuclear receptors expression the following antibodies were used: anti-ER (clone 1D5,) anti-PR (clone PgR636) (DakoCytomation, Carpinteria, CA, USA). After heating to 60°C, the sections were dewaxed and hydrated. Antigenic determinants were found by heating the sections in Tris/EDTA buffer (pH 9.0) in a microwave oven (650 W) for 20 minutes. In thus prepared sections, the endogenous peroxidase activity was blocked with 3% H_2O_2, and then they were incubated with diluted antibodies at the ratio 1:100 (anti-ER) or 1:200 (anti-PR) with TBS with 0.1% albumin at the room temperature for 30 minutes. Antibodies' binding sites were visualised using a secondary antibody coupled with a horseradish peroxidase–labelled polymer (30 minutes incubation at the room temperature) and diaminobenzidine (DAB; 5 minutes at the room temperature) in the EnVision™ Detection System and the Peroxidase/DAB+ kits (DakoCytomation, Carpinteria, CA, USA). Cell nuclei were stained with hematoxylin and preparations were embedded in a solid medium (Consul Mount; Thermo Fisher Scientific Inc. Waltham, MA, USA).

To determine HER-2 expression, HercepTest™ (Dako, Carpinteria, CA, USA) was used following the manufacturer's protocol. After sections were heated in a thermostat to 60°C, they were dewaxed and hydrated, and antigenic determinants were found in citrate buffer, pH 6.0. The endogenous peroxidase activity was blocked with 3% H_2O_2 with 15mM NaN_3, and then rabbit primary antibody anti-HER-2 (Ready-to-use) was applied on the sections and incubated at the room temperature for 30 minutes. The sections were rinsed, and then a secondary antibody coupled with dextran polymer and horseradish peroxidase was applied on them (30 minutes at the room temperature), and antibody-

binding sites were visualised with DAB (5 minutes at the room temperature). Then the sections were stained with hematoxylin and embedded in a solid medium (Consul Mount; Thermo Fisher Scientific Inc. Waltham, MA, USA). For each reaction, a positive control included in the kit and the negative control with a reagent for a negative control included in the kit used instead of the primary antibody were performed simultaneously.

Assessment of immunohistochemically-stained preparations

The immunohistochemically-stained histopathological preparations were assessed qualitatively. The hormonal nuclear receptors (ER and PR) status was assessed with the cut-off point method, and cases where at least 10% of cancer cells exhibited expression of the studied receptor were classified as positive (ER+ or PR+), regardless of labelling intensity. Photos of representative cases for ER+, ER-, PR+ and PR- are shown in Fig. 1 A-C and Fig. 2 A-C.

Immunohistochemically-determined HER-2 receptor expression was assessed using the 4-grade scale specified by the American Society of Clinical Oncology/College of American Pathologists (Wolff at al., 2007). According to these criteria, cancers with no membrane staining were classified as HER-2 0 (negative). Cancers with weak and heterogeneous HER-2 membrane expression were classified as HER-2 1+ (negative). Cancers with weak or heterogeneous membrane expression present in at least 10% of cancer cells were qualified as HER-2 2+ (ambiguous). Cancers classified as HER-2 3+ (positive) showed strong, uniform HER-2 membrane expression for at least 30% of cancer cells. Photos of representative cases classified as HER-2 0, HER-2 1+, HER-2 2+ and HER-2 3+ are shown in Fig. 3 A-D.

Statistical results evaluation

The statistical evaluation of the results was performed with the Prism 5.00 package (GraphPad Software, San Diego, CA), and results with $p<0.05$ were considered as statistically significant. Results obtained for the analysed groups were compared using the Wilcoxon rank test.

Results

Tab. 1 lists data for estrogen, progesterone and HER-2 receptors expression in the patients qualified for this study. Tab. 2 shows expression of studied receptors in the patients in each age group. Estrogen receptor expression was found in 286 cases (68.1% of the patients), progesterone receptor expression in 231 cases (55% of the patients), and HER-2 receptor expression in 91 cases (21.7%). Simultaneous estrogen and progesterone receptors expression was found in 222 (52.9%) patients.

In the studied group the triple-negative (ER-/PR-/HER-2-) status was found in 68 patients (16.2% of the cases).

The average age of patients with triple-negative cancers was 58.3 years (median 57.5 years versus 58.2 years and 57 years in groups of patients with any receptor expression, respectively). The average age of patients with HER-2 positive cancers was 55.9 years (median 54.0 years versus 58.8 and 59.0 years in patients with the HER-2 negative status).

Although no statistically significant differences were found, cancers with the ER+ and/or PR+ and HER-2+ status were least often found in the group of patients below 40 years of age (2.9% versus 6.6-8.8% for other cancer subtypes).

Discussion

From literature, in ca. 60% of invasive breast cancer cases cells contain receptors for these hormones (Pieńkowski 2000; Hayes et al. 2001; Dunnwald et al. 2007; Pawlicki 2011). In the group of patients qualified for this study, expression of these receptors was found in 52.9% of cases.

It must also be emphasised that ER receptor expression is more frequently observed (54-80 % (Thorpe et al. 1987; Kreiger et al. 1991; Nomura et al. 1992; Tavassoéli and Devilee 2003; Althuis et al. 2004) than PR receptor expression (34 - 65% breast cancer cases (Thorpe et al. 1987; Kreiger et al. 1991; Nomura et al. 1992; Althuis et al. 2004). Also in the patient population in our study, ER receptor expression was found more often (68.1% of cases) than progesterone receptor expression (55.0% of cases).

The literature data indicates that, similarly as in our analysed population where overexpression was found for 21.7% of the cases, HER-2 overexpression and/or amplification is also found in 20-30% of breast cancer cases (Dowsett et al. 2000).

The breast cancer subtype (ER+ and/or PR+, HER-2 -; ER+ and/or PR+, HER-2 +; ER- and PR-, HER-2 -; ER- and PR-, HER-2 +) distribution in the analysed population was similar to that reported in the literature (e.g.: (Kwan et al. 2009)) and we did not observe a relationship between studied receptors expression and the patients' age. Other authors found significantly more frequent occurrence of ER+ and/or PR+, HER-2– subtype and ER+ and/or PR+, HER-2+ subtype in younger patients (Kwan et al. 2009). Similarly, according to published data, HER-2 overexpression and the triple-negative subtype is more often found in younger patients (e.g.: (Dolle et al. 2009; Kwan et al. 2009)). However, these relationships were not observed in the patients' population in this study.

Conclusions

One of the breast cancer subtypes associated with the worst prognosis is the triple-negative subtype (ER(-), PR(-), HER-2(-)). Frequency of that subtype is high. In our patients' population triple-negative tumours represented 16.2% of cases. Obtained results are consistent with observations of other authors (9.3% in the study (Kwan et al. 2009) or 12% in the study (Lund et al. 2009)). The high ratio of these tumours in the population of patients with breast cancer requires a research aiming at finding new predictive and prognostic factors, to characterise their malignant potential and plan effective therapeutic scenarios. Subsequent studies will concern distribution of histological types, grades and prognoses in that subgroup of breast cancer patients. Moreover, neoplasms occurring in young patients will be studied in detail (histological type, grade, response to hormonal and chemotherapy).

Tab. 1. Estrogen, progesterone and HER-2 receptors expression in breast cancer patients.

Receptor status	Number of patients/ percentage	Age [years] (mean/media)
ER+	286/68,1	58,6/59,0
ER-	134/31,9	57,2/56,0
PR+	231/55,0	58,7/59,0
PR-	189/45,0	57,6/56,0

HER-2 + HER-2 -	91/21,7 329/78,3	55,9/54,0 58,8/59,0
ER+ and PR+	222/52,9	58,8/59,0
ER+ and PR-	64/15,2	58,3/56,5
ER- and PR+	9/2,1	56,8/53,0
ER- and PR-	125/29,8	57,2/56,0
ER+ and/or PR+, HER-2 -	261/62,1	58,9/59,0
ER+ and/or PR+, HER-2 +	34/8,1	56,0/54,0
ER- and PR-, HER-2 -	68/16,2	58,3/57,5
ER- and PR-, HER-2 +	57/13,6	55,8/54,0

Tab. 2. Estrogen, progesterone and HER-2 receptors expression in breast cancer patients grouped according to age.

Receptor status	Age group	Number of patients/ percentage
ER+ and/or PR+, HER-2 -	≤40 ys > 40 ys	17/6,5 244/93,5
ER+ and/or PR+, HER-2 +	≤40 ys > 40 ys	1/2,9 33/97,1
ER- and PR-, HER-2 -	≤40 ys > 40 ys	6/8,8 62/91,2
ER- and PR-, HER-2 +	≤40 ys > 40 ys	5/8,8 52/91,2
ER+	≤40 ys > 40 ys	11/4,0 275/96,0
ER-	≤40 ys > 40 ys	18/13,4 116/86,6
PR+	≤40 ys > 40 ys	14/6,1 217/93,1
PR-	≤40 ys > 40 ys	15/7,9 174/92,1
HER-2 +	≤40 ys > 40 ys	6/6,6 85/93,4
HER-2 -	≤40 ys > 40 ys	24/7,3 305/92,7

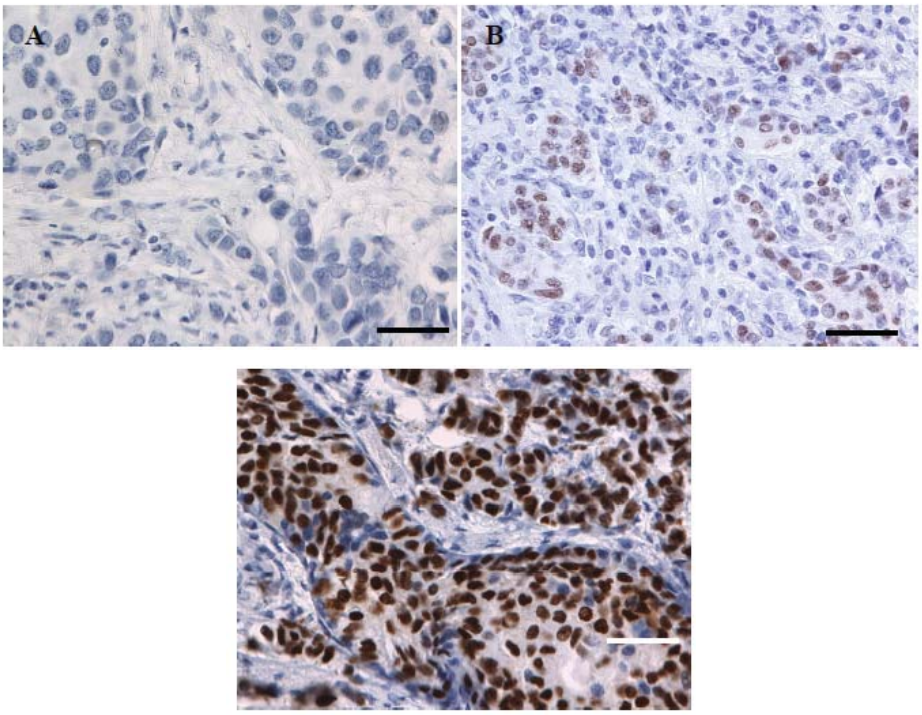
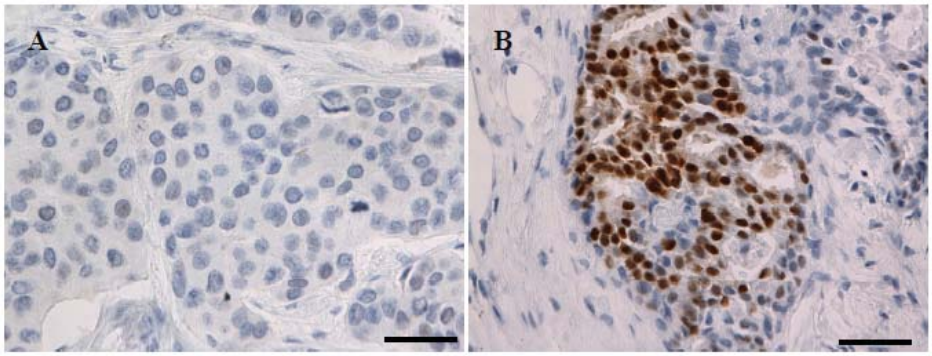


Fig. 1. The status of ER receptors detected using immunohistochemistry in the standard preparations fixed in formalin and soaked in paraffin. A) ER-negative expression (ER-) B) ER-positive expression (weak and average staining intensity in about 40% of cancer cells); C) ER-positive expression (strong and average staining intensity in about 90% of cancer cells). Scale bars - 50μm.



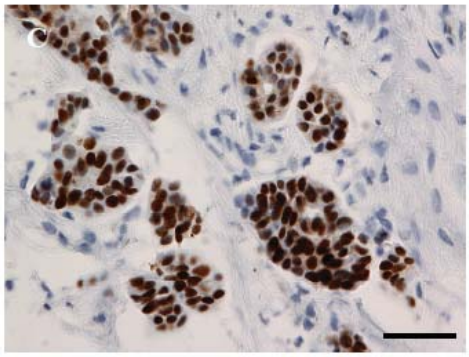


Fig. 2. The status of PR receptors detected using immunohistochemistry in the standard preparations fixed in formalin and soaked in paraffin. A) PR-negative expression (PR-) B) PR-positive expression (weak and average staining intensity in about 60% of cancer cells); C) PR-positive expression (strong and average staining intensity in about 90% of cancer cells). Scale bars - 50μm.

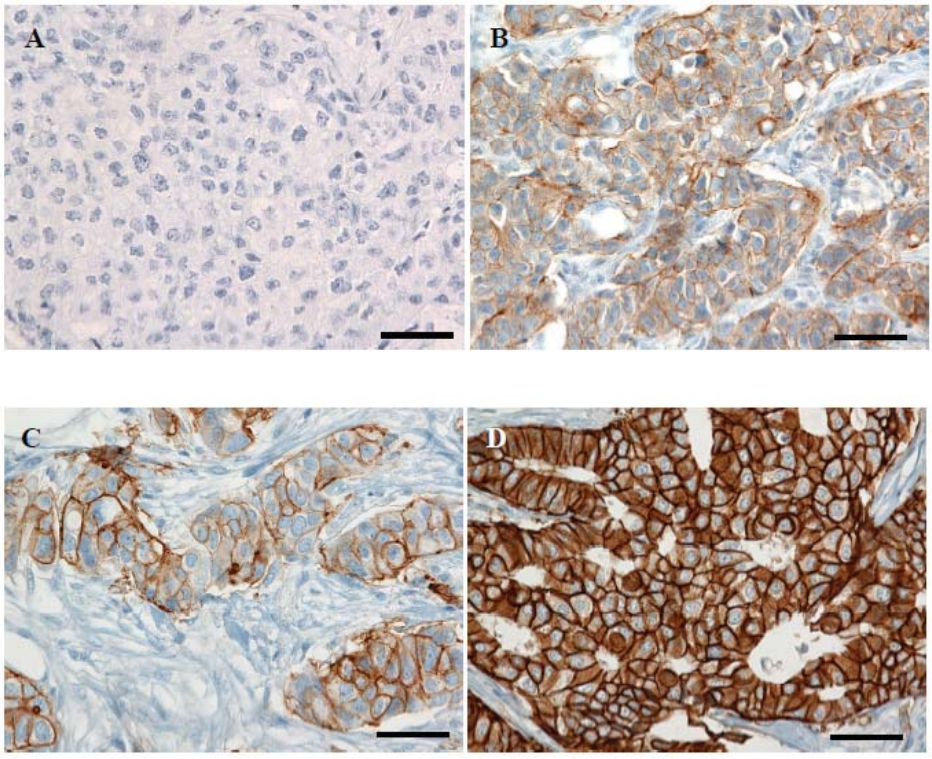


Fig. 3. A status of the HER-2 receptor in the sections labelled by immunohistochemistry in breast cancer. A) no HER-2 expression (HER-2 negative); B) HER-2 expression at 1+ (HER-2 negative); C) HER-2 expression at 2+ (HER-2 ambiguous status); D) HER-2 expression at 3+ (HER-2

positive). The blue-violet nuclei are tinted with hematoxylin. Scale bars-50 μm.

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