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HER2 Status in Breast Cancer Determined by IHC and FISH: Comparison of the Results

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HER2 (human epidermal growth factor receptor 2) status became an important prognostic and predictive factor in breast carcinoma clinical management. There are two main techniques of evaluation of HER2 status: immunohistochemistry (IHC) for the protein expression and fluorescence *in situ* hybridization (FISH) for amplification of *HER2* gene. The aim of the study was to compare the results obtained by IHC and FISH methods in determination of HER2 status in breast cancer. Three hundred and sixty breast cancer specimens were examined. Patients were operated in the Oncology Centre in Warsaw. IHC and FISH were performed in every case. IHC was performed with DAKO HercepTest and FISH with Oncor-QBiogene reagents. IHC results were classed into 4 groups, accordingly to the four-tier DAKO criteria system (0, 1+, 2+, 3+). FISH results were divided into three main categories: NA – no amplification, LA – low amplification and HA – high amplification. The number of copies of chromosome 17 was also assessed. Over 90% of cases described by IHC as 3+ exhibited amplification of *HER2/neu* gene. Remaining cases were positive with IHC, but presented no gene amplification. This might be due to the subjective assessment of the membrane staining. Another possibility is that overexpression of the protein was caused by mRNA stability or disorders in receptor degradation. The majority of cases classed by IHC as 2+ were also negative by FISH (80%). One fifth of IHC 2+ tumours were found to exhibit gene amplification. Remaining cases showed no amplification of *HER2/neu* gene, combined with aneuploidy of chromosome 17. All cases described by IHC as 0/1+ were also HER2-negative by FISH. Conclusions: IHC is well-established method of assessing HER2 status in breast cancer. Nonetheless, a group of cases described as 2+ should be additionally examined using FISH. The results obtained by the latter method are more reliable. In order to improve accuracy and gain the highest quality of HER2 status evaluation, in 2+ cases both methods should be applied.

Introduction

Appearance and maintenance of malignant phenotype is associated with the changes in quality and quantity of proteins produced. Gene amplification is one of the common mechanisms by which cancer cells gain the increased protein synthesis, which can lead to malignant transformation.

Self-sufficiency or hypersensitivity to growth signals is one of six acquired capabilities of cancer. The others include:
– insensitivity to anti-growth signals;
– tissue invasion and metastasis;
– limitless replicative potential;
– sustained angiogenesis;
– evading apoptosis [9].

Growth factors receptors (GFRs) are often overexpressed in many types of cancer. GFR overexpression enables the cell response to ambient levels of growth factors that would not trigger proliferation in normal cells. Breast, ovarian, pulmonary and other types of cancer are often characterised by overexpression of HER2 receptor [11].

HER2 is 185kDa surface protein serving as growth factor receptor carrying protein kinase activity [3]. HER2 belongs to the family of HER receptors, consisting of four members (HER1-HER4) and sharing similar characteristics. These membrane receptors transduce growth signals from growth factors into the nucleus in order to increase proliferative activity of the cell. Over-

expression of HER2 protein on the cell surface leads to hypersensitivity of the cancer cell to growth factors and improves malignant phenotype [5, 18]. In over 90% of cases overexpression of HER2 is caused by amplification of *HER2* gene [26, 28, 21].

In breast cancer there are several well-established predictive and prognostic factors, such as histological type and grade, tumour size, lymph node involvement, estrogen and progesterone receptor status [1, 6, 16]. They allow predicting disease-free survival, overall survival and the patient's reaction to the treatment. In 1987 in *Nature* a publication by Slamon et al. has appeared revealing, that *HER2/neu* gene amplification predicts independently overall survival (OS) and disease-free survival (DFS) in node-positive patients [27].

Since HER2 status has been considered as important predictive and prognostic factor in breast cancer [14, 15], right assessment of HER2 status became necessary in the management of this disease. Retrospective analysis of clinical trials proved this factor may be involved in the process of response to certain types of chemo- and hormonotherapy [19, 20, 23].

There is a number of questions concerning whom to test, which method should be applied and finally – how to interpret the results of HER2 status assessment [8, 25].

There are several methods, by which protein or gene status can be assessed. Immunohistochemistry for protein expression is commonly used and well-established laboratory technique. Gene copy number can be detected by several methods, for example Southern blot analysis or fluorescence *in situ* hybridization [12].

Two of the methods mentioned above have been successfully applied to routine diagnostic procedures in pathology laboratories: IHC and FISH [21-25].

Both methods (IHC and FISH) can be applied on archival material and used with formalin-fixed, paraffin- embedded tissue. The use of IHC arises several questions. The right choice of the antibody seems to be the main issue. Commercially available antibodies differ significantly with their sensitivity and specificity [24]. Antigen- retrieval methods and tissue fixation process also influence the quality of immunohistochemical stains, and therefore – the final result of HER2 status assessment. The latter one depends also on the subjectivity of stain assessment by the pathologist [26].

The second method applied to routine diagnostics is FISH. Fluorescence *in situ* hybridization offers the most accurate, reliable and reproducible results of HER2 status assessment. With FISH, in the majority of samples, it is possible to count gene copy number, which is directly correlated with the quantity of surface receptor level [27]. Therefore, in most cases the results are easy to interpret and do not depend on the subjective assessment of the pathologist. This method employs fluorescence microscope which is not commonly available in pathology laboratories.

The aim of the study was to compare two methods: FISH and IHC in assessment of HER2 status in breast cancer.

Material and Methods

Three hundred and sixty cases of invasive breast carcinoma were examined. Patients were operated in the Oncology Centre in Warsaw because of invasive breast carcinoma. HER2 status was examined in each case using both IHC (immunohistochemistry) and FISH (fluorescence *in situ* hybridization).

Sample preparation

The surgically resected tumour tissue was routinely fixed in 4% buffered formalin for 48 hours and embedded in paraffin. Tumour tissue blocks were cut into 4μm thick sections and mounted on positively charged slides. The slides were baked overnight (68^{o}C). In each case standard HE staining was performed in order to confirm the presence of invasive carcinoma cells.

FISH analysis

All chemicals were purchased from QBiogene (Oncor, UK). Sections were deparaffinised in xylene (2x10 minutes, RT), dehydrated in 99.98% ethanol (2x5 minutes, RT) and air-dried. Tissue sections were treated with Pretreatment Solution (15–30 minutes, 45oC, water bath), according to the manufacturer's protocol (Oncor, UK). After rinsing specimens in standard saline citrate (2xSSC), they were moved to the proteinase K (Oncor, UK) working solution (25–45 minutes, 45°C, water bath). After enzymatic digestion step the slides were rinsed with 2xSSC and dehydrated in ethanol (70, 80, 96%, 1 minute, RT), the digestion degree was examined by applying 20μl of propidium iodide (Oncor, UK). Slides were then examined under fluorescence microscope (Olympus) and digestion of invasive carcinoma was assessed. Appropriately prepared tissue sections were then rinsed in 2xSSC, dehydrated in graded series of alcohol (70, 80, 96%, 1 minute, RT) and air-dried. Dual-colour probe cocktail consisting of *HER2/neu* (rhodamine-labelled, red) and chromosome 17 (FITC- labelled, green) probes was applied (Oncor,

UK). The quantity of probe mix applied was dependent on the size of the slide (from 10 to 30ml/slide). Specimen and probe DNA was denatured by placing the samples on hot-plate (80°C, 2 minutes).

Hybridization was carried out under plastic coverslip in moist chamber (overnight, 37°C). Post-hybridization wash was performed in 2xSSC (5 minutes, 65°C) followed by wash in 1xPBD (Oncor, UK) (5 minutes, RT). Tissue sections were then counterstained with DAPI/Antifade (Oncor, UK).

Scoring criteria

Slides were evaluated for *HER2/neu* gene amplification using Olympus BX60 microscope (Olympus Polska, Poland) equipped with filters: rhodamine, FITC, DAPI monofilters and triple-bandpass (rhodamine/FITC/DAPI) filter (Olympus Polska, Poland). Samples were scanned at x200 magnification, and *HER2/neu* and CEN17 signals were counted at x1000 magnification.

The number of *HER2/neu* gene copy number and chromosome 17 (CEN17) copy number were counted in all cases in at least 60 invasive carcinoma nuclei. Lack of *HER2/neu* gene amplification (NA – no amplification) was stated in cases, in which no more than 4 *HER2/neu* copies and no more than 4 CEN17 copies were detected (Figs. 1 and 2). Aneuploidy was stated in all cases, in which average CEN17 copy number in 60 nuclei was higher than 4. Samples were assigned to low amplification (LA) group when they expressed on average 5–10 *HER2/neu* gene copies and no more than 4 CEN17 copies. Detection of more than 10 *HER2/neu* gene copies per nucleus and no more than 4 CEN17 copies meant high amplification (HA) of *HER2/neu* gene. In some cases the signal enumeration was impossible because of cluster(s) consisting of many copies of the gene. These cases were also considered to be highly amplified and assigned to HA group (Figs. 3 and 4).

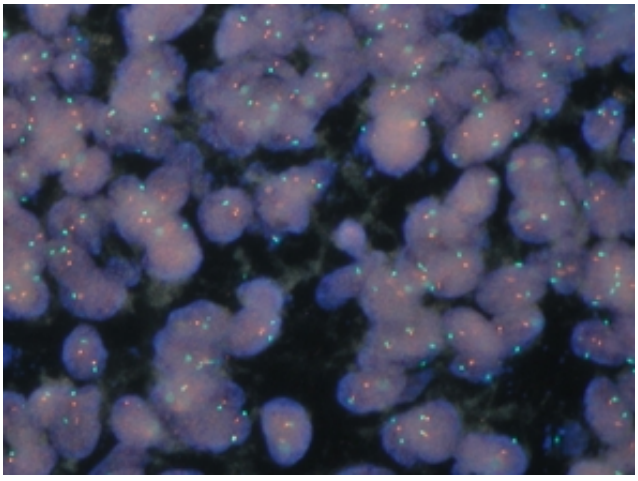

Fig. 1. Lack of amplification of *HER2/neu* gene (rhodamine-labelled, red). Centromeres of chromosome 17 are FITC-labelled (green). Counterstain DAPI. Magn. 1000x.

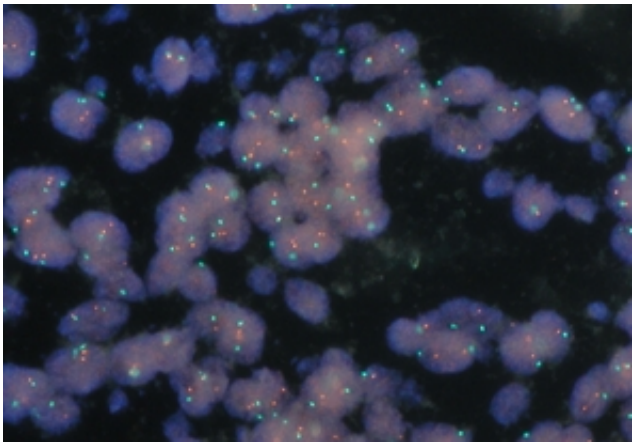

Fig. 2. Lack of amplification of *HER2/neu* gene (rhodamine-labelled, red). Centromeres of chromosome 17 are FITC-labelled (green). Counterstain DAPI. Magn. 600x.

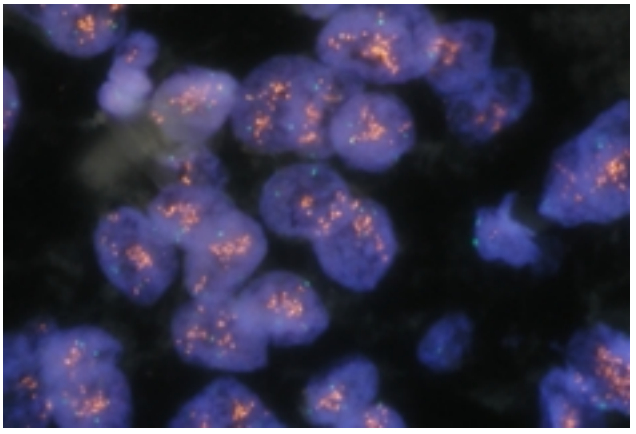

Fig. 3. High amplification of *HER2/neu* gene (rhodamine-labelled, red). Centromeres of chromosome 17 are FITC-labelled (green). Counterstain DAPI. Magn 1000x.

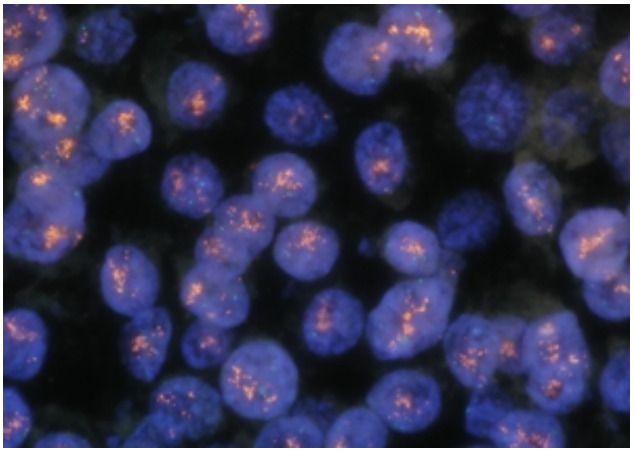

Fig. 4. High amplification of *HER2/neu* gene (rhodamine-labelled, red). Centromeres of chromosome 17 are FITC-labelled (green). Counterstain DAPI. Magn 1000x.

Immunohistochemistry (IHC)

DAKO HercepTest (Dako, Denmark) was used for immunohistochemical evaluation of HER2 status. Samples were fixed and prepared as described above. Immunohistochemistry was performed accordingly to the manufacturer's instruction. The strength of the staining was evaluated by pathologist and the stains were grouped in four categories (Dako, Danemark):

1. No staining is observed or staining is observed in less than 10% of the tumour cells (0 – negative);
2. A faint/barely perceptible membrane staining is detected in more than 10% of the tumour cells. The cells are stained only in part of their membrane (1+ – negative);
3. A weak to moderate complete membrane staining is observed in more than 10% of the tumour cells (2+ – weakly positive, Fig. 5);
4. A strong complete membrane staining is observed in more than 10% of the tumour cells (3+ – strongly positive, Fig. 6).

HercepTest is interpreted as negative for HER2 protein overexpression (0 and 1+), weakly positive (2+ staining intensity), and strongly positive (3+ staining in- tensity).

Results

Three hundred and sixty archival cases of invasive breast cancer were examined both by immunohistochemistry (IHC) and fluorescence *in situ* hybridization

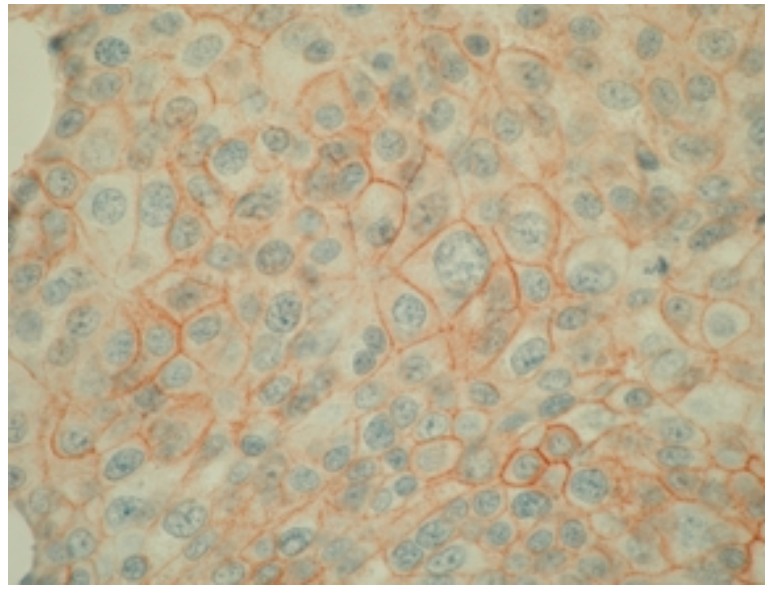

Fig. 5. HER2 2+ immunohistochemical stain. Weak to moderate positive membrane staining is visible. Magn 600x.

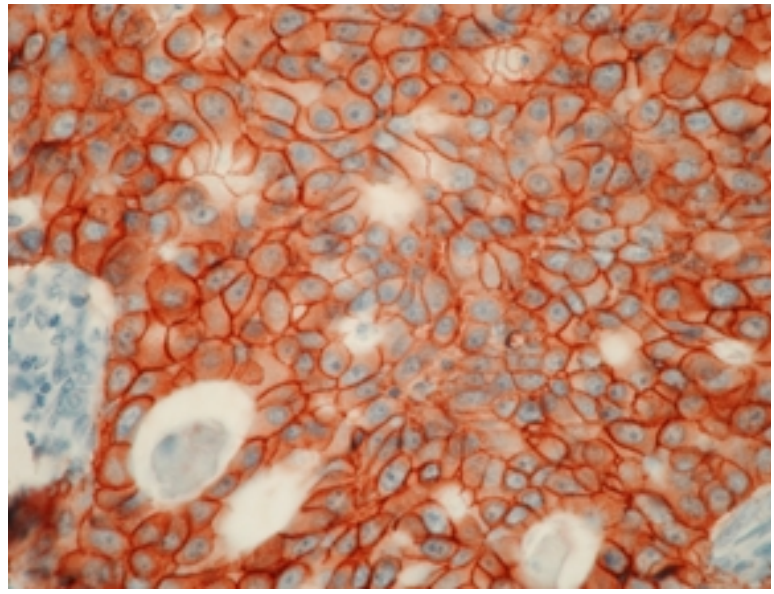

Fig. 6. HER2 3+ immunohistochemical stain. Strongly positive, complete membrane staining is visible. Magn 600x.

TABLE 1
The results of *HER2/neu* gene assessment by FISH in IHC 2+ group

2+	n	%
HA	23	7.3
HA aneupl	2	0.6
LA	39	12.4
NA	230	73.0
NA aneupl	21	6.7
	315	100.0

TABLE 2
The results of *HER2/neu* gene assessment by FISH in IHC 3+ group

3+	n	%
HA	28	80
LA	4	11
NA	3	9
	35	100.0

(FISH). Firstly, the immunohistochemistry was performed in each case. The group of specimens was selected to contain the majority of cases assigned by IHC to IHC 2+ group (n=315). Only 35 cases of IHC 3+ and 10 of IHC 0 and 1+ were selected for this study.

The cases assigned by IHC to 0/1+ group were all HER2-negative by FISH (n=10).

Among the samples described immunohistochemically as 2+, amplification of *HER2/neu* gene was discovered in 20% of cases (n=64). It includes cases, in which high and low amplification was stated. The remaining cases (n=251) were found to be negative by FISH method (Table 1).

In the group of specimen assessed by IHC as 3+ *HER2/neu* gene amplification was discovered in 91% (n=32) of the cases. Lack of gene amplification was detected in only 3 cases (9%) (Table 2).

In 7.3% (n=23) of cases aneuploidy of chromosome 17 was found. This result refers only to the IHC 2+ cancers – only in this group the excessive copies of chromosome 17 were detected.

Discussion

Applying molecular biology methods in medical diagnostics allows for the accurate analysis of changes in the phenotype and genotype, and in consequence helps in determining the complete diagnosis. Full diagnosis of breast cancer consists of well-established predictive and prognostic factors such as histological type and grade, estrogen and progesterone receptors and recently HER2 status. Having this information provided, not only disease-free and overall survival, but also the reaction to the treatment can be better predicted.

Herceptin – monoclonal anti-HER2 antibody – was introduced to the treatment of breast cancers overexpressing HER2 receptor [29]. In consequence the interest in methods enabling the most accurate screening of patients to this treatment has grown. Only patients with HER2 protein overexpression and/or *HER2/neu* gene amplification benefit from Herceptin treatment, as it was demonstrated in a number of publications [4, 2, 7]. Pre- viously, the group of patients, whose tumours in immunohistochemistry expressed HER2 protein at 3+ and 2+ levels were considered as positive and were given the Herceptin treatment. The results of the studies demonstrated, that the group of IHC 2+ does not benefit from Herceptin as much as IHC 3+ patients and in comparison is more heterogeneous than the 3+ group.

Our aim was to examine the IHC 2+ group using more accurate and reliable than immunohistochemistry method – fluorescence *in situ* hybridization (FISH). Our results indicate that IHC 2+ group is characterised by high heterogeneity. Nearly eighty percent of these cases do not have *HER2/neu* gene amplification. This percentage covers groups with no amplification (NA, 73.0%) and no amplification with chromosome 17 aneuploidy (NA aneupl, 6.7%). Tumours with the excessive copies of chromosome 17 clinically are considered as HER2-negative, but the results of recent studies indicate, they can benefit from Herceptin therapy [3]. Most importantly, among patients characterised immunohistochemically as HER2 2+, over 20% of all cases have the amplification of *HER2/neu* gene and are potential beneficiates of trastuzumab treatment.

The results of FISH tests in IHC 3+ group confirm published data and indicate that over 90% of them exhibit amplification of *HER2/neu* gene. Nine per cent of these tumours are HER2-negative by FISH. This high rate of negative tumours might be due to small number of cases included in this particular group (n=35). Another explanation of this result could be the overestimation of IHC stains, abnormal stability of HER2 receptor messenger RNA or defects in the process of receptor sequestration, which lead to enduring presence of HER2 receptor on the cell surface.

All cases assessed immunohistochemically as 0 and 1+ were HER2-negative by FISH – it confirms high concordance of these two methods in HER2 status determination in these groups.

The results demonstrate the necessity of additional testing of IHC 2+ group by more objective and reproducible method. Fluorescence *in situ* hybridization (FISH) has these advantages. Considering relatively high cost of FISH tests they should be performed in referee centres in properly selected cases.

Proper diagnosis of breast cancer requires among well-established prognostic and predictive factors also HER2 status evaluation. Immunohistochemical determination of HER2 protein should be supported by FISH in tumours expressing weak or moderate positive membrane staining – IHC 2+ group.

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