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Evaluation of HER2/*neu* Gene Amplification in Patients with Invasive Breast Carcinoma. Comparison of *in situ* Hybridization Methods

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One of the prognostic and predictive factors in invasive breast carcinomas is determination of the *HER2*/*neu* proto-oncogene amplification or HER2 protein overexpression. HER2 amplification/overexpression is associated with a more aggressive disease course, greater likelihood of recurrence and generally poor prognosis.

The authors compared the specificity, simplicity of a given procedure and method standardization, the simplicity of evaluation the results of each *in situ* hybridization method and time needed for performing the test.

Sixty-three cases of infiltrating breast carcinoma from surgically excised tumors and core needle biopsies were included in the study. The first step was the determination of HER2 status by immunohistochemistry. The patients with moderate (2+) and strong (3+) overexpression of HER2 protein were chosen for determining *HER2* amplification by three methods of *in situ* hybridization: FISH, CISH and *in situ* hybridization with silver autometallography.

The statistical analysis revealed a good agreement between IHC and ISH methods and among ISH methods.

The results indicate that all *in situ* hybridization methods are equivalent tools for evaluating *HER2* gene amplification in archival material. There is no clear answer which method is the best assay to determine HER2 marker status, although the authors present some advantages and disadvantages of all the described techniques and a proposed algorithm for choosing a method for a given laboratory.

Introduction

Breast carcinoma is the most frequent malignancy in women in developed countries [28, 33].

The behavior of breast cancer is influenced by several factors, such as histological type and grade, tumor size, proliferation index, lymph node status, lymphatic and blood vessel invasion, estrogen (ER) and progesterone (PGR) receptor status, and others [6, 10, 32, 34, 38].

Recently, several authors have shown that amplification of the *HER2*/*neu* proto-oncogene or HER2 protein overexpression is an important prognostic and predictive factor in invasive breast carcinomas [6, 9, 10, 20, 27, 32, 34, 38].

The HER2/*neu* (also known as c-*erb*B2) oncogene is a member of the epidermal growth factor receptor family, together with epidermal growth factor receptor EGFR (HER1), HER3 (c-erbB-3) and HER4 (c-erbB-4) [3, 19, 27, 30, 33, 41].

The rat *neu* oncogene was first identified in 1981 as a transforming gene in the chemically induced neuroblastoma model [2, 5, 8, 19, 30, 31, 41]. The human homolog of this gene was independently isolated by several groups through its homology with human *EGFR* or *v-erbB* and was named *HER2* [5, 7].

The *HER/neu* gene is located on the long arm of chromosome 17 (locus 17q12–21.32) [18, 39] and encodes 1255 amino acids [17, 19], 185 kDa transmembrane tyrosine kinase receptor [17, 19, 41]. This protein is partially homologous to EGFR [2, 8, 14, 17, 18]. HER2 is an important member of the HER family and plays a crucial role (cooperating with other HER receptors via a complex) in signaling network to regulate cell growth, differentiation and survival [3]. The HER2 oncoprotein consists of an extracellular domain, a transmembrane domain and an intracellular domain with intrinsic tyrosine kinase activity [2, 5, 40].

Overexpression of this receptor occurs in about 10 to 34% invasive breast carcinomas [8, 30] in 90% of these cases, where *HER2* gene amplification is present [8, 30, 39]. Some tumors with increased *erbB-2* mRNA and protein level have no detectable gene amplification. It is possible that alternative transcriptional and post-transcriptional me-

chanisms controlling *erbB*-2 expression are operating [8, 19]. Overexpression of HER2 protein may be caused by aneuploidy of chromosome 17 [27, 39], producing more copies that the two present in normal cells [18, 39].

Gene amplification or protein overexpression of HER2 is associated with a poor prognosis, more aggressive disease, a greater likelihood of recurrence, shortened overall survival and it correlates with tumor chemo-resistance [26, 27].

The clinical relevance of HER2 in breast infiltrating carcinomas has become a subject of debate since the appearance of trastuzumab. Trastuzumab (Herceptin) is a first human antibody against the external domain of HER2/*neu* approved for the treatment of HER2-positive metastatic breast cancer patients [9, 15, 23, 37]. This compound has antitumoral activity [9], blocking proliferation of tumor cells overexpressing HER2 protein [4, 15]. The interesting point of such a specific treatment is that only patients with tumors that overexpress HER2 benefit from such a therapy. For that reason, the determination of HER2 status in breast cancer is becoming of great interest [9].

To determine the HER2 status, several methods are used, e.g: immunohistochemistry [29, 37], ELISA assays [29] or Western blotting on protein level [31], determination of gene amplification by various *in situ* hybridization techniques [29], different variants of PCR methods [22] or Southern blotting [31].

As stated above, the determination of the HER2 status in invasive breast cancer is very important in choosing a proper treatment for the patient. The purpose of the present study was to compare some *in situ* hybridization methods to assess the *HER2* gene amplification degree and select the best method.

The simplicity of a given procedure and method standardization, the simplicity of evaluation the results of each *in situ* hybridization method and time needed for performing the test were compared.

Material and Methods

Material

The material included in the study consisted of 63 cases of infiltrating breast carcinoma from surgically excised tumors and core needle biopsies, embedded in paraffin blocks and originating from the files of the Pathomorphology Department and other hospitals from the Małopolska region. The median age of the patients was 54.9 (range, 31–90 years). Histopathological classification and grading was done on hematoxylin and eosin stained slides according to the standard histopathological procedure. Thirty-three cases of breast cancer patients with HER2 overexpression on 3+ level and 30 cases diagnosed as moderate expression of HER2 (2+ level) by IHC method were chosen from the patients' group diagnosed in 2000–2004.

Methods

The first step was the determination of the HER2 status by immunohistochemistry (IHC) – HercepTest (DakoCytomation, Glostrup, Denmark). The patients with moderate (2+) (30 cases) and with strong (3+) HER2 protein overexpression (33 cases) were chosen to check *HER2* amplification by thee methods of *in situ* hybridization:

– fluorescent *in situ* hybridization FISH (PathVysion, Vysis or HER-2/*neu* (ERBB2)/ AlphaSat. 17D17Z1, Qbiogene) – 63 cases,
– chromogenic *in situ* hybridization CISH (SPOT LIGHT CISH Kit, ZYMED) – 55 cases,
– and *in situ* hybridization combined with DAKO GenPoint catalyzed signal amplification system for *in situ* hybridization with silver autometallography, as previously described [36] – 63 cases.

Immunohistochemistry assay (IHC)

The immunohistochemical technique and scoring system were done according to the manufacturer's guidelines accompanying the HercepTest kit. The assay was performed on 4–5-μm-thick tissue sections. The cases were scored by two of the authors (T.R, R-S.L.).

Fluorescent in situ hybridization assay (FISH)

FISH was done on tissue sections at the Pathomorphology Department of Jagiellonian University. Amplification of HER2 was evaluated using Qbiogene reagents (probe: ERBB2)/ AlphaSat. 17D17Z1 (no. PONC1712)) or PathVysion HER-2 DNA Probe Kit (no. 30-161060**,** Vysis, Inc., Downers Grove, IL, USA). Both kits use a dual-color probe for determining the number of copies of HER2 and the chromosome 17 centromeres. In brief, 4–5-μm-thick archival formalin-fixed paraffin-embedded tissue sections were deparaffinized and dehydrated in 100% ethanol and air-dried. The slides were then pretreated with 0.2M hydrochloric acid for 20 minutes at room temperature, followed by washes in distilled water and immersed in Vysis Wash Buffer. Subsequently, they were immersed in Pretreatment Solution at 80^0C for 30 minutes. Then, they were washed in distilled water and in Wash Buffer two times. The slides were subsequently immersed in a protease solution for 15–20 minutes at 37^oC, washed in Vysis Wash Buffer and dried in air. Then, the tissue sections were immersed in 10% buffered formalin for 10 minutes at room temperature, washed in Vysis Wash Buffer twice and dried in air. Then, 8–10 μl of probe was applied

on tissue sections, which were covered with 22x22 mm coverslips, sealed with rubber cement and dried at 37°C. The slides were denatured at 80°C for 2 minutes and then hybridized at 37°C overnight in a hybridizer MP-16 (Hiperon, Genos). After hybridization, the slides were washed with Post-Hybridization Wash Buffer (2xSSC, 0.3% NP-40, pH 7.0–7.5) at 72+/– 1°C for 2 minutes. The slides were air-dried and 4'-6'-diamidino-2'-phenylindole (DAPI) counterstain was applied.

The scoring system used is described in detail in the manufacturer's protocol. In brief, a minimum of 60 nuclei were scored using a fluorescent microscope (Zeiss Axioskop, Germany). The ratio of HE2/*neu* signals (orange) to chromosome 17 centromere (green) was determined. The ratios of < 1.8 were considered as non-amplified, 1.8–2.2 as low-amplification and > 2.2 as high amplification. The manufacturer's guidelines recommend that a ratio at or near the cutoff (1.8–2.2) should be interpreted with caution and additional nuclei should be counted. In Poland, this borderline ratio was regarded as low-amplification.

Chromogenic in situ hybridization assay (CISH)

CISH was done on 4–5-μm-thick archival formalin-fixed paraffin-embedded tissue sections using SPOT-Light reagents purchased from ZYMED, Laboratories, Inc., South San Francisco, CA. Briefly, the sections were deparaffinized and incubated in pretreatment buffer in a temperature-controlled heater (92–100°C) for 15 minutes. The sections were then washed twice for 3 minutes with phosphate-buffered saline (PBS). Enzymatic digestion was done by applying 100μl FFPE digestion enzyme onto the slides for 15–20 minutes at 37°C. The slides were then washed with PBS as previously and dehydrated with graded ethanols (70%, 85%, 95% and 100%). The ready-to-use digoxygenin-labelled HER2/*neu* probe (consisting of two contig BAC clones, ZYMED) was applied onto the slides, which were covered with 22x22 mm coverslips (10μl probe mixture/slide). The slides were denatured at 95°C for 10 minutes, and then hybridized at 37°C overnight. After hybridization, the slides were washed with 0.5x standard saline citrate for 5 minutes at 75°C, followed by three washes in PBS/0.025% Tween-20, pH 7.4, at room temperature. Then, the slides were incubated with CAS-Block. The HER2/*neu* probe was detected with sequential incubation with the following antibodies: FITC-Sheep Anti-Digoxigenin (incubation for 55–60 minutes at room temperature), HRP-Goat Anti-FITC (incubation for 55–60 minutes at room temperature). The enzymatic reaction was performed with DAB (incubation 20–30 minutes at room temperature). Tissue sections were counterstained with hematoxylin and coverslipped.

Detection of the HER2/*neu* oncogene by CISH was evaluated according to the criteria of Kumamoto et al. [22] in a minimum of 50–60 of invasive breast carcinoma cells. A lack of amplification was defined as 1–4 gene copies per a nucleus, low-level amplification was defined when 5–8 gene copies per a nucleus or small gene copy clusters were found in > 50% of tumor cells; and high level amplification was defined when > 8 gene copies per a nucleus or large gene copy clusters where found in > 50% of tumor cells.

In situ hybridization assay using DAKO GenPoint Catalyzed Signal Amplification System for in situ Hybridization with silver autometallography

Archival formalin-fixed paraffin-embedded tissue sections, 4–5-μm-thick, were deparaffinized, dehydrated in three changes of absolute ethanol and then soaked in distilled water. The tissues were then cell-conditioned using a target retrieval solution (10mM citric buffer, pH 6.0) for 40 minutes at 95°C, and cooled at room temperature for 20 minutes. After a few changes of distilled water washes, the tissue sections were digested with Proteinase K (DAKO) at a 1:5000 dilution in 50mM of Tris/HCl, pH 7.6, for 5 minutes at room temperature. After 5-minute washing (3 times) in distilled water, endogenous peroxidase was blocked for 20 minutes using 3% hydrogen peroxide in absolute methanol at room temperature. After a 10-20 minute distilled water wash, the tissue sections were dehydrated with graded alcohols (100%, 95% and 70%) and then air-dried. A biotinylated cDNA probe INFORM HER-2/*neu* Probe (Ventana Medical Systems, Inc, Tucson, AZ) was applied as 10-15μl, coverslipped and sealed with rubber cement. The probe and the target were co-denaturated for 10 minutes at 90°C and hybridized at 50°C overnight. After hybridization, the tissue sections were subjected to a stringency wash of 2x SSC: 6 minutes at 50°C, then 6 minutes at 60°C. Then, the slides were washed with phosphate-buffered saline (PBS) containing 0.1% Tween-20, pH 7.6, for 5 minutes at room temperature. Streptavidin-horseredish peroxidase (DAKO SA-HRP from GenPoint Kit at dilution of 1:800), 100μl total volume, was applied for 15 minutes at room temperature. The sections were washed in PBS with 0.1% Tween-20 three times (5 minutes for each wash). Prediluted tyramide signal amplification (TSA) biotin (DAKO GenPoint Kit) was then applied to the sections for 5 minutes at room temperature. The tissues were then washed with PBS/Tween 20 three times for 5 minutes each, treated with Lugol's iodine by immersion for 5 minutes, followed by 3 rinses in distilled water, and cleared by a few-second immersion in sodium thiosulfate (2.5%) to remove contaminating heavy metals in the sections that would interfere with autometallography. The slides were then washed 5 times in double-distilled water for a total of 7 minutes and after that – immersed for 5 minutes in PBS, pH 7.6, containing 0.1% cold water fish

gelatin (Sigma). Streptavidin-NANOGOLD (Nanoprobes, Inc., Yaphank, NY) prediluted to 1:250 with PBS, pH 7.6, containing 1% bovine serum albumin was applied and then the slides were incubated for 30 minutes at room temperature. The tissues were subsequently washed with PBS, pH 7.6, two times for 5 minutes, immersed in PBS, pH 7.6, with 0.1% cold water fish gelatin for 5 minutes, rinsed in double-distilled water several times throughout 10 minutes (the last two changes using ultra pure water) and the autometallographic signal was developed using silver acetate (AMG – Silver Acetate Autometallography). Solutions A (80 mg silver acetate in 40 ml of ultra pure water) and B (200 mg hydroquinone in 40 ml citrate buffer) were freshly prepared for every run as reported by Hacker [13]. Solutions A and B were mixed just before using and the tissue sections were immersed in these solutions for 10-20 minutes. The amplification reaction was terminated by washing in distilled water. Then, the sections were counterstained with nuclear fast red, dehydrated in graded alcohols and cleared in xylene and mounted in Shandon Consul-Mount.

The slides were interpreted as non-amplified (if only one or two small discreet black signals were identified within 50–60 nuclei of the invasive carcinoma), low-level amplified (4 to 8 discreet nonconfluent signals), or amplified (massive black confluent signals) using conventional light microscopy.

Statistical analysis

The statistical analysis was performed using the Statistica 6.0 PL (StatSoft Inc., USA) and Excel 2003 (Microsoft Corp. USA). The Spearman's rank correlation coefficient, gamma correlation coefficient, Pearson χ^2 test, and kappa statistics were used when appropriate. The significance level was set to 0.05.

Results

The histological classification, grading and immunostaining results for HER2/*neu* of the 63 tumors are shown in Table 1. Table 2 illustrates the relationship of the HercepTest staining intensity with the ISH assay results. The photomicrographs exemplifying each of the methods are shown in the Figures 1–8.

In IHC 3+ group by the FISH method, there were 23 cases with high *HER2* gene amplification (69.7% concordance rate between IHC and FISH), 2 cases with low amplification (6.1%) and 8 without gene amplification (24.2%). In IHC 2+ group by FISH, there were three cases with high gene amplification (10%), there was a lack of low amplification cases (0%), and 27 cases without gene amplification (90%) were observed.

TABLE 1
Histology, grade vs HER2 immunohistochemistry of invasive mammary carcinoma

IHC / Histological type and grade	3+	2+
Ductal, Bloom I°	**2**	**4**
Ductal, Bloom II°	**13**	**13**
Ductal, Bloom III°	**17**	**9**
Lobular	**1**	**4**
Total (63)	**33**	**30**

TABLE 2
Relationship between HER2 immunohistochemistry (for weak (2+) and strong (3+) overexpression) and FISH (63 cases), SILVER (63 cases) and CISH (55 cases) amplification in invasive mammary carcinoma

IHC	FISH High amplification	FISH Low amplification	FISH Non-amplified
3+	69.7%	6.1%	24.2%
2+	10.0%	0%	90.0%
	SILVER High amplification	SILVER Low amplification	SILVER Non-amplified
3+	75.7%	18.2%	6.1%
2+	30.0%	50.0%	20.0%
	CISH High amplification	CISH Low amplification	CISH Non-amplified
3+	65.4%	23.1%	11.5%
2+	3.4%	38.0%	58.6%

In IHC 3+ group by hybridization *in situ* with the silver autometallography method (SILVER), there were 25 cases with high *HER2* gene amplification (75.7% concordance rate between IHC and SILVER), 6 cases with low amplification (18.2%) and 2 cases without gene amplification (6.1%). In IHC 2+ group by SILVER, there were 9 cases with high amplification (30%), 15 cases with low amplification (50%) and 6 cases without gene amplification (20%).

In IHC 3+ group by the CISH method, there were 17 cases with high *HER2* gene amplification (65.4% concor-

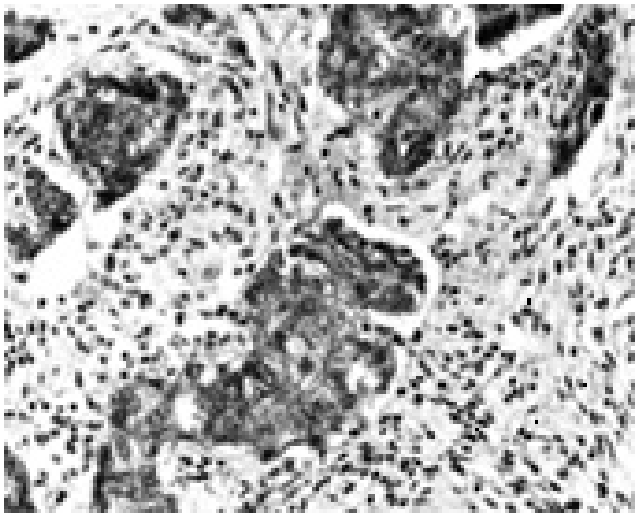

Fig. 1. IHC for HER2 in breast cancer: 2+ over expression level, distinct but variable membrane staining (objective lens 20×).

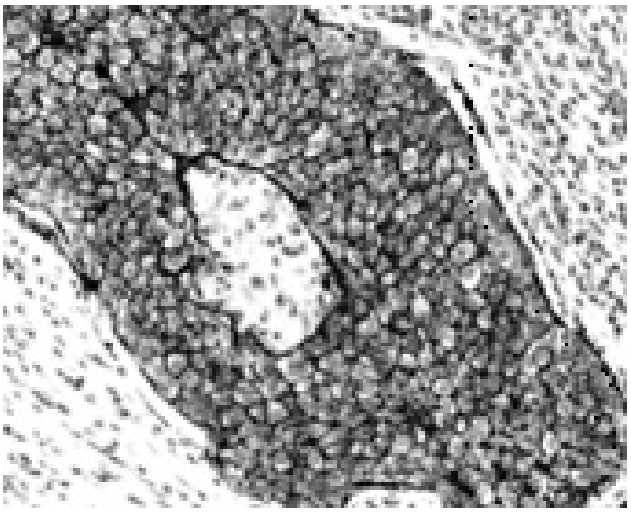

Fig. 2. IHC for HER2/neu in breast cancer: 3+ over expression level, intense membrane staining in all tumor cells (objective lens, 20×).

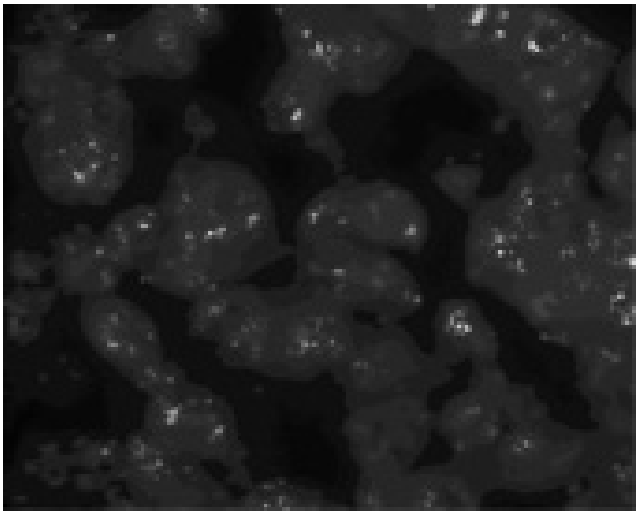

Fig. 3. FISH showing HER2 amplification - red signal, (objective lens, 100×).

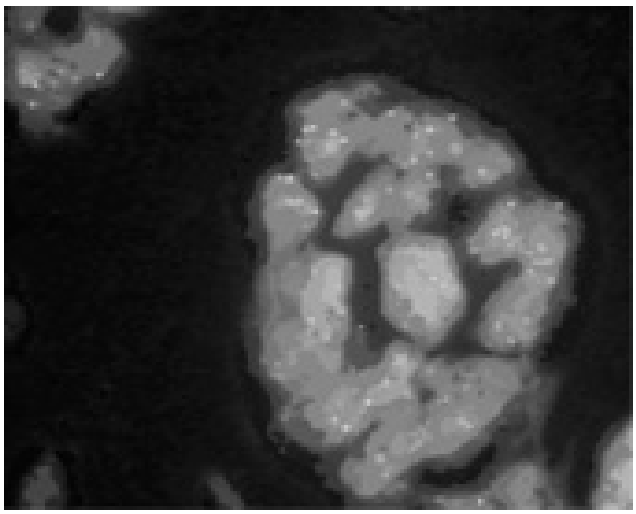

Fig. 4. FISH showing HER2 amplification - red signal for HER2 amplification, green signal for chromosome 17, (objective lens, 100×).

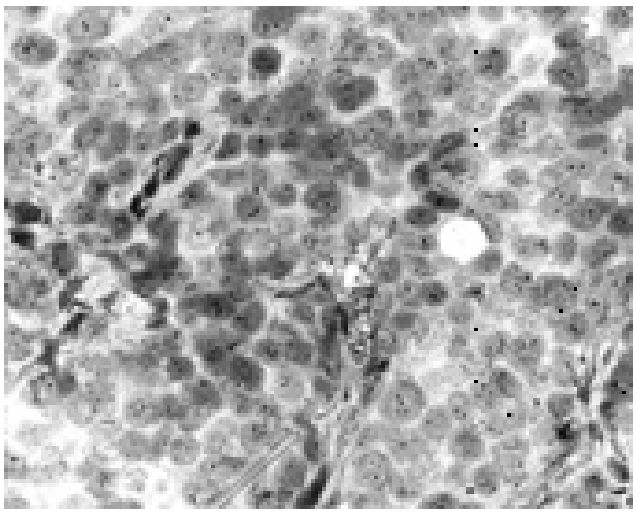

Fig. 5. CISH for HER2 in breast cancer. No HER2 amplification showing in 2–3 copies per nucleus – brown dots (objective lens, 40×).

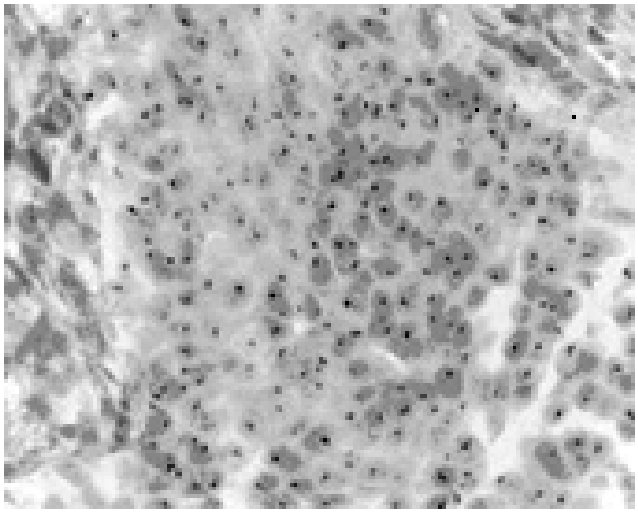

Fig. 6. CISH for HER2 in breast cancer. High-level HER2 amplification – brown clusters (objective lens, 40×).

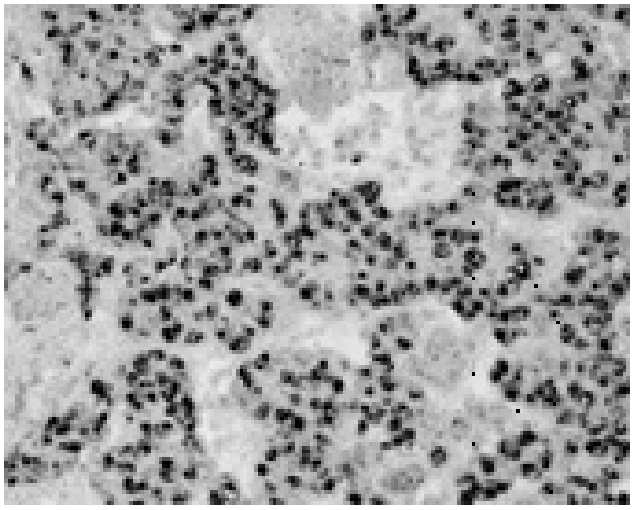

Fig. 7. Hybridization *in situ* with silver autometallography for HER2 in breast cancer. High-level HER2 amplification – black clusters (objective lens, 20×).

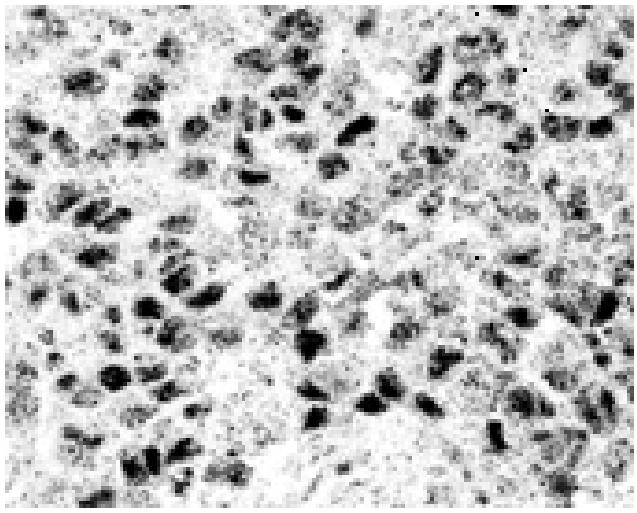

Fig. 8. Hybridization *in situ* with silver autometallography for HER2 in breast cancer. High-level HER2 amplification – black clusters (objective lens, 40×).

dance rate between IHC and CISH), 6 cases with low amplification (23.1%), and 3 cases without gene amplification (11.5%). In IHC 2+ group by CISH, there was 1 case with high amplification (3.4%), 11 cases with low amplification (38%) and 17 cases without gene amplification (58.6%).

The concordance between the IHC and ISH methods is shown in Table 3 and Table 4.

The overall concordance rate between the IHC and ISH methods was not perfect; however, the statistical analysis revealed a good agreement between the IHC and ISH methods and among the ISH methods. A moderate agreement was found using kappa statistics among all the methods.

Discussion

Evaluation of the *HER2* oncogene status has recently become an important biomarker for identifying patients who would respond to anti-HER2 therapy [21]; this is why it is so important to assess the best method for detection of amplification or overexpression of HER2.

TABLE 3
The relationship of results obtained by IHC and ISH methods

	IHC vs FISH	IHC vs SILVER	IHC vs CISH
Pearson χ^2 test	p << 0.01; 27.62	p << 0.01; 13.27	p << 0.01; 25.40
Gamma correlation coefficient	0.91	0.69	0.86
Spearman rank correlation coefficient	0.65	0.45	0.65
Kappa coefficient	0.11	0.58	0.25

TABLE 4
The relationship of results obtained among different ISH methods

	CISH vs FISH	FISH vs SILVER	SILVER vs CISH
Pearson χ^2 test	p << 0.01; 40.70	p << 0.01; 39.91	p << 0.01; 30.15
Gamma correlation coefficient	0.96	0.96	0.89
Spearman rank correlation coefficient	0.79	0.72	0.70
Kappa coefficient	0.53	0.38	0.58

The most popular method for evaluation of the HER2 status is immunohistochemistry. Generally, IHC is currently extensively used as a diagnostic tool for determining the presence or absence of particular proteins and certain carbohydrates in routinely fixed and embedded tissue specimens [16]. This method is relatively inexpensive and easy to perform in comparison with hybridization *in situ* methods. IHC visualizes cell types that may carry abnormalities, but the problem is in the intralaboratory, and especially interlaboratory standardization of this method. Because immunostaining results can directly determine therapeutic decisions, assuring the reliability and reproducibility of the method is necessary. A large number of factors influence staining results, causing a high degree of interlaboratory variability in the obtained results [16], especially when applying different, commercially available antibodies [16, 18, 43]. In the IHC method, the most discrepant results are noted in the 2+ group (weak positive) – in these cases, evaluation and confirmation by hybridization *in situ* is essential [18].

In situ hybridization (ISH) is one of the basic methods of molecular biology and provides the advantage of visualizing and even quantifying clinically relevant molecules in a morphological context. It is one of the most important techniques of visualization gene expression at the cellular level in tissues [16].

The FISH method in comparison to IHC is a more complex and more expensive technique; nevertheless, is easier for interlaboratory standardization [25]. It is also highly sensitive [29], although it does not allow for a simultaneous histological examination of tissues structure beyond basic tissue identification. Some authors consider that FISH should be a basic method for evaluation the HER2 status [15]. A disadvantage of FISH is the requirement of expensive equipment for the analysis and results archiving (a fluorescent microscope with suitable filters, a sensitive CCD camera), which not all diagnostic laboratories can afford. Moreover, fluorescent signals tend to fade in a few weeks or months, and the analysis of FISH results is time consuming, the FISH procedure takes two days (as compared to only a few hours needed for the IHC technique) [36]. Sometimes it is not possible to perform FISH in paraffin-embedded tissues, because of poorly fixed and preserved tissue or difficulties with digestion.

Taking into consideration the advantages and disadvantages of IHC and FISH, combinations of these techniques were worked out. They consist in a hybridization reaction (as a more sensitive and specific method) based on labeled antibodies and an immunoenzymatic reaction (easier to visualize and store) called CISH [43] or ISH with detection using autometallography, e.g. with silver or gold deposition (GOLDFISH) in the reaction site [36].

These methods do not require very expensive equipment for the analysis and slide archivization, result interpretation is faster (light microscope) and more user-friendly, because it does not require "working in the dark", there is also a possibility for simultaneous tissue structure verification.

In the CISH technique (elaborated by the ZYMED company), the probe is very specific and does not require repetitive sequence blocking [32, 43]. But in both techniques – CISH and hybridization with autometallography – it is essential to confirm DNA ploidy, because it is impossible to detect two or more different signals as the reaction is monochromatic [32]. Some authors consider that CISH is more sensitive than FISH [22], but an indisputable advantage of CISH over FISH is the possibility of morphological tissue verification in light microscopy and non- bleaching signals [32].

The presently tested hybridization *in situ* with silver autometallography is the most laborious among the presented methods, requires more tests for proper standardization, as it uses a lot of reagents from different manufacturers. Often, intense background staining makes the interpretation of results difficult or even impossible. Nevertheless, it is a very sensitive method due to additional amplification step with biotin [42].

In the present study, the HER2 marker was evaluated by the ISH methods for 63 patients with invasive carcinoma (only the group with 2+ and 3+ overexpression level by IHC). The size of the investigated group is not very large, but it allows for receiving statistically significant results.

In our series, the best concordance rate was in 3+ group by IHC and SILVER – 75.7%, then for IHC and FISH – 69.7%; the poorest concordance result was observed between IHC and CISH methods – 65.4%. The highest rate of cases with 3+ overexpression and without gene amplification was observed in the FISH method – 24.2%, the lowest for the SILVER method – only 6.1%.

In the present study, the overall percentage of concordance is not so very high, but Hammock et al. showed that strong HER-2/*neu* protein overexpression by immunohistochemistry often does not predict oncogene amplification by fluorescence *in situ* hybridization [14]. They investigated 102 invasive breast cancer cases comparing IHC (HercepTest, DAKO) and FISH (Vysis). Only 22 of 45 tumors with 3+ positivity (49%) did show amplification by FISH, what was less than in our study (almost 70% concordance rate between IHC and FISH by Vysis). Here, we obtained 10% cases with high HER2 amplification for 2+ expression level by IHC; however, Hammock et al. obtained 6% cases in the same group. They stated that more than 50% of breast tumors with strong 3+ HER2/*neu* overexpression did not show oncogene amplification by FISH, and most tumors with 2+ and negative IHC also

failed to amplify. After the analysis of their research, they postulated that in their experience, FISH studies should be performed on all 3+ and 2+ staining tumors to avoid inappropriate and toxic treatment [14].

The cases qualified to the 3+ IHC group should show a strong complete membrane staining observed in more than 10% of the tumor cells, according to the published scoring guidelines of the HercepTest. Perhaps we should take into consideration stricter criteria and increase the threshold of tumor cells for all staining patterns. Sometimes an overstated percentage of 3+ IHC group was misclassified because of cytoplasmic staining [24]. In the CISH scoring system, the commonly recommended threshold criterion is 50% of tumor cells [22, 32]. The immunohistochemical scoring system might be verified and the criterion should be moved to 40–50% of tumor cells. Hammock et al. believe that the decision to perform FISH on IHC-negative tumors should be guided by additional parameters, including tumor grade and estrogen receptor status as well [14].

In his review [15], Hayat quotes many research results from various laboratories. According to some authors (Persons DL. et al. [26]), he affirmed that the existing discrepancies between IHC and FISH results were not uncommon. Grushko et al. confirmed that overexpression estimated on 2+ and 3+ level by IHC did not have to be related to gene amplification [11], what was evident in the present study as well.

Other researchers (Gupta et al.) showed that the overall concordance rate was 83.9% between CISH (ZYMED) and FISH (PathVysion, Vysis), but only for 31 cases with invasive breast carcinoma [12]. Zhao et al. obtained a complete agreement between CISH and FISH (62 cases), but first they performed FISH and then they continued with CISH detection [43]. Similar results concerning the equivalency of FISH (PathVysion and HER2 INFORM, Ventana Medical Systems) and CISH (ZYMED) were obtained by Arnould et al. (96% concordance) [35].

Hoang et al. obtained high concordance between IHC (HercepTest) and FISH (PathVysion) – 91% cases in 3+ group by IHC showed high gene amplification, 99% cases were without overexpression and with a lack of gene amplification [18]. A somewhat higher concordance rate between IHC (HercepTest) and FISH (PathVysion) – 97% – was achieved in the 3+ group and high *HER2* amplification by Tsuda et al. in 215 cases of ductal and lobular invasive breast carcinoma [35].

In some cases (1–7%) protein overexpression without the *HER2* gene amplification may be present. It may be caused by alternative transcriptional or post-transcriptional mechanisms controlling *erbB*-2 expression [8, 19]. Also, a reverse situation is possible, with gene amplification without protein overexpression, but it is very rare (1–2%). Such a result might be caused by tissue fixation [18].

In conclusion, results interpretation in the IHC method is rather subjective and the ISH methods appear to be more unambiguous. In practice, it is not so clear if we analyze each hybridization *in situ* technique, what was showed in the current study.

We believe that it is essential to apply stricter criteria for scoring in the immunohistochemistry method. It may be especially appropriate for cases, which are classified as 2+ level of HER2 overexpression. The threshold of 40–50% tumor cells in a tissue section with a weak to moderate complete membrane staining would give more reliable results than considering the threshold of only 10% of tumor cells. It should be also considered to verify by FISH all cases, which are classified as 3+ level protein overexpression if the number of positive tumor cells only marginally exceeds 10%. However, we believe that the first stage for evaluation of the HER2 marker status should remain immunohistochemistry, and that the 2+ group and some patients with 3+ score, as stated above, should be verified by some ISH method.

We think that CISH is the method with the easiest and the fastest results interpretation; the FISH result interpretation is the most time consuming (although its internal control for chromosome 17 should be emphasized), while the greatest challenge is presented by background staining often appearing in hybridization *in situ* with silver autometallography.

The present results indicate that all *in situ* hybridization methods are equivalent tools for evaluating *HER2* gene amplification in archival material. There is no clear answer which method is the best assay to determine the HER2 marker status, although here some advantages and disadvantages of all the described techniques are presented and an algorithm is proposed for deciding which method is to be selected for a given laboratory.

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