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Original article

Comparison of *in situ* hybridization methods for the assessment of HER-2/neu gene amplification status in breast cancer using a tissue microarray

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ABSTRACT

Background: This project compared HER-2/neu gene status in breast cancers, as demonstrated by FISH (fluorescent *in situ* hybridization) and CISH (chromogenic *in situ* hybridization) and using a tissue microarray (TMA). The study also aimed to show whether the TMA technique could be used in clinical diagnostics, rather than remain a scientific tool.
Materials and methods: A TMA was constructed using 121 breast cancer specimens, 6 cores from each specimen. Demonstration and assessment of HER-2/neu gene status was by FISH (Vysis Path) and CISH (DAKO Duo CISH).
Results: The 121 breast cancer specimens were divided into 3 groups by HER-2 status, as determined by immunohistochemistry. In the HER-2 negative group no amplification was observed in 36 out of 40 cases. 3 cases showed amplification by both methods and one by CISH alone. The equivocal HER-2 group showed no amplification in 30 out of 41 cases and amplification in 9 cases. One case was FISH negative CISH positive and one was discarded. In the HER-2 positive group, amplification was confirmed in 37 of the 40 cases by both methods. 3 cases were unsuitable for assessment.
Conclusions: This study indicated that CISH is a sensitive alternative to FISH in detecting HER2 gene amplification and may replace FISH in HER2 testing. Good agreement was observed between methods (98.5% – 119 out of 121 cases).

Furthermore, as only 4 out of 121 cases were unsuitable for assessment (no signal or missing TMA cores) – it may be feasible to use TMA in diagnostics.

1. Background

One of the most important prognostic and predictive markers in breast cancer is Human Epidermal Growth Factor Receptor 2 (HER2), whose overexpression is associated with an aggressive disease course. In many Polish oncology centres, including the Greater Poland Cancer Centre in Poznan, HER2 status testing has been included in a routine diagnostic procedure applied to all new cases of breast cancer since 2001.[9] The assessment is carried out according to an algorithm (Fig. 1). A routinely obtained result is verified, using the *in situ* hybridization method, only in the event when the result is borderline positive (15–30% of all cases),[4] that is the result

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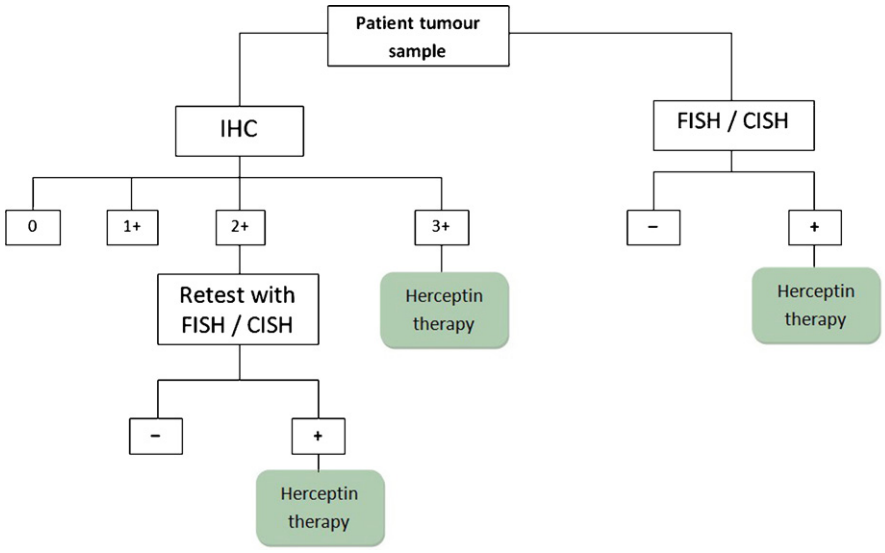

Fig. 1 – The algorithm for the determination of HER2 status in breast cancers.

of immunohistochemical HER2 assessment is graded 2+. Amongst patients with metastases to the lymph nodes, overexpression of HER2 is associated with a median survival period of 3 years, while a 6–7 year survival period is an average for patients without overexpression of HER2 in their cancer cells.[7,10,18]

The value of HER2, both as a prognostic and as a predictive factor, requires practical, repeatable, reliable and easily accessible assessment methods. In practice, methods for the assessment of HER2 status include immunohistochemistry (IHC) and *in situ* hybridization (fluorescent – FISH and chromogenic – CISH). Their value arises from the possibility of assessing the parameter of interest within preserved cancer specimens (IHC) or within the nuclei of cancer cells (FISH, CISH) by means of an optical microscope (or a fluorescence microscope in the case of the FISH method). Besides, these methods allow a simple testing of archival neoplastic tissues stored in paraffin blocks. HER2 testing should be performed routinely in the case of patients with a diagnosis of invasive breast cancer. Some controversy exists, however, as to the best method for the determination of HER2 status. This concerns not only the choice of test, but also the optimal method for the application of that test to every case.[11,16–18]

In spite of efforts on the part of the international pathology community aimed at improving the status of HER2 testing in routine practice,[6] inadequacies in immunohistochemical and *in situ* hybridization tests remain a serious problem.[16,17]

For the purpose of saving both time and money, it seems reasonable to test as many samples as possible on a single slide. The introduction of the tissue microarray (TMA) technique in 1986, with further subsequent improvements, helped achieve this goal. This approach allows the assessment of many sections at a time by putting tens of tissue cores into one paraffin block (up to 200 per block) and simultaneously reduces the amount of reagent required to test each specimen.[1–3,13]

2. Aim

The aim of this study was to confirm that:

1. The CISH method is concordant with the currently most widely used FISH method, for the assessment of HER2/neu status in breast cancer cells.
2. The TMA technique is a reliable and inexpensive approach to the simultaneous assessment of HER2/neu status in breast cancer samples taken from many different patients.

3. Materials and methods

The study group comprised of 121 women diagnosed in the Greater Poland Cancer Centre in 2009, aged between 34 and 87 years, with a clinical diagnosis of invasive breast cancer.

Cases were excluded from the study if the originally diagnosed material had been obtained by fine needle aspiration biopsy or if the fixation of the tissue had been insufficient. On the basis of a histopathological assessment of immunohistochemically stained slides (stained for overexpression of the HER2 receptor), the group of 121 breast cancer cases was subdivided into 3 groups (by IHC):

1) negative (IHC based HER2 receptor status: score 0/1)
2) equivocal (IHC based HER2 receptor status: score 2)
3) positive (IHC based HER2 receptor status: score 3)

Subsequently, on the basis of a histopathological assessment of traditionally stained haematoxylin and eosin (H&E) stained slides, areas of tissue containing cancerous cells were selected. These areas defined source material for the collection of tissue cores to be used in the construction of tissue microarray (TMA) blocks.

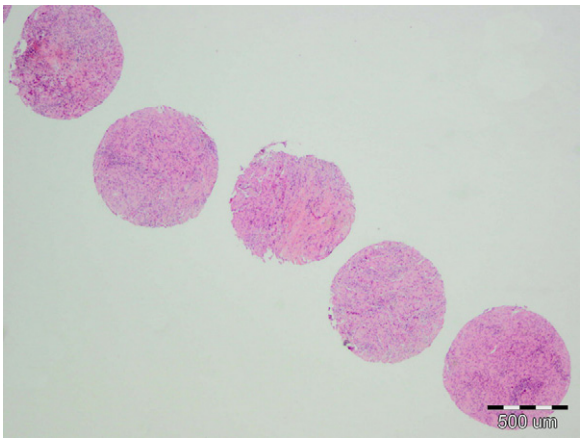

Fig. 2 – Tissue cores stained by haematoxylin and eosin (magnification ×20).

3.1. *Construction of the tissue microarray (TMA)*

From each of the selected paraffin blocks tissue cores were sourced, as described above, and collected by means of suitable needles (Beecher Instruments). These cores were transferred to a special multi-tissue TMA block. Six such cores, of 0.6 mm in diameter, were selected from each specimen for the purpose of reducing the risk of data loss through destruction of cores during subsequent sectioning and staining procedures. The TMA blocks were cut at a thickness of 4 μm, and the sections were mounted onto "Superfrost Plus" microscope slides (Thermo Scientific). In total, 6 TMA blocks were prepared, each containing 6 tissue cores (thus ensuring the presence of representative material) from each of the 20 cases of breast cancer.

The slides with sections from the TMA blocks were deparaffinized and stained according to a traditional H&E method (Fig. 2) and by immunohistochemistry (Fig. 3) for the purpose of confirming the presence of cancer tissue in each core of the TMA. Identical TMA sections were then stained according to appropriate FISH and CISH (duo-CISH) procedures.

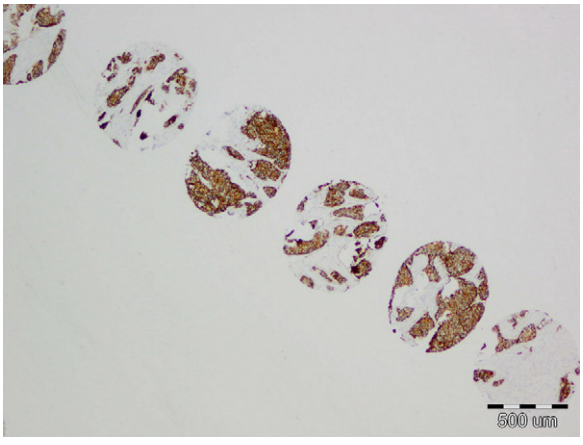

Fig. 3 – Tissue cores stained by immunohistochemistry (magnification ×20).

3.2. *FISH staining according to the "Path Vysion" method for the "Vysis" HER-2/neu probe kit*

Slides bearing TMA sections were deparaffinized and rehydrated prior to incubation in "Pre-Treatment Solution" at 80 °C in a water bath for 30 min. Subsequently, the samples were subjected to protease enzymatic digestion in a water bath at 37 °C for 15 min. In later phases of the procedure we carried out denaturation and hybridization with a molecular probe in a hybridizer (denaturation at 72 °C for 5 min. and hybridization at 37 °C for 14–20 h). The following day the samples were rinsed in post-hybridization buffer to remove unbound probe (72 °C for 5 min. in a water bath). The complex was visualised using DAPI fluorescent complex. The specimens were mounted under cover-slips and examined using a fluorescence microscope.

3.3. *Assessment system for fluorescent in situ hybridization*

The FISH tests were carried out using proprietary probes from PathVysion™ (Abbott/Vysis: LSI® HER2 Spectrum Orange™ and CEP 17 Spectrum – Green™).

Analysis of signals was performed by 2 independent observers using a fluorescence microscope (magnification ×1000). Hybridization signals were counted in 20 cell nuclei from each core. A negative result was recorded if the FISH coefficient was <1.8 or when the copy number of the Her-2/neu gene was <4.0. A positive result for amplification of the Her-2/neu gene was recorded if the FISH coefficient was >2.2 or the gene copy number was >6.0. In the event when the FISH coefficient was found to be between 1.8 and 2.2, or the copy number was found to be between 4.0 and 6.0, cases were deemed to be equivocal.

3.4. *CISH staining according to the "Dako Duo CISH" method for the "HER2 CISH pharmDx™" probe kit*

Slides bearing TMA sections were deparaffinized and rehydrated prior to incubation in "Pre-Treatment Solution" at 95–99 °C in a water bath for 10 min. Subsequently, the samples were subjected to enzymatic digestion in pepsin at 37 °C in a hybridizer for 6 min. This was followed by denaturation and hybridization with a molecular probe in a hybridizer (denaturation at 82 °C for 5 min and hybridization at 45 °C for 14–20 h). The following day, unbound probe was rinsed away using Stringent Washing buffer (65 °C for 10 min) in a water bath. In later phases of the procedure, endogenous peroxidase activity was blocked, followed by incubation with CISH antibody mixture. For visualisation, the Red and Blue Chromogen complexes were applied. Cell nuclei were stained in haematoxylin. The slides were sealed using a sealing mountant (Tissuemount) and coverslipped prior to assessment using an optical microscope.

Table 1 – FISH and CISH results for 121 cases.

Result	Number of cases tested by FISH	Number of cases tested by CISH
Negative	68	66
Amplification	49	55
No result	4	0
Total	121	121

3.5. *Assessment system for chromogenic in situ hybridization*

CISH tests were performed using proprietary DNA probes from Dako (HER2 CISH pharmDx™. HER2 Spectrum Red and CEP 17 Spectrum – Blue).

An analysis of the signals was carried out by 2 independent observers using an optical microscope (magnification ×1000). Hybridization signals were counted for 20 cell nuclei in each core. A result for amplification of the Her-2/neu gene was counted as negative if the FISH coefficient was <1.8 or the copy number of the Her-2/neu gene was <4.0. A positive result for amplification of the Her-2/neu gene was recorded if the FISH coefficient was >2.2 or if the Her-2/neu gene copy number was >6. Cases where the FISH coefficient was between 1.8 and 2.2 or where the Her-2/neu gene copy number was between 4.0 and 6.0 were deemed to be equivocal.

4. Results

121 cases of breast cancer from patients diagnosed in the Greater Poland Cancer Centre in Poznan in 2009 were analysed. These cases were divided into 3 groups (according to their HER-2 status, as determined by immunohistochemistry (IHC)). Six tissue microarrays were constructed using 6 tissue cores from each patient. The results of all FISH and CISH tests are shown in Table 1.

From among the 121 cases analysed, the FISH method showed no amplification of the HER2 gene in 68 cases (Fig. 4) and amplification of the gene in 49 cases (Fig. 5). Due to technical issues, 4 cases could not be analysed using the FISH method (either there were no signals seen during microscopic examinations or insufficient TMA spots were available for that patient). Using the CISH method, 66 cases showed no amplification (Fig. 6), while the remaining 55 cases showed amplification of the HER-2 gene (Fig. 7). In the 4 cases where FISH could not be used, the CISH procedure showed amplification. CISH also showed amplification in 2 cases which FISH had shown to be negative. The results broken down into the tested groups (HER2 negative, equivocal and positive) are shown in the Tables 2–4.

5. Discussion

During this project it was noted that the CISH technique is both a sensitive and specific method for the detection of HER-2 and could be used in the same role as FISH in the HER2 testing algorithm. The observed level of agreement between the two methods was 98.5% (119 cases out of 121). The *in situ*

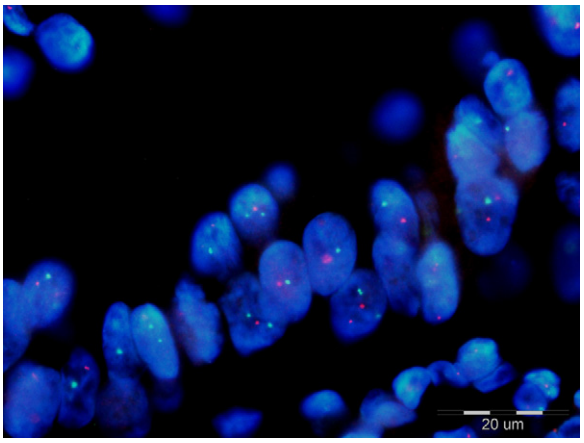

Fig. 4 – FISH showing no amplification of the HER2 gene in invasive breast cancer. The HER2 gene is seen as a red signal while the centromeres of chromosome 17 are seen as green signals. This test is negative for amplification of the HER2 gene as the ratio HER2/CEP17 is less than 1.8.

hybridization method was compared with immunohistochemistry in other studies, where the level of agreement ranged from 75% to 99%.[2,5,8,10,14,15,19,20] For example, comparing our results with those presented by Hannah et al.,[10] the level of agreement between FISH and CISH in the three groups stratified according to HER2-IMH status was slightly lower in our study. The explanation for this difference is probably the number of patients in the group studied by Hannah, which was more than a half larger than our group. In general, the discrepancies between the methods (FISH negative, CISH positive) could be explained by the occurrence of chromosome 17 polysomy or incorrect counting of unspecific deposits in the cell nuclei. Hannah et al. also described the most frequent difficulties in the interpretation of results – the possibility of having several copies of a gene visible in one place when cell nuclei are aligned in stacks in thick sections.[10] Despite those shortcomings, the CISH method still has many advantages. In

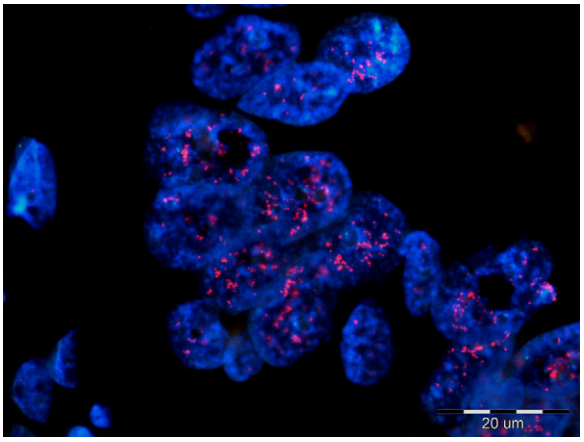

Fig. 5 – FISH showing amplification of the HER2 gene in invasive breast cancer. Gene HER2 is seen here as a red signal while the centromeres of chromosome 17 are green. A high level of amplification of the HER2 gene is evident from the HER2/CEP17 ratio which is higher than 1.8.

Table 2 – Correlation between FISH and CISH in the 40 cases of breast cancer of the HER-2 negative group (as assessed by immunohistochemistry).

	CISH−		CISH+		Unsuitable for CISH assessment
FISH−	36	90%	1	2.5%	0
FISH+	0	0	3	7.5%	0
Unsuitable for FISH assessment	0	0	0		0

Table 3 – Correlation between FISH and CISH in the 41 breast cancer cases of the HER-2 equivocal group (as assessed by immunohistochemistry).

	CISH−		CISH+		Unsuitable for CISH assessment
FISH−	30	73%	1	2.5%	0
FISH+	0		9	22%	0
Unsuitable for FISH assessment	0		1	2.5%	0

Table 4 – Correlation between FISH and CISH in the 40 cases of breast cancer of the HER-2 positive group (as assessed by immunohistochemistry).

	CISH−	CISH+		Unsuitable for CISH assessment
FISH−	0	0		0
FISH+	0	37	92.5%	0
Unsuitable for FISH assessment	0	3	7.5%	0

comparison to FISH, the CISH method does not require access to expensive equipment for analysis of results. Interpretation of the results is done using an ordinary optical microscope and does not require work in the dark (as is the case when using a fluorescence microscope for the FISH method). The CISH method also offers the possibility to make a simultaneous verification of the structure of tissues as well as to archive slides for longer periods than is possible with the FISH method.[10,15,19]

The question of whether or not tissue cores can be representative of whole tumours is frequently raised in regard to the TMA technique. The majority of tumours are of heterogeneous nature and a small sample of tissue will not always display the same biological characteristics as a whole tumour. For this reason, the TMA technique is also frequently questioned as a useful diagnostic tool and is usually used only in scientific studies. In order to increase the credibility of the TMA technique while reducing the likelihood of error associated with the difficulty of obtaining a representative sample, many authors use several cores from the same donor tissues. There are several papers discussing the issue of the number of cores that are needed to produce comparable results to those obtained from whole sections. Some of the authors, like Jourdan et al.,[12] found that only a few (up to four) cores are required to achieve a 100% agreement. Others, like Camp et al.,[3] found, however, that even when as many as 10 tissue cores are taken from a tumour, some disagreement may be

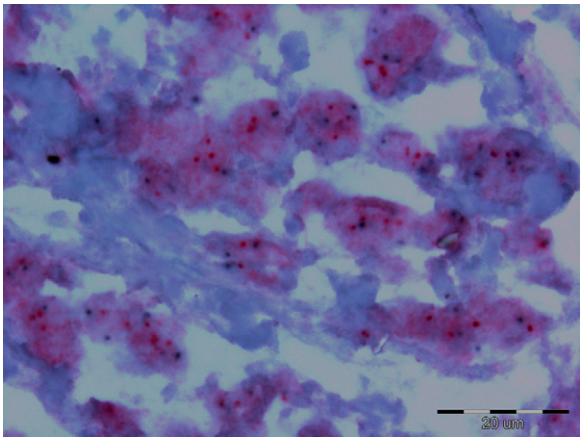

Fig. 6 – CISH showing no amplification of the HER2 gene in invasive breast cancer. The HER2 gene is seen as a red signal while the centromeres of chromosome 17 are seen as dark-blue/black signals. This test is negative for amplification of the HER2 gene as the ratio HER2/CEP17 is less than 1.8.

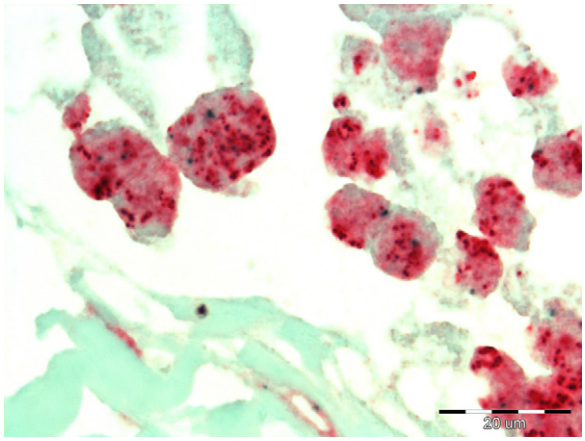

Fig. 7 – CISH showing amplification of the HER2 gene in invasive breast cancer. Gene HER2 is seen here as a red signal while the centromeres of chromosome 17 are dark-blue/black. A high level of amplification of the HER2 gene is evident from the HER2/CEP17 ratio which is higher than 1.8.

noted in the results. In our opinion, it should be noted that the experience of the person responsible for collection of material from the source samples is of significance. In order to ensure that the material used in this study would be representative, 6 cores of tissue were collected from each case.

In our study the agreement between the HER2 receptor using FISH on microarray material and on full sections amounted to 95% and was slightly better than that achieved in a study by O'Grady et al. 14 They showed the advantages of the tissue microarray technique, such as having various primary tumours in a single paraffin block which allows a simultaneous analysis of a great many cases with the use of very few slides. These slides also have the advantage of ensuring that all the tissue samples are treated using exactly the same conditions for staining or marker demonstration while using significantly less tissue for the test.

In the results of this study, only 4 out of 121 cases tested by the TMA method could not be assessed (owing to a lack of signal or TMA cores) – which suggests that it is possible to apply tissue microarrays to clinical diagnostics. At the same time, the use of TMA allows the reduction of costs associated with testing for the amplification of the HER-2/neu gene using *in* situ hybridization techniques. This in turn could allow the introduction of the TMA technique to routine clinical diagnostics in all cases of breast cancer, not only borderline cases (those where immunohistochemistry for the HER2 receptor was scored as 2+).

6. Conclusions

In conclusion, the results of this study show that CISH may be an alternative to the FISH method in the assessment of HER2 gene amplification for all patients with invasive breast cancer, using equipment widely available in diagnostic pathology laboratories. We also confirmed the suitability of the tissue microarray technique for a simultaneous assessment of HER2 gene amplification status in tumours arising from tens of different patients, while maintaining a representative tissue sample in each case, with good reproducibility and credible results. It would not be unreasonable to assume that further improvements to the techniques described in this study may soon lead to a reduction in costs and improved turnaround time in the diagnostic process.

Conflict of Interest

None declared.

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