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ARTICLE

Detection of HER-2/neu Gene Amplification in Breast Carcinomas Using Quantitative Real-time PCR – A Comparison with Immunohistochemical and FISH Results

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The aim of our study was to evaluate the value of quantitative real-time-PCR (qPCR) in the determination of HER-2/neu amplification status of human breast carcinomas by comparing qPCR, FISH and immunohistochemistry results from the same samples. A total of 210 breast carcinomas were examined. Ready-to-use CB11 antibody was applied to detect HER-2/neu oncoprotein expression. In 76 out of 210 cases FISH was performed, and 162 cases were investigated with qPCR. Seventy-five tumors were 2+ or 3+ positive with immunohistochemistry, while 135 samples were either completely negative or 1+. In 45 cases results from all three methods were available. Out of these, in twenty negative and sixteen positive cases both FISH and qPCR led to similar results. The mean qPCR amplification ratio in the concordant positive cases was 5.424 while in the qPCR+/FISH- group the mean ratio was 2.765. Out of 121 samples with scores of 0 or 1+ immunohistochemical result, analyzed also with qPCR, 26 showed HER-2/neu gene amplification. In these cases the mean amplification ratio was 2.53. Comparison of FISH and qPCR together with immunohistochemistry shows that qPCR is more sensitive to detect HER-2/neu gene amplification in tumors scored as 2+ with immunohistochemistry, but the diagnostic cut-off ratio should be defined above 2.7 to avoid high number of false positive cases. Amongst the immunohistochemistry score 2+ cases, 10 of 18 showed gene amplification by qPCR while 10 of 26 by FISH. In conclusion, a well calibrated HER-2/neu qPCR assay may serve as useful alternative to FISH in breast cancer patients. (Pathology Oncology Research Vol 12, No 4, 197–204)

Key words: HER-2/neu protein, HER-2/neu DNA amplification, breast carcinoma, immunohistochemistry, FISH, quantitative RT-PCR, LightCycler RT-PCR system

Introduction

The HER-2/neu gene, member of the EGF receptor family, was discovered independently in four different laboratories, and therefore it has alternative names like neu, HER-2 or c-erbB-2.[5] HER-2/neu protein overexpression is observed in approximately 25% to 30% of breast carcinomas.[1,29,38] In humans, overexpression of HER-2/neu has generally not been found in normal breast tissue or in benign lesions including hyperplasia,[29] but it is commonly seen in ductal carcinoma in situ (DCIS), almost twice as frequently as in invasive carcinomas.[15,42]

The clinical importance of HER-2/neu came in focus of research and therapy following the recognition that amplification of the gene and overexpression of the protein in breast tumors were associated with poor prognosis.[35,36] Based on these observations, breast cancer has become the major focus of HER-2/neu studies.

In 1998 a new drug (Herceptin®, generic name trastuzumab) was approved by the US Food and Drug Administration (FDA) for the treatment of female patients with advanced breast cancer. For women with HER-

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2/neu-overexpressing breast carcinomas, who may not be responsive to standard breast cancer treatment, Herceptin® has shown great promise by increasing survival time. However, the drug is only effective if breast cancer cells carry extra copies of the HER-2/neu gene.[21]

In Hungary, Trastuzumab (Herceptin®) therapy at present is available via the National Health Insurance when it is indicated by both immunohistochemistry and FISH. Conclusive local data are not yet available about the outcome of patients receiving this treatment.

The two most commonly used methods for testing HER-2/neu status are immunohistochemistry, which measures semi-quantitatively the expression of the HER-2/neu protein on the cell surface, and fluorescent in situ hybridization (FISH), which detects amplification of the HER-2/neu gene. Although immunohistochemistry and FISH techniques work with well established parameters, sometimes there is discordance between results from different laboratories or after retesting the same case.[9,30]

In 2001 immunohistochemistry performed with HercepTest (Dako) has been approved by the FDA for diagnostic use. The Oncology Advisory Drug Committee recommended similar approval of FISH, because studies have shown that most carcinomas with 2+ immunohistochemistry score did not have HER-2/neu gene amplification.[19,20]

Although immunohistochemistry is readily available and the scoring system (Dako) based on the assessment of the intensity and completeness of membrane staining is also approved, the results are still subjective, while FISH assay is expensive and laborious. The necessity of HER-2/neu gene amplification testing is demonstrated by Kauraniemi et al[21] who found that Herceptin treatment induces a dose-dependent growth reduction only in breast cancer cell lines with HER-2 amplification, whereas non-amplified cell lines are practically resistant.

Considering the patient's life expectancies, the cost of the different tests and that of the specific treatment itself, it is highly important to use the most sensitive and cost-effective diagnostic method.

In the near future it will be important to analyze in full details the results of the anti-HER-2 therapy, and to correlate the methods used for the detection of HER-2/neu status with the effectiveness of the therapy.

Our aim was to evaluate the value of the highly sensitive and quick qPCR method and to correlate it with the results of immunohistochemistry and FISH.

Materials and Methods

A total of 210 consecutive breast carcinomas diagnosed in the years 2002-2003 were examined for HER-2/neu status. For each case the type and the (Nottingham) histological grade[12] were recorded. Routinely processed, paraffin-embedded surgical biopsies were investigated.

HER-2/neu protein expression was tested with immunohistochemistry on paraffin-embedded samples in all 210 cases. In 162/210 cases, samples were analyzed with qPCR, and FISH validation was performed in 76/210 cases. In 45/210 cases parallel results were available from all three different methods.

Immunohistochemistry

Sections were deparaffinized, followed by antigen retrieval for 6 min in antigen retrieval solution (Vector Laboratories Inc., Burlingame, CA, USA). HER-2/neu protein was detected with the CB11 ready-to-use monoclonal antibody (Novocastra Laboratories, Newcastle, UK). Following 1 h incubation at room temperature, the secondary antibody and the avidin-biotin peroxidase complex from a Vectastain Kit (Vector Laboratories) were applied on the samples. In each case, by omitting the primary antibody, a negative control was included. The chromogen substrate was VIP (Vector Laboratories) or 3,3'-diaminobenzidine (DAB) (Dako, Glostrup, Denmark). Each slide was counterstained with hemalaun, and scored on a 0 to +3 scale by two experienced pathologists according to approved guidelines and described by many authors.[16,40] Routinely, cytoplasmic, confuse staining are considered as HER-2/neu negative as recommended by previous studies.[40] For the purpose of the present study these few cases were analyzed as a separate group and considered as false reaction.

HER-2/neu copy number determination with qPCR from paraffin-embedded samples

In 162/210 cases HER-2/neu gene amplification was analyzed with qPCR using the LightCycler technology system (Roche Diagnostics GMBH, Mannheim, Germany).

DNA isolation. Paraffin-embedded material was prepared as follows: Two 5-μm-thick tissue sections, cut from the block used previously for immunohistochemistry, were independently extracted. After deparaffinization in xylene, tissue sections were digested in Lysis Buffer (Roche) containing 10 μg/ml proteinase K, overnight at 65ºC. The DNA was separated from other cellular components using phenol:chloroform:isoamyl alcohol (25:24:1) and then precipitated with cold ethanol, dried and resuspended in sterile distilled water in 100 m1 final volume. The DNA was stored at -20ºC until use.

Quantification using the LightCycler technology. qPCR was performed with the LightCycler-HER-2/neu DNA Quantification Kit provided by Roche Pharmaceuticals. A 112-bp fragment of HER-2/neu gene and a 133-bp fragment of the reference gene were amplified by LightCycler

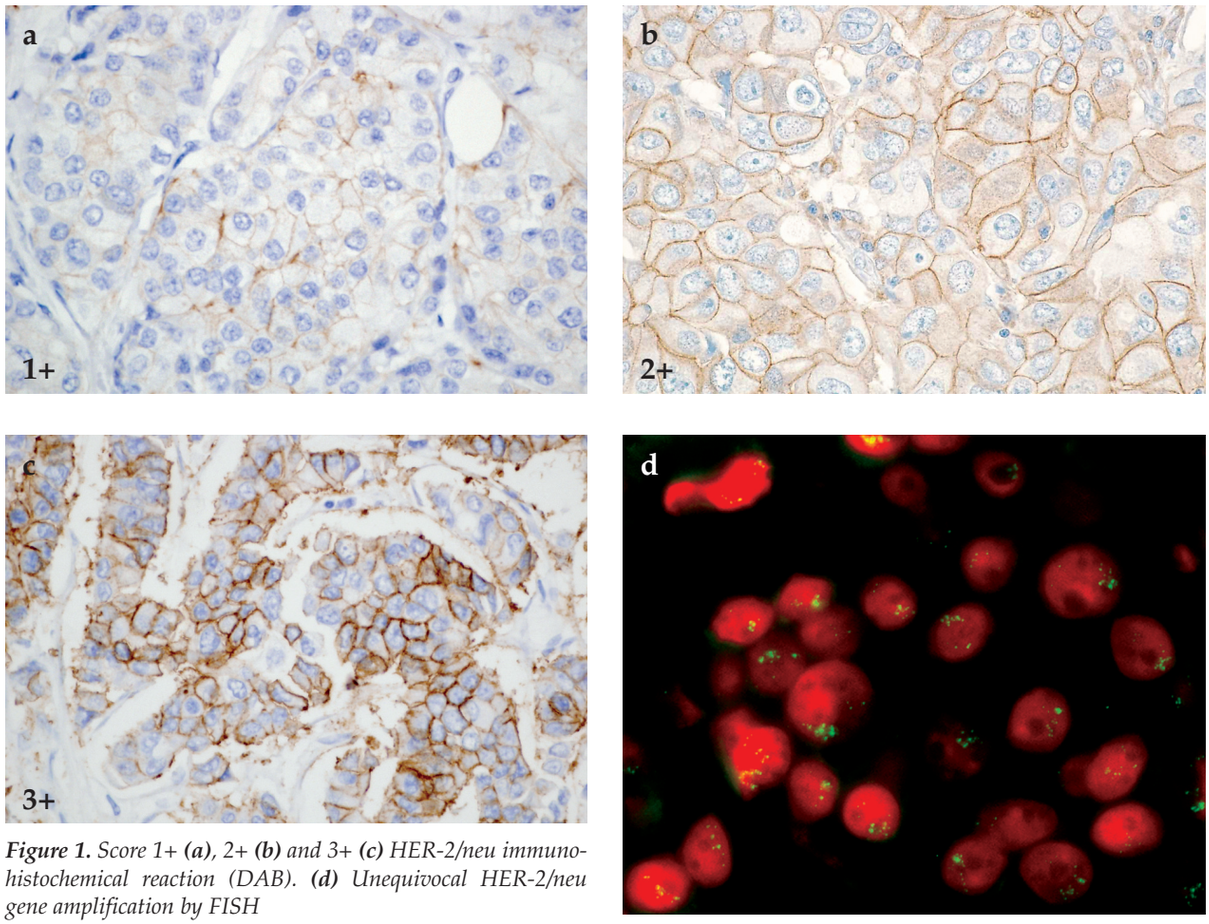

Figure 1. *Score 1+* ***(a)****, 2+* ***(b)*** *and 3+* ***(c)*** *HER-2/neu immunohistochemical reaction (DAB).* ***(d)*** *Unequivocal HER-2/neu gene amplification by FISH*

qPCR specific primers.[7,8] Both genes are localized on chromosome 17. A positive control, provided with the kit, and a template-free negative control were included at each qPCR cycle. qPCR reaction was performed according to the manufacturer's instructions.[8] Two µl of DNA extracted from tissue were added to each reaction mix. qPCR conditions were as follows: after an initial 6-min pre-incubation step at 95ºC, 45 amplification cycles were performed, each consisting of 95ºC for 10 sec, 58ºC for 10 sec, and 72ºC for 10 sec. The fluorescent signals were measured after each primer-annealing step at 58ºC.

The calculation of the relative amounts of HER-2/neu DNA compared to the reference gene DNA was done by Relative Quantification Software from Roche Molecular Biochemicals. Final results were expressed as a ratio of HER-2/neu and reference gene copies in the sample, normalized with ratio of HER-2/neu and reference gene copies in the calibrator DNA set to one. A ratio above 2.0 was regarded as being positive for HER-2/neu amplification. Positive cases showing a ratio of amplification slightly over 2 were re-examined.

Fluorescence in situ hybridization

Typically, the sections analyzed by FISH were adjacent to the section used for immunohistochemistry and the same areas of the tumors were evaluated. FISH was performed using a fluorescein-labeled HER-2/neu probe, automated technique (Ventana Medical Systems, Pty Ltd, Victoria, Australia).[4,25] In brief, paraffin-embedded 4-5-µm-thick sections were mounted on silanized slides. Sample preparation, denaturation, hybridization and post wash were performed in the automated system Ventana BenchMark. After protein digestion, to improve the probe penetration to the target DNA, the breast tissue and the HER-2/neu probe are denatured simultaneously at 90ºC for 10 min, followed by hybridization for 11 h at 50ºC. After biotin-labeled HER-2/neu probe hybridizes to the target, a linker antibody (FITC-labeled mouse anti-biotin) is added to the reaction. Amplification was performed with FITC-labeled sheep anti-mouse IgG. Slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI) in antifade solution (Vectashield, Vector Laboratories). A positive

control section was included in each batch. Fluorescence signals were counted using a Nikon-Microphot-SA fluorescence microscope equipped with an appropriate (FITC and DAPI) filter set. For each specimen, gene copy level was assessed in two areas of at least forty non-overlapping tumor cell nuclei. A tumor was diagnosed as being amplified if there were more than 4.5 copies of HER-2/neu per cell on average.

Results

Histopathological classification of the samples

Invasive ductal carcinoma of the breast of no special type (NST/NOS) was the predominant cancer type 179/210 (85.2%), while 14/210 (6.6%) were infiltrating lobular carcinoma of classical or variant type and 17/210 (8.09%) were special/rare types (cribriform, mucinous, tubular, apocrine, metaplastic). Grades of the 210 invasive carcinomas were as follows: 49/210 (23.3) were grade 1, 91/210 (43.3%) grade 2 and 70/210 (33.3) grade 3.

Immunohistochemistry for HER-2/neu

Immunohistochemistry was performed in all 210 cases. Using CB11 antibody and scoring system according to approved guidelines, 104 cases (49.5%) did not show HER-2/neu protein expression, 23 samples (10.9%) were scored as 1+ (*Figure 1a*), 31/210 (14.7%) as 2+ (*Figure 1b*), 44/210 (20.9%) as 3+ (*Figure 1c*) and 8/210 (3.3%) as false reaction.

Quantification using the LightCycler qPCR technology

qPCR was performed in 162 cases, 53 of which were considered amplified and 109 non-amplified. Of the 162 cases, 92 tumors were score 0 by immunohistochemistry, 22 were 1+, 18 were 2+, 23 were 3+ and 7 showed false reaction. The mean HER-2/neu DNA amplification ratio using qPCR compared to the results of immunohistochemistry is presented in *Table 1*. In 26/121 cases with 0, 1+ or false immunoreaction, qPCR has shown gene amplification (mean 2.53). Twelve of the 92 IHC score 0 cases, and 12 of the 22 IHC score 1+ ones showed gene amplification by qPCR, with a mean amplification ratio of 2.75 and 2.80, respectively. However, in 9 of the 12 amplified IHC score 0 cases and 9 of 12 amplified IHC score 1+ ones the value of gene amplification ratio was between 2.05 and 2.47.

Quantification using FISH

FISH for HER-2/neu amplification detection was performed in 76 cases. At the beginning of our study each case was analyzed by FISH for validation of our immunohistochemical "pre-testing" method and for comparison between results of immunohistochemistry and FISH. Later on FISH was performed only on the 2+ and 3+ cases. Therefore, in this group tumors considered negative with immunohistochemistry (0, 1+) as well as 2+ and 3+ cases were included. The result of a FISH reaction showing unequivocal HER-2/neu gene amplification is presented in *Figure 1d*. Of the 76 cases analyzed with FISH, 44 were considered amplified and 32 non-amplified. The immunohistochemical score was 0 in 9 tumors, 4 cases were 1+, 26 cases 2+, 35 cases 3+ and 2 cases showed false positive reaction. The results are presented in *Table 2*.

FISH and qPCR versus immunohistochemistry

In 45 cases immunohistochemistry as well as FISH and qPCR were performed. Analyzing the results of FISH and qPCR in 36/45 cases both methods showed the same results: 20 cases were negative and 16 positive. In 9 cases FISH and qPCR results were discordant: 6 cases were qPCR+/FISH-, while 3 cases were qPCR-/FISH+. The

Table 1. **The results of HER-2/neu DNA amplification using qPCR compared to the results of immunohistochemistry**

HER-2/neu protein expression by immunohistochemistry		*HER-2/neu DNA amplification status by qPCR – number of cases*	
Score	*Number of cases*	*Amplified (mean ratio)*	*Non-amplified*
0	92	12 (2.75)	80
1+	22	12 (2.8)	10
2+	18	10 (6.06)	8
3+	23	17 (7.78)	6
False reaction	7	2 (2.23)	5
Total	162	53	109

Table 2. **The results of HER-2/neu amplification using FISH in comparison with the results of immunohistochemistry**

HER-2/neu protein expression by immunohistochemistry		*HER-2/neu DNA amplification status by FISH – number of cases*	
Score	*Number of cases*	*Amplified*	*Non-amplified*
0	9	0	9
1+	4	1	3
2+	26	10	16
3+	35	31	4
False reaction	2	2	0
Total	76	44	32

Table 3. **Results of immunohistochemistry, FISH and mean ratio of qPCR amplification in the cases where the results of all three methods are available** (n = 45)

Immunohistochemistry		*qPCR – number of cases*		*FISH – number of cases*	
Score	*Number of cases*	*Amplified (mean ratio)*	*Non-amplified*	*Amplified*	*Non- amplified*
0	9	0	9	0	9
1+	4	2 (2.32)	2	1	3
2+	16	8 (3.38)	8	5	11
3+	16	11 (5.17)	5	12	4

mean amplification ratio of qPCR in the concordant amplified cases was 5.424 while in the qPCR+/FISH- group this ratio was 2.765.

Comparing the results of FISH and qPCR versus immunohistochemistry, it seems that the majority of discordant results are among the cases scored as 1+ and 2+ by immunohistochemistry. Although the number of cases analyzed by all three methods is relatively low and the differences are not significant, it seems that qPCR technique detects more amplified cases amongst the cases scored as 2+ by IHC. These results are summarized in *Table 3*.

Discussion

Since it was described that HER-2/neu amplification in breast carcinoma correlates with poor prognosis, this protein has become the subject of several studies.[10,35,36] The HER-2/neu gene and its protein product, HER-2/neu receptor plays a key role in carcinogenesis. The HER-2/neu receptor transfers signals via the Ras/MAPK signaling pathway and has a role in mitosis induction, proliferation, and invasion.[13,24,43] It is not surprising that in many human malignancies, including breast carcinomas, very often there is a "baseline" increase of the gene copy number and consequently a slight overexpression of the HER-2/neu receptor protein.[36,37]

The selection of patients for Herceptin® therapy relies on the demonstration of HER2/neu protein overexpression and on the presence of HER-2/neu gene amplification. Therefore the need of an accurate determination of HER-2/neu status has gained major importance. Immunohistochemistry and FISH are currently the most widely used methods to measure protein overexpression and gene amplification.[9,23] Immunohistochemistry is generally considered the more subjective assay as it relies on the assessment of the staining intensity and percentage of positive tumor cells. A research group analyzing 1536 breast cancer cases as part of the HER2000 international study showed that there is a moderate level of agreement among pathologists in scoring the immunohistochemical results.[9]

Using immunohistochemistry, the rate of HER-2/neu overexpression has been recorded between 2% and 60%,[18,34] but it is generally accepted that HER-2/neu protein is overexpressed in 15-25% of invasive breast carcinomas.[1,16,38] The wide range of protein overexpression rate, described in reports, is most likely due to differences in the antibodies used and in the patient selection, but different antigen retrieval, tissue fixation methods and interpretation could also contribute to it.

Several studies have noted a significant level of discordance with immunohistochemical tests depending on the antibodies used.[6,19] Accepting the scoring system (0, false reaction and 1+ are considered negative and 3+ is considered positive, 2+ cases are considered equivocal for HER-2/neu status), our results are in concordance with the majority of studies published.[6] However, some laboratories have found the number of positive cases to be lower compared to that observed by us. Bilous[9] et al., analyzing 1536 cancers using HerceptTest, have found 13% of the tumors to be 2+ and 12% to be 3+. Studies have been performed comparing the specificity of different antibodies,[3,9,28,31,39] and it was strongly suggested by many authors and guidelines[9] that immunohistochemical HER-2/neu testing should be performed in accredited, approved central laboratories to avoid inconclusive results.

It is worth mentioning that in a recent study Elkin and co-workers[11] described a thorough and thoughtful cost-effectiveness analysis of HER-2/neu testing and Trastuzumab therapy. They concluded that it is more cost-effective to use FISH alone or as confirmation of all positive HercepTest results rather than using FISH to confirm only the weakly positive HercepTest assays or to use HercepTest alone.

Both immunohistochemistry and FISH are FDA-approved methods of HER-2/neu assessment, but FISH is a specialized technique not available in most laboratories, and it is relatively expensive. The majority of academic centers consider 3+ HerceptTest positivity to be predictive of HER-2/neu gene amplification without necessity of FISH testing.[9] Moderate (2+) staining has not been shown to correlate strongly with gene amplification, so in these cases testing by FISH is needed. In the literature, concordance rates between immunohistochemistry and FISH range from 79% to 100% for 3+ cases[6,17,22] and between

12% to 36% for 2+ cases.[30,33] It is difficult to compare published FISH studies with each other because of the differences in their criteria used to define amplification. The majority of studies used the ratio of gene copies to chromosome 17 copies.[16] Other groups calculated the absolute number of gene copies[32] or, as we and others did,[2] the mean of gene copy number (counted in at least 40 tumor cell nuclei). Ma et al.[26] observed a very good level of concordance between the two methods of FISH analysis (the centromere-17-corrected and the net HER-2/neu gene copy number criteria). They also concluded that increase in HER-2/neu gene copy number consecutive to polysomy 17 in the absence of specific gene amplification might not lead to a strong protein overexpression in the majority of breast carcinomas. In our study FISH was performed in 76/210 cases including cases not expressing HER2/neu protein at all as well as cases scored 1+, 2+ and 3+. The results indicate that, in this series, using CB11 antibody 100% concordance is achieved between immunohistochemistry and FISH in tumors without HER-2/neu protein expression, scored as 0. The concordance between IHC and FISH in tumors unequivocally considered 3+ was 88.6%. In the group recommended to be analyzed by immunohistochemistry as well as by FISH (immunohistochemistry score 2+), the concordance between immunohistochemistry and FISH was 38.5%. It is interesting to note that the two cases considered as false reaction by immunohistochemistry has shown gene amplification by FISH.

These results suggest that HER-2/neu protein overexpression detected by immunohistochemistry does not predict oncogene amplification by FISH in all cases. Since it was demonstrated that Herceptin treatment has beneficial effect only in HER-2/neu-amplified tumors,[21] these results also suggest that alternative techniques are sought for analyzing HER-2/neu oncogene amplification.

Quantitative PCR represents an alternative method for determining gene amplification and has been shown to be a reproducible technique given the automation, sensitivity, specificity and quickness of this technique. Some studies planned to evaluate the concordance between qPCR for HER-2/neu testing and the conventional tests such as immunohistochemistry and FISH [3,31] but the results were discordant too. Our results indicate that using CB11 antibody for the detection of HER-2/neu protein expression and qPCR technique for testing HER-2/neu gene amplification in tumors without HER2/neu protein expression, scored as 0 concordances of 87.0% can be achieved between immunohistochemistry vs. qPCR. The concordance between immunohistochemistry and qPCR in tumors unequivocally considered 3+ was 73.9%. In the group recommended to be analyzed by immunohistochemistry as well as by other (molecular) techniques (score 2+), the concordance between immunohistochemistry and qPCR was 55.6%. In the group considered as false reaction 2 of 7 has shown amplification by qPCR, but the mean value only slightly exceeded 2.0.[2,23] (A ratio above 2.0 was regarded as being positive for HER-2/neu amplification.)

We conclude that by using qPCR more HER-2/neu-amplified cases can be detected in the group pf IHC score 2+ cases, although in this relatively small study we could not prove a statistically significant difference. The concordance between immunohistochemistry and qPCR or FISH in tumors scored 2+ was 55.6%, and 38.5% respectively. In the 3+ cases the concordance between immunohistochemistry and FISH was 88.6%, compared with immunohistochemistry and qPCR (73.9%). Comparing the results of FISH and qPCR we found a concordance of 80%. With case by case comparison, in 36/45 cases both methods showed the same results: 20 cases were negative and 16 positive. The mean amplification ratio using qPCR in the concordant amplified cases was 5.424, while in the qPCR+/FISH-group this ratio was 2.765. In 26 of the 121 cases with 0, 1+, false immune-reaction qPCR has shown weak gene amplification ratio of 2.53.

In earlier studies[7,8] HER-2/neu gene amplification was determined by the same method, using LightCycler technology (ratio between HER-2/neu gene amplification and reference gene located to chromosome 17), and ratio above 2 was considered amplified. Tse[41] et al. compared the HER-2/neu gene copy number quantified by real-time quantitative LightCycler qPCR with FISH and immunohistochemistry and found good concordance between LightCycler qPCR versus immunohistochemistry and/or FISH. The same group described that the LightCycler-qPCR reaches the accuracy of FISH, but it has great advantages: it can be relatively simply performed and fully automated, but it is cheaper than FISH.[41]

Comparing the results mentioned by Tse[41] and by Beyser[7] with our results, we found that using qPCR for the detection of HER-2/neu gene amplification, „specific" or "clinically relevant" amplification ratio probably begins above 2.7. Including this observation qPCR may be an alternative to FISH since in the majority of cases results obtained with the different techniques are concordant.

In a recent study[14] microdissected areas of paraffin-embedded breast carcinoma samples were investigated with qPCR. The results correlated well with the immunohistochemical and FISH results. The authors propose HER-2/neu PCR analysis as an alternative to FISH or immunohistochemistry. Recently, a Canadian group published similar results and concluded that qPCR may be an "effective and efficient" strategy in testing HER-2 status.[27]

In conclusion, our study reports data of HER-2/neu assessment by three different methods, immunohistochemistry, FISH and qPCR. Our results confirm previous reports which demonstrate the discordance between immunohistochemistry and FISH or other methods. For accuracy of HER-2/neu determination beside the FISH

method, additional HER-2/neu tests may be required. When using qPCR for the detection of clinically relevant HER-2/neu gene amplification, we suggest the cut-off level of the gene amplification ratio to define at least above 2.7. Testing by qPCR, a proportion of the immunohistochemically 2+ cases showed a better concordance as compared to the results of FISH.

Considering our results we believe that qPCR is a promising method in routine detection of HER-2/neu gene amplification and may serve as a feasible alternative to FISH. We suggest the formation of a "clinically significant amplification ratio" value to be used in HER-2/neu qPCR.

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