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ORIGINAL RESEARCH

Methods for evaluating HER2 status in breast cancer: comparison of IHC, FISH, and real-time PCR analysis of formalin-fixed paraffin-embedded tissue

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Abstract: The human epidermal growth factor receptor 2 gene (*HER2*) is amplified in approximately 15%–20% of all breast cancers. This results in overexpression of the HER2 protein, which is associated with worse clinical outcomes in breast cancer patients. Several studies have shown that trastuzumab, a monoclonal antibody that interferes with the HER2/neu receptor, can improve overall survival in patients with HER2-positive breast cancer. Immunohistochemistry (IHC), combined with different methods for in situ hybridization, is currently used for routine assessment of *HER2* status. The aim of the present study was to determine whether real-time polymerase chain reaction (PCR) can serve as a supplementary method for evaluation of *HER2* status in primary breast cancer. For this purpose, 145 formalin-fixed paraffin-embedded primary breast cancer samples were tested by real-time PCR amplification of *HER2*, using amyloid precursor protein as a reference. The results were compared with *HER2* status determined by fluorescence in situ hybridization (FISH) and IHC. The specificity, sensitivity, and reproducibility of real-time PCR were evaluated, and a comparison of formalin-fixed and fresh-frozen samples was performed. This showed concordance of 93% between real-time PCR and FISH, and 86% between real-time PCR and IHC. Therefore, we suggest that real-time PCR can be a useful supplementary method for assessment of *HER2* status.

Keywords: 17q, breast cancer, HER2, real-time PCR

Introduction

The human epidermal growth factor 2 gene (*HER2*) is overexpressed and/or amplified in 15%–20% of all breast cancers. Earlier studies have suggested that there is *HER2* amplification in up to 30% of patients with breast cancer,[1–3] but this higher rate can probably be explained by the selection of patients in those investigations. It has been shown that amplification of *HER2* is related to tumor size, lymph nodes metastases, a high S-phase fraction, aneuploidy, and low levels of steroid hormone receptors, and those are factors that might increase the rate of proliferation of tumor cells.[4] It has also been reported that angiogenesis and expression of vascular endothelial growth factor increase when *HER2* is amplified.[5] Furthermore, it has been observed that the degree of *HER2* overexpression is higher in early forms of breast cancer than in more advanced invasive carcinomas,[4] which suggests that alterations in *HER2* alone cannot lead to progression from a relatively benign to a more malignant phenotype in breast tumors.

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Several studies have demonstrated a strong correlation between *HER2* amplification and resistance to tamoxifen.[6,7] However, Rydén et al[8] investigated premenopausal estrogen receptor-positive breast cancer patients and noted that determination of *HER2* status did provide some prognostic information, but it could not predict the outcome of tamoxifen treatment. Rydén et al also found evidence that *HER2* amplification is associated with the rates of response to different chemotherapeutic agents. Retrospectively, the results reported in the literature suggest that *HER2* amplification is correlated with response to treatment with anthracyclines, even though this effect may be secondary to co-amplification of topoisomerase II, which is the direct target of these agents.[9]

Patients with *HER2*-amplified breast cancers have a poorer prognosis in terms of shorter periods without relapse and shorter survival.[10] Anti-*HER2* therapy with the humanized monoclonal antibody trastuzumab is effective in both the metastatic and the adjuvant setting, and this agent can improve the response rate and even survival when administered alone or in combination with chemotherapy.[5]

For adequate management of breast cancer patients, it is essential to achieve accurate assessment of *HER2* status, which can be done at the DNA, mRNA, or protein level by several different methods. In breast cancer with *HER2* amplification, patients who are treated with trastuzumab have a better prognosis, and this underlines the need for highly reproducible and cost-effective methods for evaluating *HER2* status. In most cases today, patients are selected for *HER2*-targeting therapy on the basis of *HER2* status determined by immunohistochemistry (IHC) combined with in situ hybridization (ISH). IHC is a staining technique that can be performed on paraffin-embedded or frozen tumor samples, and it is the most widely applied method for determining *HER2* status.[11] IHC uses a semi-quantitative scale of positive staining ranging from 0 to 3+. It is inexpensive, fast, and easy to carry out, but there are still some problems with reproducibility.[12] A number of ISH methods, such as fluorescence ISH (FISH) and silver ISH (SISH), can be used to determine *HER2* gene amplification. In ISH, the *HER2* gene is marked with one probe, and the centromere on chromosome 17 is marked with another as a reference. The *HER2* gene signals are quantified and divided by the signal from the chromosome 17 reference probe. In Sweden, ISH is frequently used to evaluate tumor samples that have an IHC score of 2 or 3+. *HER2* overexpression is currently defined according to the 2007 ASCO/CAP guidelines in order to achieve reproducible assay performance.[13,14]

Real-time polymerase chain reaction (PCR) is based on detection of DNA amplification. The *HER2* gene is amplified in parallel with a reference gene that has a low risk of copy-number variation in breast cancer, and then the copy-number ratio between *HER2* and the reference gene is determined. Real-time PCR is cost-effective, and many samples can be analyzed at the same time.[15]

The aim of the present study was to investigate the usefulness of real-time PCR for evaluating *HER2* status in primary breast cancer, and to compare the results with the corresponding findings obtained in IHC and FISH analyses.

Materials and methods

Tumor material

We investigated samples of formalin-fixed paraffin-embedded (FFPE) primary breast cancer tumors obtained from the Pathology Department of Linköping University Hospital, Linköping, Sweden. The samples were routine surgical specimens that had been fixed in formalin, processed, and stored according to standard histological protocols. They were collected from 1993 to 2002. During that period, it was not routine practice to perform *HER2* testing in all cases of newly diagnosed breast cancer. Thus, the selection of tumor material was based on previous clinical testing of *HER2,* accomplished either solely by FISH (145 tumors) or by both IHC and FISH (127 tumors). The FISH and IHC analyses were performed and evaluated by standardized routine methods at the central pathology laboratory responsible for all such investigations in this health care region of Sweden. In the real-time PCR series, fresh-frozen tumor samples collected from a subgroup of the cohort (16 tumors) were used for comparison with the FFPE tumor samples. The study was approved by the local medical ethics committee at Linköping University.

DNA preparation

On the original slides of each of the samples, a representative area containing tumor cells was marked by a pathologist (HO). Thereafter, three tissue microarray cores (diameter 0.8 mm) were obtained from the corresponding area in each paraffin block, and DNA was isolated using a Puregene® DNA purification kit (Gentra®, Minneapolis, MN, USA). The quality and concentration of the DNA were determined using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Real-time PCR

Real-time PCR for the target gene *HER2* and the reference gene *APP,* encoding amyloid precursor protein (APP), were

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run in triplicate in separate wells. The 15-μL reaction mixture contained 1× TaqMan® Fast Universal PCR Master Mix (Applied Biosystem, Foster City, CA, USA), 0.1 μM reverse primer, 0.1 μM forward primer, 0.1 μM probe (all three from Sigma-Aldrich, St Louis, MO, USA), and 15 ng of DNA. An epMotion 5070 (Eppendorf, Hamburg, Germany) system was used for automated pipetting of templates and master mixes into a 96-microwell plate. The PCR program started with one cycle at 95°C for 20 seconds. The amplification was run for 40 cycles with denaturation at 95°C for 3 seconds and annealing and extension at 60°C for 30 seconds. The 5 - and 3 -end nucleotides of the probe were labeled with the reporter FAM (6-carboxy-fluorescein) and the quencher dye TAMRA (6-carboxy-tetramethylrhodamine), respectively. All reactions were performed in an ABI Prism® 7700 Sequence Detection System (Applied Biosystems AB, Stockholm, Sweden). The primers and probes used in this analysis are presented in Table 1. The content of the target in tumor samples was quantified by using standard curves to determine a relative measure of the starting amount. Cells of the breast cancer line T47D show normal gene copy numbers at 21q21 and 17q12–21, and those two locations were used as a template for the standard curves. Each sample was normalized on the basis of its content of the reference gene *APP*; this gene is located at 21q21, which has not been found to exhibit alterations in breast cancer patients. Standard curves for both the target gene and the reference gene for each run were constructed using fivefold serial dilutions ranging from 242.4 to 0.95 ng/μL DNA and 7500 Fast System software (Applied Biosystems AB, Stockholm, Sweden). The theoretical value for the slope was −3.32 because each fourfold dilution in the standard curve had a C_t difference of 2. The standard curves for *HER2* and *APP* had mean slopes of 3.19 and 3.08, with coefficients of variance of 3.9% and 2.4%, respectively. DNA was prepared using a Puregene® DNA purification kit. All samples were run in triplicate. All real-time PCR analyses were performed without knowledge of the *HER2* status shown by the FISH and IHC analyses.

Table 1 Design of primers and probes used in real-time PCR

Gene	Oligonucleotide	Sequence	Product size
HER2	Forward primer	5′-GGT CCT GGA AGC CAC AAG G-3′	80 bp
	Reverse primer	5′-GGT TTT CCC ACC ACA TCC TCT-3′	
	Probe	5′-FAM-AAC ACA ACA CAT CCC CCT CCT TGA CTA TCA TCA A-3′	
APP	Forward primer	5′-TTT GTG TGC TCT CCC AGG TCT-3′	72 bp
	Reverse primer	5′-TGG TCA CTG GTT GGT TGG C-3′	
	Probe	5′-FAM-CCC TGA ACT GCA GAT CAC CAA TGT GGT AG-3′	

Abbreviations: *APP*, encoding amyloid precursor protein; *HER2*, human epidermal growth factor receptor 2; PCR, polymerase chain reaction.

IHC analysis of HER2

IHC staining for HER2 had been performed on 127 FFPE tumor samples, and this was achieved using the HercepTest™ (Dako, Glostrup, Denmark) exactly as stipulated by the manufacturer. Scoring of the results of HercepTest™ IHC was done according to the instructions at that time period: 0, no staining at all or membrane staining in $<10\%$ of cells; 1+, weak or barely perceptible staining in $>10\%$ of cells, and only part of the membrane stained; 2+, weak to moderate staining of the entire membrane in $>10\%$ of cells; 3+, strong staining of the entire membrane in $>10\%$ of cells (Figure 1). In all cases, interpretation was limited to invasive tumor.

Fluorescence in situ hybridization

FISH was performed on 4 μm thick sections of FFPE tissue using the Vysis LSI *HER2*/neu (spectrum red)/CEP 17 (spectrum green) DNA probe (Abbott Laboratories, Abbott Park, IL, USA) as recommended by the manufacturer. The numbers of red and green signals were counted in a minimum of 60 tumor cell nuclei in each section, and a signal ratio was obtained. A *HER2*/CEP 17 ratio score of ≤ 2.0 was classified as unamplified and a score of >2.0 as amplified.

Statistics

The relationships between grouped variables were analyzed by the χ^2 test or Fisher's exact test. All *P*-values were

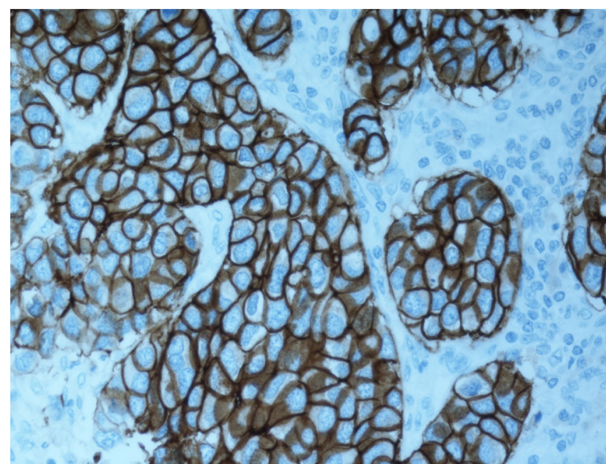

Figure 1 HER2 immunohistochemical staining with a score of 3+.
Abbreviation: *HER2*, human epidermal growth factor receptor 2.

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two sided, and $P < 0.05$ was considered to be statistically significant. All of the tests were included in the statistical package InStat (GraphPad Software, La Jolla, CA, USA).

Results

Assessment of *HER2* status by real-time PCR

FFPE samples of 145 primary tumors were analyzed by real-time PCR, and the *HER2/APP* ratio ranged from 0.01 to 17.1 (Figure 2). Most tumor cells have two copies of these two genes, with a modal peak at a ratio of 0.80, and thus a "normal" tumor cell will have two copies each of *HER2* and *APP*. *HER2* can be considered to be amplified when four or more gene copies are present, and hence a reasonable cut-off value is a ratio of 1.6. This means that a sample with a ratio <1.6 is considered negative, and a sample with a ratio >1.6 is regarded as positive. Sixteen tumors (collected as a subgroup of the original 145 tumors) were used to compare analysis of formalin-fixed and fresh-frozen samples, and the results obtained by these two approaches were almost identical (91% agreement). To test the reproducibility, we analyzed ten samples three times, and the results were essentially the same each time.

Comparison of real-time PCR and FISH

The real-time PCR ratio was >1.6 for a majority (82.5%) of the tumors with *HER2* amplification demonstrated by FISH, and it was <1.6 for all but two (97.8%) of the tumors determined by FISH to be unamplified. The results of some of the FISH tests were difficult to evaluate, and a number of tumors could not be scored and were consequently excluded. Ninety-eight percent of the tumors that were not amplified according to FISH were also negative (ratio <1.6) by real-time PCR (specificity), and 83% of those that were shown to be *HER2* amplified by FISH were also positive (ratio >1.6) by real-time PCR (sensitivity). The results of the comparison are presented in Table 2.

Comparison of real-time PCR and IHC

In the comparison of *HER2* status determined by real-time PCR and IHC, all cases that were positive by real-time PCR (ratio >1.6) had an IHC score of 2+ or 3+, whereas the tumors that were negative by real-time PCR (ratio <1.6) showed any of the different IHC scores (0, 1+, 2+, or 3+). No tumors with an IHC score of 0 or 1+ had a real-time PCR ratio above 1.2. Real-time PCR and IHC identified the tumors as belonging to the same group in 86% of the cases. Furthermore, 94% of IHC-negative tumors (0, 1+, and 2+) were also negative (ratio <1.6) by real-time PCR (specificity), and 69% of the IHC-positive tumors (3+) were also positive by real-time PCR (sensitivity). The results of the comparison are presented in Table 3.

Comparison of IHC and FISH

A majority of tumors with an IHC score of 3+ were FISH amplified (three out of 42 IHC 3+ samples were referred to as being FISH unamplified). The majority (94%) of the cases

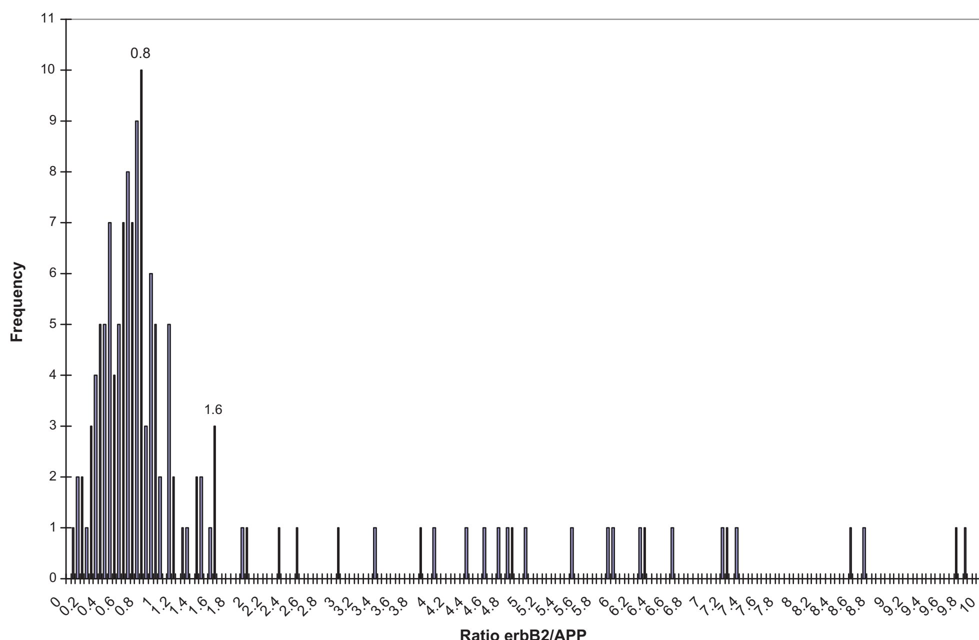

Figure 2 Histogram of the frequencies of *HER2/APP* ratios in tumor samples analyzed by real-time PCR (n = 145).
Abbreviations: *APP*, encoding amyloid precursor protein; *HER2*, human epidermal growth factor receptor 2; PCR, polymerase chain reaction.

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Table 2 Comparison of *HER2* status detected by real-time PCR and FISH, including the intermediate results (n = 145)

FISH	Real-time PCR			
	Ratio <1.2	Ratio ≥1.2 but <1.6	Ratio ≥1.6 but <2.0	Ratio >2.0
Not amplified	88	3	2	0
Excluded	9	3	0	0
Amplified	6	1	3	30
	103	7	5	30

Abbreviations: FISH, fluorescence in situ hybridization; *HER2*, human epidermal growth factor receptor 2; PCR, polymerase chain reaction.

Table 4 Comparison of *HER2* status detected by real-time PCR and by IHC (n = 127)

IHC	Real-time PCR		
	Ratio <1.2	Ratio ≥1.2 but <1.6	Ratio >1.6
0	23	0	0
1+	13	0	0
2+	41	3	5
3+	10	3	29
	87	6	34

Abbreviations: *HER2*, human epidermal growth factor receptor 2; IHC, immunohistochemistry; PCR, polymerase chain reaction.

that were IHC negative (0, 1+, and 2+) were not amplified in the FISH test (specificity), and the majority (79%) of the cases that were IHC positive (3+) were FISH amplified (sensitivity). The results of the comparison are presented in Table 4.

Discussion

In this study, we evaluated the possibility of using real-time PCR to determine *HER2* status in FFPE breast tumors. We found 93% concordance between the results of real-time PCR and FISH. By comparison, Gjerdrum et al[16] reported the corresponding rate to be 83% in their analysis of micro-dissected tumors, and those authors suggested that real-time PCR might serve as a supplement to FISH and IHC, or even as an alternative method. Similar results were obtained by Schlemmer et al[17] in an investigation using fresh-frozen breast cancer tissue and real-time PCR. Most tumors are stored as FFPE tissue specimens, and thus methods that allow the use of such samples are useful in the clinical diagnostic setting as well as in research. We found substantial agreement between the results of real-time PCR performed on FFPE tissue and on fresh-frozen breast tumors. This indicates that DNA fragmentation may not be a significant problem, if PCR amplicons are kept relatively short.

Real-time PCR is a fairly rapid technique that does not require any sophisticated models to interpret the results. Furthermore, it is cost-effective, and many samples can be analyzed simultaneously. One drawback is related to the presence of non-malignant cells in the tumor sample, which leads to a much smaller quantity of amplified genes compared to what might be achieved in the absence of this dilution factor. Theoretically, it should be possible to eliminate this problem by using laser capture microdissection to isolate tumor cells,[18] although that seems to be contradicted by a study in which Williams et al[19] obtained similar results for microdissected and non-microdissected tumor samples in a comparative analysis. To improve the options for a representative tumor sample, we tested an approach in which a pathologist initially examined tumor sections and on each slide marked a representative area with a high percentage of malignant cells. Thereafter, samples were taken from the same area in the corresponding paraffin blocks, although the exact proportions of malignant cells were not known. Lenhard et al[20] found good correlation between the results of IHC, FISH, and real-time PCR, even though the tumor cell content was as low as 20% in some of the samples they analyzed, which indicates that dilution of malignant cells with normal tissue is seldom a serious problem in evaluation of *HER2* status.

Real-time PCR is an efficient and reproducible method that can be standardized. Techniques that are to be used to determine *HER2* status must be fast, reproducible, and cost-effective, and some studies have indicated that IHC and ISH do not fulfill these requirements.[21] The advantage of both IHC and ISH is that it is possible to determine whether the analyzed samples contain invasive breast cancer tissue. Notwithstanding, there is evidence that IHC and FISH offer insufficient reproducibility, as demonstrated by a study

Table 3 Comparison of *HER2* status detected by real-time PCR and by FISH (n = 133)

FISH	Real-time PCR	
	Negative (ratio <1.6)	Positive (ratio >1.6)
Not amplified	91	2
Amplified	7	33
	98	35

Abbreviations: FISH, fluorescence in situ hybridization; *HER2*, human epidermal growth factor receptor 2; PCR, polymerase chain reaction.

Table 5 Comparison of *HER2* status detected by FISH and IHC (n = 127)

FISH	IHC			
	0	1+	2+	3+
Not amplified	22	12	41	3
Excluded	0	1	3	6
Amplified	1	0	5	33
	23	13	49	42

Abbreviations: FISH, fluorescence in situ hybridization; *HER2*, human epidermal growth factor receptor 2; IHC, immunohistochemistry.

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showing that approximately 20% of IHC and FISH assays of *HER2* carried out in small, local pathology laboratories prove to be incorrect when the same specimens are re-evaluated in a high-volume central laboratory.[22,23] However, Fernö et al[24] compared IHC and FISH assessments of *HER2* status performed at different pathology laboratories in Sweden, and their results showed high reproducibility and few errors. In our investigation, there was rather low concordance between the FISH and IHC results, which might be explained by differences in *HER2* staining due to disparities in the length of storage of the tissue blocks, the type of fixative used, the fixation time, and the processing conditions.

It should also be kept in mind that real-time PCR reflects gene amplification, whereas IHC shows protein expression. In our study, there was 86% concordance between real-time PCR and IHC, and the sensitivity was only 69%. The results of earlier attempts to compare expression of *HER2* are conflicting. Bergqvist et al[25] used fresh-frozen breast cancer tissue and RNA expression profiles and found slightly higher sensitivity compared to IHC/ISH, and Cuadros et al[26] concluded that measuring mRNA expression is not a suitable alternative to the traditional IHC/FISH methods. On the other hand, Lehmann-Che et al[27] observed good correlation between IHC and Q-RT-PCR when using fresh-frozen tissue. However, RNA is susceptible to degradation and fragmentation, and the method used to prepare the cDNA, as well as the tumor dilution of normal cells, can result in different outputs. Some studies have shown better agreement between IHC and real-time PCR than was observed in our study, but various aspects of the analyzed tumors might have differed between the investigations. Dissimilarities in the results might also depend on the use of different primers and probes in the real-time PCR, or different antibodies in IHC. In general, the methods must be more robust and valid to be clinically valuable. Earlier studies have used mRNA, and if fresh-frozen tissue is to be evaluated in the future, it will be necessary to change routine practice at many pathology departments.

In the majority of cases, overexpression of HER2 protein indicates amplification of the *HER2* gene, which might also reflect polysomy of chromosome 17. In an attempt to eliminate that, we used FISH with a dual probe system comprising one centromeric probe and one *HER2* probe.

It can be difficult to definitively determine *HER2* status by FISH, and Wolff et al[14] have reported that the same applies to assessment of *HER2* status by IHC. However, it is obvious that the results of *HER2* analysis are derived from continuous data and, in some cases, will inevitably fall into a "gray zone". There are variations in the intermediate ranges of both IHC and FISH, and patients with such results constitute a poorly studied subgroup in which it is not certain whether anti-*HER2* therapies will be successful. Ithimakin et al[28] recently proposed that treatment with trastuzumab has the potential to help patients with *HER2*-negative luminal breast cancer because *HER2* is expressed in breast cancer stem cells in non-amplified tumors and thus targeting this cell population with trastuzumab may be beneficial. This new observation regarding *HER2* implies that there are different mechanisms of *HER2* expression, and it is possible that patients with intermediate results can be regarded as a subgroup that would benefit from anti-HER2 therapy. However, for testing *HER2* in the future, it may be more appropriate to use an additional technique or a combination of methods. Essentially, the main goal is to identify one or more techniques that can achieve optimal selection of patients that will benefit from anti-HER2 therapy with trastuzumab. This agent targets the HER2 protein, although studies have shown that analysis of expression of the *HER2* gene has a greater predictive value, and Mass et al[29] have suggested that, compared to a positive IHC assay, positive FISH results can better identify patients who will benefit from trastuzumab therapy.

Conclusion

The present results show that real-time PCR can be used to detect *HER2* amplification in DNA from FFPE breast cancer tissue. Accordingly, in the future this method may prove to be useful in combination with, or as an alternative to, the IHC and FISH assays that are in broad clinical use today. However, due to the limited size of our study, it will be necessary to confirm our results in a larger study that also includes information about the response to anti-HER2 therapy.

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Disclosure

The authors declare no conflicts of interest in this work.

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