Her-2/neu Expression in Breast Cancer –
A Comparison of Different Diagnostic Methods

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Abstract. Background: Determination of Her-2/neu overexpression in breast cancer has previously been shown to be of prognostic significance. In this study, Her-2/neu expression in breast cancer was characterised by real-time PCR (RLT-PCR) based LightCycler-HER-2/neu DNA Quantification with immunohistochemistry (IHC) and fluorescence in situ hybridisation (FISH). Material and Methods: Fifteen specimens of invasive breast cancer – whole tissue sections as well as microdissected tumour cells – were subjected to RLT-PCR. Additionally, IHC and FISH were performed. Results: Her-2/neu overexpression was detected by FISH and by real-time PCR in the same tumours. In contrast, IHC revealed discordant results. Conclusion: Determination of Her-2/neu amplification by real-time PCR is a sensitive and specific method with some advantages over FISH. This method is simple and reliable and has the potential of categorizing those tumours with borderline Her-2/neu overexpression as determined by IHC.

The Her-2/neu oncogene and its gene products (p185 kd Her/neu) both occur early in breast carcinogenesis and are almost exclusively restricted to transformed mammary ductal epithelium. Her-2/neu is a member of the membrane spanning type I receptor tyrosine kinase family, including four closely related members: EGFR, Her-2/neu, Her-3 and Her-4. Her-2/neu acts as a non-liganded signaling subunit of one of the other receptors. Binding of one of the other receptors results in a heterodimerization of the receptor and Her-2/neu, thus activating Her-2/neu tyrosine kinase and other downstream signaling molecules, e.g. ras/ MAP kinases (1, 2).

Her-2/neu overexpression has previously been detected in nearly 30% of all breast cancer types (3) and has been shown to be an important prognostic marker in many clinical studies (4-9). Her-2/neu overexpression is associated with resistance to treatment with tamoxifen alone and in many CMF-chemotherapeutic regimens (10, 11), whereas adriamycin-based therapies are favourable (12, 13). The introduction of trastuzumab (Herceptin®, Genentech, Inc., San Francisco, CA, USA), a humanized monoclonal antibody against Her-2/neu, into the armamentarium of breast cancer treatments was a remarkable achievement during the past decade. However, only patients with amplification or overexpression of Her-2/neu are likely to benefit from trastuzumab applied as first-line treatment for metastatic breast cancer in a single agent regimen or in combination treatment protocols with cytotoxic drugs (14). Furthermore, current clinical trials (e.g. BCIRG-006; Intergroup N9831 and NSABP B-31) are designed to evaluate the efficacy of trastuzumab in an adjuvant setting. Thus, the Her-2/neu status remains a critical factor for adequate therapeutic interventions.

To detect Her-2/neu expression, different diagnostic procedures have previously been established. However, characterization of patients’ Her-2/neu status still remains a methodological problem.

Immunohistochemical staining (IHC) using different antibodies on paraffin-embedded tissue represents the standard procedure for routine diagnostic analysis. However, several clinical studies have revealed that this method may produce discordant results (15-17). Many commercially available antibodies against Her-2/neu have a wide range of sensitivity and specificity. IHC staining is subject to variations in tissue fixation and processing and,
cancer cells and homogenized tissue. Fluorescence
in situ hybridisation (FISH), evaluating gene
amplification, offers several advantages over
conventional IHC assays: the cut-off value for positivity has previously
been standardized, results are quantitative and internal
positive controls are included in each assay. Additionally,
DNA is less subject to the effects of variations in tissue
fixation and processing than protein (19). Nevertheless,
FISH represents a time-consuming and expensive assay and
may be dependent upon the pathologist’s interpretation (7,
15, 17). Characterization of patients’ Her-2/neu status thus
remains a major technical problem.

In this pilot study, 15 specimens of breast cancer were analyzed for Her-2/neu gene expression by application of the
conventional techniques, IHC and FISH. The results were compared with findings of a new real-time PCR kit for
quantitative detection of Her-2/neu gene expression relative to a reference gene in a single reaction by dual color
fluorescence methodology. In addition, laser capture microdissection was performed to compare PCR results between microdissected homogenous populations of breast
cancer cells and homogenized tissue.

Materials and Methods

Fifteen cases of invasive breast cancer were included in this study. Prior to surgery, all patients signed a standardized form that was
approved by the Ethical Council of the Medical Faculty, allowing the collection of tumour tissue for research (AZ 266/98). Tissue
preservation was performed by the following standardized protocol: immediately after tumour extirpation, the tumour specimen was
transferred to the pathology laboratory adjacent to the operating room under sterile conditions. An experienced pathologist
cut tissue blocks containing macroscopically visible tumour and
surrounding tissue. One block was cut twice for research purposes with each cut side representing the same part of the tumour. One
part was routinely fixed in buffered formalin (12-18 hours) and
embedded in paraffin, while the other was transferred in a cryotube, frozen in liquid nitrogen and stored at −170°C until experiments started.

Routine pathological variables of the tumours, including tumour
size, grading and immunohistochemical determination of oestrogen receptor (ER), progesterone receptor (PR) and Her-2/neu status, are listed in Table I.

The following procedures were performed on the two tumour
specimen sets collected.

Immunohistochemistry. IHC was performed and interpreted from the archival paraffin block by two pathologists in a blinded fashion.
Both pathologists prepared serial 2-µm sections that were placed on Superfrost Plus slides (Menzel Gläser, Braunschweig,
Germany). IHC Her-2/neu overexpression was determined with the commercial HercepTest™ (DAKO, Hamburg, Germany),
according to manufacturer’s instructions and the DAKO-scoring systems, taking into consideration only the membrane staining
pattern and intensity of invasive tumour cells. For HercepTest™
staining, the control slides were used as supplied with the kit.

Fluorescence in situ hybridisation. FISH Her-2/neu amplification
was determined with the Path Vision HER-2/neu DNA Probe kit,
according to the manufacturer’s instruction (Vysis, Bergisch-
Gladbach, Germany). FISH was considered successful if its
hybridisation was uniform, single copy status could be identified and
no detectable DNA loss occurred. Tumours containing up to 4
Her-2/neu signals per nucleus in more than 90% of the analysed
nuclei were interpreted as not amplified, more than 4 signals
indicated amplification. As described above, results were
interpreted by two independent pathologists.

Real-time quantitative PCR. Her-2/neu PCR quantification was performed on: a) whole tissue sections (i.e., tumour cells and
stroma), and b) laser capture microdissected tumour cells of the
cryoconserved tumour samples. Both methods required different
slide preparation and DNA-extraction methods.

Six-µm frozen sections were prepared from each tumour sample by a cryotome (LEICA CM 3050) and subsequently subjected to
DNA isolation using the DNA Isolation Kit I (Roche Diagnostics,
Mannheim, Germany). For laser capture microdissection of tumour
cells, frozen sections were mounted on uncoated slides, immediately fixed and stained with hematoxylin and eosin,
according to a standard protocol. After dehydration in a graded
alcohol series, approximately 1000 tumour cells of each tumour
were dissected with a Laser capture microdissection unit (PixCell-II,
Arcturus, Mountain View, CA, USA). Transfer films containing
dissected cells were placed in a microfuge tube containing 150 µl
lysis buffer (Puregene Tissue Kit, Biozym, Hess-Oldendorf,
Germany) and incubated at 65°C for 15 minutes. DNA isolation
was carried out according to the manufacturer’s protocol. DNA
was stored at −20°C until use. Quantitative PCR was performed by using the LightCycler-HER2/neu DNA Quantification Kit (Roche Molecular Biochemicals, Germany) (20), providing specific primers for a 112-bp fragment of the Her-2/neu gene and a 133-bp
fragment of a reference gene, both localized on chromosome 17.
The PCR conditions were as follows: after an initial 6-minute pre-
incubation step at 95°C, 45 amplification cycles were performed,
each consisting of 95°C for 10 seconds, 58°C for 10 seconds and
72°C for 10 seconds. Fluorescence signals were measured after
each primer annealing step (58°C). Each sample was analysed
twice and the median value was used for quantification. PCR grade
water served as negative control. For quantification of the results,
a calibrator DNA supplied in the kit was used. Calculation of the
relative amounts of Her-2/neu DNA as compared to the amount of
the reference gene was performed by the LightCycler Relative
Quantification Software®. The results were expressed as a ratio of
Her-2/neu: reference gene copies in the sample, normalised to the
ratio of Her-2/neu: reference gene copies in the Calibrator DNA. The ratio Her-2/neu: reference gene copies in the Calibrator DNA was set to one. A ratio of <2.0 was assumed to be negative for Her-2/neu amplification, a ratio of ≥2.0 was assumed to be positive for Her-2/neu overamplification.

Results

In this study, 15 specimens of breast cancer were analysed for Her-2/neu expression and gene amplification status by IHC, FISH and real-time quantitative PCR. The results, receptor status and histological classification of each tumour are summarized in Table I.

Her-2/neu expression analysis with the HercepTest™ -Kit (IHC) and the DAKO-Score (Figure 1) were performed separately by two pathologists in a blinded fashion and revealed different scoring results: pathologist A classified only 3 tumours as +3, the others as +2 and +1, whereas pathologist B classified all 5 tumours which showed gene amplification by FISH as +3. Both pathologists classified 1 tumour that showed no gene amplification by FISH as +3 (tumour #7). Furthermore, 4 other tumours were judged differently by HercepTest™ analysis (0 versus +1).

FISH analysis (Figure 2) was performed by two independent pathologists in a blinded fashion. The results revealed gene amplification of Her-2/neu in 5 of 15 tumours including 3 invasive ductal carcinomas, 1 lobular invasive carcinoma and 1 medullary invasive carcinoma.

Real-time PCR analysis of total tissue specimens of all 15 tumours identified gene amplification in 5 tumours. These tumours were identical with breast cancer specimens positive for Her-2/neu expression as detected by FISH. The PCR index indicating amplification ranged from 2.63 – 5.15. The PCR index of all tumours without Her-2/neu gene amplification ranged from 0.85 – 1.43. Real-time PCR, with pure tumour cells harvested by laser capture microdissection, resulted in PCR indices only slightly different from the indices determined in homogenized tissue specimens. Due to lack of material, only 12 tumours were included in the laser capture microdissection analysis.

Discussion

In this study, we compared different diagnostic methods of determining Her-2/neu overexpression in breast cancer with special regard to real-time PCR. Selection of patients representing potential candidates for treatment with trastuzumab is usually performed by immunohistochemistry. Scoring of immunohistochemical assays is standardized based on a system (0, +1, +2, +3) for the membrane-staining pattern and staining intensity of tumour cells. Only patients with a score of +3 by IHC on paraffin-embedded specimens are considered for treatment or are candidates for clinical trials. However, Her-2/neu overexpression analysis by IHC often yielded discordant results due to the application of different antibodies (17, 21, 22) and variable formalin fixation as well as paraffin-embedding procedures (5, 6, 23). In addition, interobserver discrepancies were evident because our pathologists agreed on the interpretation of the IHC results in only three out of five specimens.
Due to the fact that Her-2/neu overexpression is caused by amplification of its oncogene in more than 90% of all cases, FISH is also used as an entry criterium to trastuzumab-based clinical trials. Several studies comparing FISH and IHC-based methods for characterization of the Her-2/neu status have previously been performed (7, 15, 17). Pauletti et al. analysed 900 breast cancer specimens by IHC as well as FISH (7) and concluded that FISH remains superior in segregating low-risk from high-risk patients. Additionally, comparison of FISH and IHC methods in 117 breast cancer samples with known Her-2/neu gene amplification and overexpression status as determined by solid matrix blotting methods revealed that the Vysis® FISH assay was the most accurate assay (24). Alternative assays for the detection of Her-2/neu expression include Western and Southern blot analysis, as well as enzyme immunoassays (EIA) and PCR methods (5, 9, 23, 25). Western blots and EIA need fresh-frozen tissue and are,
therefore, not suitable in conventional clinical practice. In order to detect gene amplification reliably, quantification is mandatory. Comparative quantification methods, which express the target concentration in relation to a reference gene, have previously been used by other investigators (23). However, problems within these studies included differences in PCR efficacy as well as time-consuming handling as a potential source for non-systematic errors e.g. ethidium bromide staining and densitometric analysis. In our study, we used the LightCycler technology in combination with the LightCycler-HER2/neu DNA Quantification Kit and LightCycler Relative Quantification Software, thus allowing simultaneous amplification and quantification in a combined procedure (26). Application of the method identified all patients with Her-2/neu amplification as indicated by FISH. Merkelbach-Bruse et al. have previously demonstrated similar results comparing IHC, FISH and real-time PCR, however, the concordance rate between PCR and FISH was only 92% (27). Additionally, cases were not selected in a random manner, and IHC and FISH were not analysed by a reference pathologist, thereby explaining the different results between the studies. Although the number of samples analysed is too small for any statistical validation at this time, several findings warrant further evaluation.

Real-time PCR analysis of Her-2/neu gene expression was evaluated in this study in relation to a reference gene in a single reaction by dual colour fluorescence methodology. The procedure of real-time quantitative PCR covers many of the advantages of FISH: the cut-off value for positivity can be standardized, results are quantitative and internal positive control are always included. In addition, variations in tissue fixation and processing will not influence the results due to the DNA-based technique. The kit ensures standardization of reagents with quality control performed by the manufacturer. In contrast to FISH, the assay is easy to perform, the results can be evaluated within 70 minutes, it yields reliable results and may detect even low levels of amplification.

The problem that surrounding non-cancerous tissue, e.g. stroma, might have an important impact on real-time PCR results in comparison to microdissected tumour cells has previously been analysed by Lehmann et al. (28). In this study, Her-2/neu amplification was detected in two of five cases within the microdissected samples. In our study, comparison of real-time PCR results between tissue homogenates and microdissected tumour cells revealed little discrepancy. However, it should be noted that real-time PCR, as employed in our study, was carried out in a different manner than by Lehmann et al.

Discordant results were only detected in the case of tumour #2. Overexpression was detected in both the tissue homogenate and the microdissected tumour cells; however, the amplification determined in the microdissected cells was over 3 times higher within the tissue homogenate. This finding might be due to the fact that tumour #2 was a lobular carcinoma, a histological subtype in which the tumour cells "march single file" through the stroma. The dilution effect of the stroma may be considerably greater for these diffuse lobular carcinomas than for invasive ductal carcinomas characterized by accumulating tumour cells. However, further studies analysing a greater number of tumours with this histological subtype must evaluate whether microdissected cells yield more precise results in the case of distinct histological tumour subtypes.

Conclusion

The importance of a reliable, reproducible and objective test for Her-2/neu expression cannot be overemphasized. All patients suffering from breast cancer types with Her-2/neu overexpression should be offered trastuzumab-based therapy. However, costs for diagnostic methods must always be considered. Currently, tumours evaluated 2+ by IHC are generally sent for FISH analysis to identify false-negative IHC results. If further studies and data from larger tumour specimens confirm that real-time PCR represents a reliable and accurate assay, this PCR assay should be considered as the complementary test to IHC rather than FISH. Its advantages over FISH are presented in our study.

In addition, our data indicate tissue homogenates adequately reflect the Her-2/neu status of the tumour. If further studies in a larger cohort of tumours samples could reproduce this finding, microdissected cells need not be employed for routine analysis.

References


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