Quantitative real-time PCR analysis and microarray-based RNA expression of HER2 in relation to outcome


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Background: Our aim was to use quantitative real-time PCR (Q-PCR) and RNA expression profiles (RNA-EPs) to investigate HER2 status in relation to outcome.

Patients and methods: Cut-off levels for Q-PCR and RNA-EP were established in relation to immunohistochemistry (IHC) validated by FISH in a test set of frozen tissue samples from 40 primary breast cancers. The HER2 status was subsequently studied in another validation set of 306 tumors, where Q-PCR and RNA-EP results were compared with previously carried out IHC that we had validated by chromogenic in situ hybridization (CISH).

Results: Q-PCR and RNA-EP offered similar sensitivity (90% versus 77%), specificity (93% versus 95%), and negative (99% versus 98%) and positive (63% versus 61%) predictive values for HER2 determinations. Analyses of relapse-free survival (RFS) and overall survival on the basis of 5 and 10 years of follow-up indicated equivalent hazard ratios for all three techniques. In contrast to IHC/CISH, both Q-PCR and RNA-EP analyses of HER2 also gave statistically significant results regarding RFS and breast cancer-corrected survival after 10 years of follow-up.

Conclusion: The use of RNA-EP and Q-PCR to analyze HER2 in frozen and formalin-fixed breast cancer samples may be an alternate approach to IHC in combination with FISH/CISH.

Key words: breast cancer, CISH, diagnostic methods, HER2, quantitative real-time PCR, RNA expression profiles

Introduction

The increasing number of patients with breast cancer whose survival has been improved by trastuzumab therapy, in the metastatic setting [1, 2] and probably also in the adjuvant setting [3, 4] underlines the need for highly reproducible yet cost-efficient methods for evaluating HER2. Selection of patients with HER2-overexpressing tumors for trastuzumab treatment is usually done on the basis of immunohistochemistry (IHC) combined with FISH or CISH. The HER2 status is then validated by FISH in a test set of frozen tissue samples from 40 primary breast cancers. The HER2 status was subsequently studied in another validation set of 306 tumors, where Q-PCR and RNA-EP results were compared with previously carried out IHC that we had validated by chromogenic in situ hybridization (CISH).

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Conclusion: The use of RNA-EP and Q-PCR to analyze HER2 in frozen and formalin-fixed breast cancer samples may be an alternate approach to IHC in combination with FISH/CISH.

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Introduction

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The new quantitative real-time PCR (Q-PCR) and RNA expression profile (RNA-EP) analyses show promise as possible independent methods of HER2 assessment. Both methods require small amounts of tumor tissues and deliver quantitative estimates of the HER2 DNA and RNA expression. Microarray technology is commonly used in basic medical and biological research but not yet used for routine analysis in clinical practice. Today various microarray platforms are used for generating RNA-EPs and there are no golden standards, which probably are two possible explanations to conflicting results [11, 12]. Real-time PCR is by many considered the 'gold standard' for gene expression analysis and commonly used for validation of microarray results [13].

Accordingly, our primary aim was to investigate the usefulness of these techniques for HER2 gene evaluation. To accomplish that goal, we compared Q-PCR and RNA-EP with the standard methodology comprising IHC supplemented with FISH or CISH. Our secondary objective was to investigate the prognostic impact of HER2 status analyzed by all three methods at 5 and 10 years of follow-up.

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patients and methods

patients and tumor samples

We used two different population-based patient materials collected in Sweden. The first of these originated from study population A consisting of 279 women who had primary breast cancer and received therapy for that disease 1994–1996 in the Stockholm region. The HER2 status of the tumors from these patients had previously been analyzed by IHC combined with FISH in a retrospective study [14]. Frozen tumor tissue stored in liquid nitrogen from this population was used to design what we here refer to as ‘the test set’, in which we could evaluate different arbitrarily chosen cut-off levels for HER2 positivity in Q-PCR and RNA-EP analyses. To create this test set, we selected 20 consecutive tumors with HER2 protein overexpression and 20 HER2-negative tumors (determined by IHC), and then we examined the HER2 status of these 40 specimens by both Q-PCR and RNA-EP. The 20 tumors with HER2 protein overexpression were in addition retested with FISH, after which 16/20 were found to have HER2 amplification.

To verify and further investigate the cut-off points established in our test set, we used frozen tumor tissue (stored in −80°C) from study population B comprising a patient cohort in Uppsala County (Sweden), which initially consisted of 315 women with primary breast cancer diagnosed from 1987 to 1989 [15]. We were able to retrieve 306 of the original 315 tumors, and we refer to these as the validation set.

To be able to compare the DNA- and RNA-based methods with combined IHC/CISH, we used two subsets of tumors in which HER2 had been successfully analyzed by IHC/CISH and Q-PCR (N = 244) and by IHC/CISH and RNA-EP (N = 232), respectively. These two subsets are referred to in the text as the Q-PCR set and the RNA-EP set. Dropout as well as methods used for these two subsets of the validation set are described in Figures 1 and 2.

We successfully analyzed HER2 by real-time PCR in 262 breast cancer tumors in the validation set. Of these 262, 18 IHC-positive (2+ and 3+) tumors had to be excluded because they were not available for CISH or CISH analysis failed, which left 244 tumors in the Q-PCR set, presented in Figure 1. RNA-EP analysis on the other hand was successful in 251 tumors, 44 of which showed a moderate to strong HER2 protein overexpression. Nineteen of these 44 had to be excluded from the analyses of the RNA-EP set, because they were not available for CISH or such assessment failed, presented in Figure 2.

immunohistochemical analysis of the test set

The analysis was carried out as previously described [14]. In brief, we used formalin-fixed paraffin-embedded (FFPE) sections of 4 μm as templates and the automatic immunostaining was subsequently carried out in a TechMate instrument (DAKO, Glostrup, Denmark) using the CB11 antibody (HER2/neu, diluted 1 : 300; Novocastra Laboratories Ltd, Newcastle, UK). All slides were reviewed by one pathologist (GPE) who had no knowledge of the IHC, Q-PCR, RNA-EP, and FISH scores provided by the other methods.

All tumors in the Q-PCR and RNA-EP sets that had previously shown a positive IHC staining with the mAb CB11 [15], irrespective of intensity, were collected for CISH analysis. In addition, all tumors determined to be HER2 positive by Q-PCR (cut-off 2.0) and/or RNA-EP (cut-off 6.0) were selected for CISH analysis. All CISH assessments were carried out at the Department of Pathology, University of Tampere, Finland, as described previously [17]. In brief, FFPE tissue specimens were evaluated for the HER2 DNA probe (10 μl of a mixture consisting of two contiguous bacterial artificial chromosome (BAC) clones; Zymed Inc., South San Francisco, CA). Unamplified gene copy number was defined as one to five signals per nucleus, and amplification was classified as six or more signals per nucleus in >50% of cancer cells, or observation of a large gene copy cluster. In tumors with a borderline copy number count (4–8 gene copies per cell), CISH of an adjacent section was hybridized with a chromosome 17 centromere probe (Zymed) for comparison. The pathologist (J) evaluated all CISH hybridizations without knowledge of the IHC, Q-PCR, RNA-EP, or clinical outcome.

FISH analysis of the test set

FISH analyses were carried out using the PathVysion HER2 DNA probe kit (Vysis Inc., Downers Grove, IL) as described in a recent publication [16]. Briefly, FFPE tissue specimens were mounted on slides and evaluated for HER2/neu gene copy number using a Leica DMLB microscope equipped with appropriate filters, according to the manufacturer’s recommendations. A ratio of HER2 to chromosome 17 >2.0 is considered amplified.
The MicroSpin affinity columns in the Qiagen kit were used for RNA isolation, and the quality of the RNA was estimated in an Agilent 2100 bioanalyzer (Agilent Technologies, Rockville, MD). The initial lysate was applied to the RNeasy MicroCartridge, and the first centrifugate was stored at −70°C for later extraction of genomic DNA. Such DNA was purified from the above-mentioned frozen sample by following the QIAmp Mini Kit protocol (Qiagen) according to the instructions of the manufacturer. As in the RNeasy protocol, the DNA extraction involves purification in an affinity spin column and subsequent washing and ethanol precipitation of the DNA. A NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used to assess the quality and quantity of DNA from each sample.

**Q-PCR analysis of the Q-PCR set**

A LightCycler-HER2/neu DNA quantification kit (discontinued kit; Roche Applied Biosciences, Penzberg, Germany) was used according to the manufacturer’s instructions to quantify HER2 amplification in an aliquot (100 ng) of the DNA extracted from each tumor tissue sample. The final results were expressed as the ratio of HER2 to reference gene copies in the sample, normalized against the ratio of HER2 to reference gene copies in the calibrator DNA, which was set to one. The manufacturer specifies that a ratio <2.0 is presumably negative for HER2 overamplification and that a ratio equal to 2.0 is probably positive.


The Affymetrix (Santa Clara, CA) protocol was used to prepare in vitro transcription products for oligonucleotide array hybridization and scanning as described in a recent publication [19]. In brief, we used the human HG-U133 (chips A and B, respectively) and scanning was done according to the manufacturer’s instructions (Affymetrix Genechip® Technical Manual, 2001), followed by global mean normalization of the expression data. Three criteria were used to exclude samples from additional analyses: (i) a scaling factor more than four times greater than the average scaling factor for all chips, (ii) present calls <30%, and (iii) $R^2$ value of the Pearson correlation coefficient of the expression data compared with all other arrays <0.6. The probe sets for HER2 were X216836_s_at, X210930_s_at, and X234354_x_at. In the statistical analyses, we used the results generated by X210930_s_at due to the favorable association of this probe set with FISH, as compared with the other two probe sets, when used in the test set.

**statistical methods**

We used the Cox regression model to analyze HER2 status estimated by IHC, FISH/CISH, Q-PCR, and microarray expression profiling in relation to relapse-free survival (RFS), breast cancer-correlated survival (BCCS), and overall survival (OS). The RFS was calculated from date of diagnosis to date of first metastasis (local or distant). Contralateral and/or new primary breast cancer was not counted as a relapse, but patients were censored at the time of contralateral breast cancer. OS was calculated from date of diagnosis to date of death from any cause compared with BCCS indicating death from breast cancer only. The distribution of patient and tumor characteristics in the different populations was tested by Student’s $t$-test and chi-square tests, respectively. SPSS 13.0 software (SPSS Inc., Chicago, IL) was used to carry out all statistical analyses, including generation of receiver operating characteristic (ROC) curves comparing HER2 determined by Q-PCR and RNA-EP with HER2 assessment by our reference IHC/CISH.

**results**

**cut-offs investigated in the test set**

The chosen cut-offs of 2.0 for Q-PCR and 6.0 for the RNA-EP technique were obtained from analyses of the test set. On the basis of Figure 3A and B, we chose two different cut-off values for each method. The results from the comparison of the two chosen cut-off values for Q-PCR and RNA-EP analyses, respectively, are presented in Table 1. For Q-PCR in relation to IHC/FISH, the most favorable cut-off level with respect to sensitivity, specificity, negative predictive value, and positive predictive value was 2.0.

Concurrently, we found 6.0 to be the best cut-off point in RNA-EP evaluation of HER2 in the test set, displayed in Table 1.

**validation set**

In the validation set, the overall concordance between Q-PCR and IHC/CISH was 93%. The levels for sensitivity and specificity were 90% and 93%, respectively (Table 2).
The RNA profile analysis in the validation set showed a high degree of specificity (95%) but lower sensitivity (77%) compared with IHC/CISH (Table 2). Furthermore, the overall concordance between RNA-EP and IHC/CISH was 93%.

Moreover, for 238 tumors with successful Q-PCR and RNA-EP analyses, we found a statistically significant correlation between the HER2 evaluations conducted with the two methods (Spearman correlation coefficient 0.6, \( P = 0.01 \)).

**ROC curves**

ROC curves for Q-PCR (2.0) and RNA-EP (6.0) analyses agreed very well with the results obtained using our reference method IHC/CISH (Figure 4). The area under the curve was 0.91 for Q-PCR versus IHC/CISH and 0.86 (\( P < 0.001 \)) for RNA-EP versus IHC/CISH.

**survival analyses in the Q-PCR set**

One of our aims was to compare survival analysis results obtained by Q-PCR and IHC/CISH. No obvious differences between Q-PCR and IHC/CISH were revealed by hazard ratios (HRs) for RFS, BCCS, and OS at 5 or 10 years of follow-up (Table 3). The HRs for RFS and BCCS at 5 years of follow-up were similar, regardless of the method used, whereas at 10 years of follow-up only HER2 estimated by Q-PCR maintained statistical significance and only with regard to RFS [HR 1.9, 95% confidence interval (CI) 1.2–3.0, \( P = 0.008 \), Table 3].

**survival analyses in the RNA-EP set**

RNA-EP and IHC/CISH evaluation of HER2 gave similar HR values at 5 and 10 years of follow-up with regard to HER2 status in relation to RFS, BCCS, and OS (Table 4). At 10 years of follow-up, however, only RNA-EP still gave statistically significant results for RFS and BCCS

### Table 1

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Screening by IHC/FISH</th>
<th>NPV/PPV (%)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
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<td>2.0 7 14 89/100 81 100</td>
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<td></td>
<td>3.0 0 13 84/80 75 87</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA-EP</td>
<td>&lt;5.5 8 3 73/45 81 33</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.0 16 13 98/61 90 93</td>
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<td></td>
<td>6.0 11 17 98/61 90 93</td>
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</tbody>
</table>

*Two different cut-off points were used in both the RT-PCR and the RNA-EP analysis in the test set (\( N = 40 \)).

Q-PCR, quantitative real-time PCR; RNA-EP, RNA expression profile; IHC, immunohistochemistry; NPV, negative predictive value; PPV, positive predictive value.

### Table 2

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Screening by IHC/CISH</th>
<th>NPV/PPV (%)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>2.0 15 26</td>
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<td></td>
<td>6.0 11 17</td>
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</table>

Q-PCR, quantitative real-time PCR; RNA-EP, RNA expression profile; IHC, immunohistochemistry; CISH, chromogenic \textit{in situ} hybridization; NPV, negative predictive value; PPV, positive predictive value.

### Table 3

<table>
<thead>
<tr>
<th>Survival</th>
<th>Follow-up (years)</th>
<th>Method</th>
<th>HR</th>
<th>95% CI</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
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<td>1.0–3.2</td>
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<td>1.3–3.7</td>
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<td>Q-PCR</td>
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<td>0.9–2.7</td>
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<td>1.2–3.0</td>
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<td>0.9–3.0</td>
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Q-PCR, quantitative real-time PCR; IHC, immunohistochemistry; CISH, chromogenic \textit{in situ} hybridization; HR, hazard ratio; CI, confidence interval; RFS, relapse-free survival; BCCS, breast cancer-corrected survival; OS, overall survival.
significant results with regard to HER2 in correlation with RFS (HR 1.8, 95% CI 1.0–3.1, \( P = 0.04 \)) and BCCS (HR 2.1 95% CI 1.1–4.0, \( P = 0.03 \), Table 4).

**Discussion**

Our objective was to ascertain whether the Q-PCR and RNA-based microarray methodologies could achieve HER2 status evaluations equivalent to the results provided by the IHC combined with FISH or CISH. Our findings indicate that both the Q-PCR and RNA-EP methods do indeed deliver high-quality HER2 determinations with corresponding or even improved prognostic information compared with IHC combined with FISH or CISH. Although the results were similar after 5 years, the IHC/CISH combination was inferior to the two challengers in prognosticating survival after 10 years of follow-up. In other words, with the new techniques, the prognostic value of HER2 status was still statistically significant with regard to RFS (both Q-PCR and RNA-EP) and BCCS (only RNA-EP) even at 10 years of follow-up. This indicates that methods other than the recommended techniques on the basis of IHC and FISH can generate even more precise data without being more difficult and time consuming to carry out.

The increasing demands on future techniques for HER2 determinations call for them to be rapid, reproducible, and cost-effective, and yet still have high throughput capabilities. The present techniques comprising IHC and FISH do not fulfill all these requirements [20, 21].

Nevertheless, analysis generated by IHC, FISH, and CISH are visualised in the histological preserved tissue, why it is possible to confirm that the analysis is made in invasive breast cancer tissue. This information is not available when we use Q-PCR or RNA-EP for HER2 determination using frozen tissue as a template.

**Table 4.** The RNA-EP set: comparison of 5- and 10-year survival by Cox proportional hazards modeling of IHC/CISH and RNA-EP results (\( N = 232 \))

<table>
<thead>
<tr>
<th>Survival</th>
<th>Follow-up (years)</th>
<th>Method</th>
<th>HR</th>
<th>95% CI</th>
<th>( P )</th>
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<td>0.8–2.5</td>
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**RNA-EP, RNA expression profile; IHC, immunohistochemistry; CISH, chromogenic in situ hybridization; HR, hazard ratio; CI, confidence interval; RFS, relapse-free survival; BCCS, breast cancer-corrected survival; OS, overall survival.**

In this study, we only address the prognostic value of HER2 generated by different methods, as none of the included patients have received trastuzumab therapy. Tumors of the validation set were collected in 1987–1989 and trastuzumab therapy was not offered to patients until 1998 in Sweden. Our reference method IHC/CISH could be limited by us using the mAb CB11 for evaluation of HER2, which was proved to be inferior to other antibodies according to a recent study [22]. But no antibody has consistently been demonstrated to be superior with regard to sensitivity and specificity (23), why we consider it appropriate to use CB11 in our study. Besides the tumors that were not available for CISH, we had to exclude 25%–30% of the tumors that were HER2 positive according to IHC or the new techniques, because they failed the CISH analysis, probably because the tissue samples were too old (collected in 1987–1989).

RNA is highly sensitive to degradation and fragmentation, and such breakdown continues even in FFPE [24]. However, the expression profiles of 48 genes elucidated by RT-PCR are essentially identical when using FFPE tissue or frozen tissue (from the same tumor) as template [24]. It is important to employ optimized and standardized protocols to correct for differences in the quality of the input RNA; in the present study, this was achieved using Agilent chromatographic methodology [19]. Correction and/or normalization relative to included reference genes can be done to reduce differences in quantities of RNA [25]. Furthermore, dilution of RNA from nonmalignant tissue can be overcome by use of precise tissue extraction procedures such as laser-assisted microdissection [26]. The laborative microdissection step, however, might not be needed to achieve accurate results according to a report on sequence-based TP53 analysis from microdissected tumor samples compared with tumor samples not microdissected [27].

HER2 amplification occurs at the chromosomal level, and thus, it can be evaluated by Q-PCR using DNA as template. DNA is more stable than RNA, which might also be a reason why our Q-PCR assay correlates so well with the established IHC/C(F)ISH protocol. The better long-term prognostic indication brought by Q-PCR can possibly be explained by the continuous nature of Q-PCR data along with the wholly automated quantization process used in this methodology, which contributes to a higher degree of accuracy, compared with IHC which can be said to be more vulnerable to intra- and interobserver errors.

It was recently reported that there is 83% concordance between HER2 mRNA expression and HER2 DNA amplification, resulting in a statistically significant association of 0.67, \( P < 0.02 \) (Spearman correlation test) [25]. Moreover, our data confirm studies demonstrating substantial agreement between the results of HER2 status evaluation at the DNA, mRNA, and protein levels [28, 29].

Since most tumors have previously been and are still stored as FFPE tissue specimens, methods allowing the use of FFPE samples are highly interesting in a clinical context for both diagnostic assays and research. IHC, FISH, and CISH can be used on FFPE tissues, which is a great advantage with these techniques, in addition to that the methods are well known, quite easy to setup, and run in most pathology departments. Although we used DNA and RNA from freshly frozen samples
in our investigation, several other studies have, however, indicated that Q-PCR data exhibiting similar consistency can be generated from FFPE specimens [24, 28, 29].

Most RNA-EP techniques require intact, full-length RNA. This observation can explain why we have not found any comparable results obtained by applying expression array methodology to FFPE, since heavy fragmentation of RNA occurs during storage in formalin. By comparison, RNA-EP offers obvious advantages, because HER2 evaluation can be done as part of a clinical breast cancer—specific multi-gene chip and thereby reduce scores of individual evaluations to a single assessment. Two recently published reports indicate that such a scheme may prove to be beneficial [30, 31].

The present results indicate that DNA-based Q-PCR and RNA-based gene expression chip methodology are comparable to, and in certain clinical aspects better than, the commonly used IHC-based analysis. Although similar observations have been made in previous investigations, our work comprises by far the largest population combined with the longest clinical follow-up time. Also, ours is the first study to present clear cut-offs for RNA-EP data, which provides solid validation of this methodology. Both RNA-EP and dual-hybridization probe Q-PCR show promise as clinically useful techniques that can give more accurate and, hopefully, more cost-effective analysis of HER2 in the future.

acknowledgements

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references


