Expression of HER2 and the Coamplified Genes GRB7 and MLN64 in Human Breast Cancer: Quantitative Real-time Reverse Transcription-PCR as a Diagnostic Alternative to Immunohistochemistry and Fluorescence In situ Hybridization

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Abstract

Purpose: Accurate testing of HER2 is centrally important for breast cancer therapy and prognosis. Immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) are current standard testing methods. As a potential alternative for assessment of HER2, we explored quantitative real-time reverse transcription-PCR (RT-PCR), a fast and inexpensive method yielding quantitative results insensitive to interobserver variability and amenable to standardized scoring.

Experimental Design: We assessed HER2 status at the DNA, mRNA, and protein levels with FISH, quantitative RT-PCR, and IHC in 136 tumor samples from 85 breast cancer patients. Expression of GRB7, MLN64, and p21, genes coregulated with HER2, was also quantified with quantitative RT-PCR and correlated with the overall survival (OS) and disease-free survival (DFS) individually and in combination with HER2.

Results: Twenty-nine percent and 19% of the patients scored HER2 positive with IHC and quantitative RT-PCR, respectively. In 18 of 19 cases, HER2 statuses in tumors and lymph node metastases were identical. HER2 status significantly correlated with DFS when determined by IHC (P < 0.01), quantitative RT-PCR (P < 0.003), but not with FISH (P = 0.09). The combination of HER2 with MLN64, but not with GRB7 or p21, enhanced the prognostic power for the DFS (P < 0.00005) and OS (P < 0.0008).

Conclusions: Quantitative RT-PCR seems to be clinically as useful in the assessment of HER2 status as IHC and FISH, yielding comparable correlations of HER2 status with the OS and DFS. Thus, quantitative RT-PCR analysis of HER2 or HER2 plus MLN64 is a promising complement or alternative to current methods for HER2 testing, particularly in laboratories lacking FISH or IHC technology.

The gene for the human epidermal growth factor receptor 2 (EGFR2/herbB2/HER2/neu), hereafter referred to as HER2, is located on the long arm of chromosome 17 (17q21.1) (1–3). HER2 encodes for a 185-kDa cytoplasmic transmembrane receptor protein (HER2-neu) with intrinsic receptor tyrosine kinase activity and 50% homology to epidermal growth factor receptor 1 (1, 4). Amplification of HER2 is among the clinically most relevant genetic aberrations in breast cancer (5). HER2 is overexpressed or amplified in 15% to 30% of all cases, which is associated with a poor prognosis, a shorter disease-free survival (DFS) and overall survival (OS), and a more aggressive cancer phenotype (5–7). It is also connected with unresponsiveness to endocrine therapy even in hormone receptor–positive patients (8, 9) and with responsiveness to anthracyclines (10–13). Furthermore, it is associated with estrogen receptor and progesterone receptor negativity, high histologic grade, high proliferation index, and a higher number of lymph node metastases (7, 10, 14). In HER2-positive patients, a combination of chemotherapy with anti-HER2 monoclonal antibodies (e.g., trastuzumab) has been shown to improve the time to progression, the response rate, and the survival rate when compared with chemotherapy alone (15).

Kauraniemi et al. (16) recently found a minimal core region of amplification of about 280 kb containing not only HER2 but also GRB7, MLN64, and six further genes. GRB7 belongs to the GRB7 family of cytosolic adaptor and signaling molecules, which link tyrosine kinase receptors to downstream effectors (17). GRB7 is tightly coamplified and coexpressed with HER2 and the
GRB7 protein strongly binds to HER2 via its SH2 domain (18). It was suggested that the coamplification of these two genes up-regulates a HER2 signaling pathway and that their coexpression is related to extramucosal tumor invasion in esophageal cancer (19). GRB7 has also been implicated in tumor progression and the regulation of cell migration (20, 21). MLN64 belongs to the START-domain family of proteins (22). Several properties of MLN64 have been ascribed to its COOH-terminal START domain, such as cholesterol binding, stimulation of movement of free cholesterol, and augmentation of steroid biosynthesis (23). Presence of excess MLN64 in breast tumors could therefore contribute to intratumoral steroidogenesis and, subsequently, to cancer progression. Like GRB7, MLN64 amplification as well as expression in human breast carcinomas closely correlates with HER2 (24). MLN64 and HER2 share similar transcriptional regulatory elements and both are activated by transcription factor SP1 (25). Genomic colocalization of nonhomologous genes that are coexpressed and involved in common biological processes has already been described in several organisms (24, 26, 27). To achieve a complex biological function, the coordinate expression of several clustered genes may be advantageous. Hence, it has been speculated that HER2, GRB7, and MLN64, located in the same chromosomal region, are involved in a common biological pathway (25). Accordingly, aberrant activation of each of these proteins in a subset of breast tumors might contribute to their poor clinical prognosis and outcome. Overexpression of HER2 has also been shown to transcriptionally up-regulate the cyclin-dependent kinase inhibitor p21 (28).

The current method of choice for HER2 testing is immunohistochemistry (IHC; refs. 29, 30), which assesses HER2 (over-)expression directly at the protein level. However, the results of IHC are not quantitative and there are no standardized scoring system and no uniformly accepted threshold for positivity. Moreover, there is a lack of standardization with regard to primary antibodies, detection systems, as well as interpretation and reporting of the results. Interpretation of IHC results is subjective and prone to interobserver variability, especially at intermediate levels of protein expression. Accordingly, it is strongly advisable to reevaluate IHC results for HER2 scored as 2+ by fluorescence in situ hybridization (FISH) because only those 2+ samples with an amplification of the HER2 gene as detected by FISH have been reported to respond to trastuzumab (31, 32). Thus, we investigated whether quantitative real-time reverse transcription-PCR (RT-PCR), an inherently quantitative method insensitive to interobserver variability and easily amenable to standardization, represents a useful alternative diagnostic approach for scoring of the HER2 status in human breast cancer. mRNA expression of HER2, GRB7, MLN64, and p21 was evaluated by quantitative RT-PCR and correlated with clinical outcome data. We show that combined scoring of HER2 and MLN64 with quantitative real-time RT-PCR outperforms the prognostic accuracy of HER2 IHC and FISH for human breast cancer.

**Patients and Methods**

**Study population.** One hundred thirty-six tumor, lymph node, and adjacent normal tissue specimens were collected from 85 female breast cancer patients who underwent surgery in 1988 to 1994 and analyzed retrospectively under protocols approved by the institutional review board of the Medical University of Vienna (Table 1). Sixty-nine patients had invasive ductal and 16 had infiltrating lobular mammacarcinomas. Thirty-one had a negative, 42 a positive, and 12 an undetermined estrogen receptor status. Fifty-seven patients had lymph node–positive disease (pN+), 27 had lymph node–negative disease (pN0), and 1 was undetermined. Two tumors were grade 1, 45 grade 2, 37 grade 3, and 1 grade 4. The tumor stages were T1 (n = 26), T2 (n = 47), T3 (n = 2), and T4 (n = 10). The median age was 53.5 years. The median OS of all patients was 6.54 years; the median follow up for the patients still alive was 10.95 years (range, 0.1-16.2; Table 1). Paraffin sections of 56 patients were analyzed both by IHC and by FISH (see below); however, no interpretable IHC results were obtained for 1 patient and no interpretable FISH results for 2 patients. Due to logistical and technical problems, no intact RNA could be obtained for 10 of these 56 patients; thus, the only remaining 46 patients plus an additional 29 patients (75 patients in total) were analyzed by quantitative RT-PCR (see below). Thus, 46 patients were analyzed by all three methods, 10 by IHC and FISH but not by quantitative RT-PCR, and 29 by quantitative RT-PCR but not by IHC or FISH (Fig. 1).

**Immunohistochemistry.** Expression of the HER2 protein was determined on 5-μm paraffin sections by a two-step immunohistochemical staining per instructions of the manufacturer (HERcepTest, DAKO, Glostrup, Denmark). Following incubation with the primary rabbit antibody (DAKO) to human HER2 protein, a secondary goat anti-rabbit immunoglobulin conjugated with horseradish peroxidase was applied for visualization.

**Fluorescence in situ hybridization analysis.** Five-micrometer sections of formalin-fixed, paraffin-embedded tissues were used. FISH was done with a SpectrumOrange-labeled HER2 probe that spans the entire HER2 gene and a SpectrumGreen-labeled probe for centromere 17 (Vysis, Downer’s Grove, IL). Two-hundred tumor cells were analyzed...
for each sample. Tumors were scored as harboring a HER2 amplification when the ratio of the average HER2 copy number to that of centromere 17 was ≥2.0, as required by the U.S. Food and Drug Administration for the approval of this method (33, 34).

RNA extraction and cDNA synthesis. Snap-frozen breast tumor tissue was homogenized in a micro-dismembrator (B. Brown Biotech International, Melsungen, Germany) and total RNA was extracted with TRIreagent (Sigma) according to the instructions of the manufacturer. RNA concentration was determined by spectrophotometry and the RNA quality assessed using the Bioanalyser 2100 and the RNA 6000 nano LabChip Kit (Agilent, Palo Alto, CA). Total RNA (10 μg) was reverse transcribed in a final volume of 100 μL using the high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to the instructions of the manufacturer.

Quantitative real-time reverse transcription-PCR. Quantitative PCR analysis was done on the ABI Prism 7000 sequence detection system (Applied Biosystems). The following gene expression assays (Assay-on-Demand, Applied Biosystems) suitable for this system were used, which are based on 5′ nucleic chemistry and consist of two unlabeled PCR primers for amplification and a 6-FAM dye-labeled TaqMan MGB probe for detecting the sequence of interest: HER2, Hs00170433_m1; GRB7, Hs00180450_m1; MLN64, Hs00199052_m1; p21, Hs00355782_m1; and β-actin (control), Hs99999903_m1. Three microliters of template cDNA (equivalent to 100 ng of total RNA) were added to 17 μL of PCR reaction mix containing 10 μL TaqMan Universal PCR Master Mix (Applied Biosystems) and 1 μL assay-on-demand specific for the indicated transcripts. Samples were incubated for 5 minutes at 50°C and for 10 minutes at 95°C, after which, target amplification was carried out with 45 two-staged cycles with 15 seconds at 95°C and 1 minute at 60°C. Duplicate samples were analyzed for each data point. Duplicate assays of serial dilutions of a cDNA standard (cultured normal breast epithelial cells) were included in each experiment. Transcript levels were normalized to those of β-actin to account for variability in the amount of cDNA in each sample and relative expression levels were calculated using the ΔΔCT method (35). β-Actin was chosen as a housekeeping gene for normalization after thorough evaluation of 13 different housekeeping genes in a panel of representative samples using the TaqMan Human Endogenous Control Plate (Applied Biosystems).

Statistical methods. All statistical analyses were done with the open source statistical programming environment R (http://www.r-project.org/). Poor and good prognosis groups were discriminated by maximally selected log-rank statistics (36) and the P value was approximated as described (37). Kaplan-Meier plots were computed with the R survival package. OS was defined as the time from diagnosis to time of breast cancer-related death. DFS was defined as the time from diagnosis to the date of affirmation of a distant metastases, a second primary breast tumor, or a recurrent primary tumor. To correlate the expression data from quantitative RT-PCR, a two-sided Pearson product moment correlation was used. The Pearson coefficient varies from −1, describing a perfect negative correlation, to +1 for a perfect positive correlation. To combine the quantitative RT-PCR results of two or three genes, the expression levels of these genes were first normalized to each other by dividing each expression value by the mean expression value of four normal breast samples.

Results

Comparison of different methods for assessment of HER2 status. A total number of 136 samples derived from 85 female breast cancer patients were analyzed for HER2 overexpression/amplification using quantitative real-time RT-PCR, IHC, and FISH analysis (Fig. 1). Forty-eight of 75 patients (64%) analyzed by quantitative RT-PCR showed a >2-fold increased relative expression level of HER2 compared with the median of four healthy controls. Seventeen of 75 patients (23%) were scored as HER2 positive, as defined by a relative expression level of ≥80 as determined by quantitative RT-PCR; column patterns, IHC results. Arrow, the cutoff dividing between tumors scored as HER2 positive (relative expression ≥80) and tumors scored as HER2 negative by quantitative RT-PCR. Relative expression levels are represented above respective columns. Arrowhead, mean relative expression level of four specimens of normal breast tissue (2.38), na, not available. B, FISH results and selected clinicopathologic variables for each of the tumors in (A). Presence (black) or absence (white) of HER2 amplification determined by FISH; LN, lymph node involvement; ER, estrogen receptor; PR, progesterone receptor expression; and OS in years (y). Black, positive; white, negative; gray, censored or unavailable data. For example, the patient second from the left had low levels of HER2 mRNA expression, was IHC negative, had no HER2 amplification, no lymph node involvement, was estrogen receptor and progesterone receptor negative, and the OS was 22 to 45 years.

Fig. 1. Determination of HER2 expression and amplification by quantitative RT-PCR, IHC, and FISH in 74 breast tumors. A, columns, relative expression levels (arbitrary units) of HER2 as determined by quantitative RT-PCR; column patterns, IHC results. Arrow, the cutoff dividing between tumors scored as HER2 positive (relative expression ≥80) and tumors scored as HER2 negative by quantitative RT-PCR. Relative expression levels are represented above respective columns. Arrowhead, mean relative expression level of four specimens of normal breast tissue (2.38), na, not available. B, FISH results and selected clinicopathologic variables for each of the tumors in (A). Presence (black) or absence (white) of HER2 amplification determined by FISH; LN, lymph node involvement; ER, estrogen receptor; PR, progesterone receptor expression; and OS in years (y). Black, positive; white, negative; gray, censored or unavailable data. For example, the patient second from the left had low levels of HER2 mRNA expression, was IHC negative, had no HER2 amplification, no lymph node involvement, was estrogen receptor and progesterone receptor negative, and the OS was 22 to 45 years.
above a cutoff of 30.0 calculated with log-rank statistics. As determined by IHC using the HercepTest, 16 of 55 patients (29%) were positive (i.e., 2+ or 3+) for HER2 expression; 11 of them (20%) scored 3+ and 5 (9%) scored 2+. Thirty-nine patients (71%) were HER2 negative, composed of 3 (5.5%) scored 1+ and 36 (65.5%) scored 0 (Fig. 1). In FISH analysis, HER2 gene copy numbers and centromere 17 copy numbers were determined in 200 tumor cell nuclei for each of the 98 evaluable breast cancer specimens from 54 patients. The average number of HER2 gene signals divided by the average number of centromere 17 signals was determined. The resulting values ranged from 0.7 to 15, and values \( \geq 2.0 \) were scored amplified as required by the U.S. Food and Drug Administration for the approval of this method of assessment (33, 34). In 24 (44.4%) patients, HER2 was observed to be amplified in this assay, 9 (16.7%) did not have a HER2 amplification but showed an aneuploidy of chromosome 17, and 21 (38.9%) exhibited two signals for the centromere of chromosome 17 and were not amplified for HER2. The concordance of the three methods was calculated based on those 43 patients for whom a HER2 score was available for all three methods. The overall concordance of the quantitative RT-PCR with the HercepTest was 86.4%; HercepTest with FISH analysis, 81.4%; and FISH analysis with quantitative RT-PCR, 76.7% (Fig. 1).

**Comparison of HER2 status in primary tumors and lymph node metastases.** For 19 patients, both the primary tumors and corresponding lymph node metastases were analyzed with IHC, FISH, and quantitative RT-PCR (five representative samples are shown in Fig. 2). For all but one patient, the results for primary tumors and lymph node metastases matched perfectly well. FISH analysis revealed that for primary tumors with HER2 amplifications, HER2 was also amplified in lymph node metastases. Likewise, patients with high HER2 expression in primary tumors also displayed high expression in the corresponding lymph node metastases in both IHC and quantitative RT-PCR analyses. Only one patient was an exception (Fig. 2, patient #3). The primary tumor of this patient was scored as IHC 1+ (i.e., HER2 negative) whereas the corresponding lymph node metastasis was 2+ and thus HER2 positive. In FISH analysis, the primary tumor displayed two signals for centromere 17 and three signals for HER2 whereas the corresponding metastasis exhibited two signals for centromere 17 and five for HER2 (Fig. 2). Thus, the ratio of the HER2 signal divided by the centromere 17 signal equals 1.5 in the primary tumor, indicating no amplification according to the Food and Drug Administration guidelines (33, 34), whereas the HER2/centromere 17 ratio was >2 in the lymph node metastases, denoting an amplification. Because the primary tumor already had three copies of the HER2 gene, it is conceivable that a clonal evolution towards a more aggressive tumor type has occurred, which already started in the primary tumor and which progressed even further during the process of metastatic dissemination to the lymph node. Taken together, primary tumors and corresponding lymph node metastases do not show significant differences in HER2 expression or amplification.

**Correlation of overall survival and disease-free survival with HER2 status determined by immunohistochemistry, fluorescence in situ hybridization, and quantitative reverse transcription-PCR.** Tumors were classified into two subgroups for each method, one defined as negative and one as positive for HER2 overexpression or amplification. For IHC, 1+ and 0 tumors were scored HER2 negative and 2+ or 3+ tumors were scored positive. For FISH, tumors were scored as HER2 positive (amplified) when the ratio of the average HER2 copy number to that of centromere 17 was \( \geq 2.0 \); all others were scored negative. The combination of IHC and FISH analysis is currently the recommended “gold standard” of HER2 diagnostic evaluation (30, 38, 39). For this analysis, tumors that either were IHC 3+ or were IHC 2+ and scored as amplified in the FISH analysis were assigned to the HER2-positive group; all others were scored as negative. For quantitative RT-PCR, log-rank tests were done to separate the tumors into HER2-positive (relative expression level above 30; see Fig. 1) and HER2-negative ones. HER2 overexpression, as determined by quantitative RT-PCR, positively correlated with high tumor grade, positive lymph node status, estrogen receptor negativity, and progesterone receptor negativity, consistent with published IHC results for HER2 (7, 10, 14). Moreover, a combination of quantitative RT-PCR and IHC as well as quantitative RT-PCR and FISH was analyzed. To combine quantitative RT-PCR and FISH, tumors that had a quantitative RT-PCR expression level above 30 or which had an expression level above 20 and scored as amplified in the FISH analysis were assigned to the HER2-positive group, analogous to the well-established combination of IHC and FISH. In the IHC + quantitative RT-PCR combination, all tumors with a quantitative RT-PCR expression level above 30 or an IHC score of 3+ were scored positive so that potential false-negative results obtained in one method would be compensated by the other method. Next, Kaplan-Meier analyses for OS and DFS were done to visualize the differences in the prognostic power of the various HER2 assessment methods (40). Except FISH alone and in combination with quantitative RT-PCR, all methods showed a significant correlation of HER2 status with the OS and DFS (Fig. 3). The \( P \) values for separation of the poor (HER2 positive) versus the good (HER2 negative) prognosis group as determined by quantitative RT-PCR were \( P < 0.006 \) for OS and \( P < 0.003 \) for DFS. IHC analysis yielded a \( P \) value of \( <0.003 \) for OS and \( P < 0.01 \) for DFS (Fig. 3). HER2 status determined by FISH analysis did not correlate significantly with OS (\( P = 0.13 \)) or DFS (\( P = 0.09 \)). This may be due to the higher number of patients scored as HER2 positive by FISH (44%) than by IHC (29%) or quantitative RT-PCR (19%). This rather high fraction of FISH positive cases is consistent with previous studies (41, 42) and may include some false-positive cases which negatively influence the prognostic accuracy of HER2 FISH. However, a clear trend towards longer OS and DFS in nonamplified cases was detected. Combination of IHC with FISH resulted in a significant correlation of HER2 status with OS (\( P < 0.03 \)) but not with DFS (\( P = 0.17 \)). The combination of quantitative RT-PCR with either FISH or IHC did not increase but even decreased the prognostic power of each method used individually, and is not recommended based on our results. In conclusion, quantitative RT-PCR did at least as well as IHC, FISH, and IHC combined with FISH in predicting the disease outcome of breast cancer based on HER2 status (Fig. 3).

**GRB7, MLN64, and p21 are prognostic biomarkers for breast cancer.** Expression of GRB7, MLN64, and p21 was quantified by quantitative RT-PCR and the cutoff values discriminating between tumors with a low or a high expression level were determined with log-rank statistics (Fig. 4). Of the 75 patients analyzed, 19%, 31%, 17%, and 85% were scored positive (i.e.,...
expression above the cutoff) for HER2, GRB7, MLN64, and p21, respectively. The Pearson correlation coefficients of HER2 with MLN64, GRB7, and p21 were 0.87, 0.86, and 0.76; each P value was markedly below 0.001, indicating a tight coregulation of these genes with HER2. The Pearson coefficient for the correlation of MLN64 with GRB7 was 0.99; for MLN64 with p21, 0.96; and for GRB7 with p21, 0.97 (all P < 0.001). Poor OS and DFS in breast cancer patients overexpressing HER2 has been repeatedly shown (5–7, 43). Because we found that HER2 overexpression closely correlates with GRB7 and MLN64 expression, and p21 seems to be a potential mediator of HER2 signaling (28), we investigated whether GRB7, MLN64, and p21 are also useful prognostic marker genes for breast cancer. Accordingly, we correlated the OS and DFS with the expression levels of these genes in our cohort of breast cancer patients. High HER2 expression correlated significantly with a short OS (P < 0.006) and DFS (P < 0.003) in Kaplan-Meier analyses (see above). Comparison of high versus low GRB7 expression yielded a P value of <0.02 for OS and P < 0.02 for DFS (Fig. 5). Likewise, the correlation of high MLN64 expression with a short OS (P < 0.0008) and DFS (P < 0.003) was highly significant (Fig. 5). Conversely, high expression levels of p21, a negative regulator of cell cycle progression, significantly correlated with a good prognosis (OS, P < 0.02; DFS, P < 0.04). Thus, GRB7, MLN64, and p21 are powerful predictors of breast cancer outcome as illustrated in Kaplan-Meier survival curves in Fig. 5. To test whether the prognostic power of these molecular markers could be further enhanced, Kaplan-Meier curves for several biologically relevant combinations of two or three genes were calculated. Highly significant correlations with disease outcome were obtained for combinations of HER2 + GRB7 (OS, P < 0.006; DFS, P < 0.003), HER2 + MLN64 (OS, P < 0.0008; DFS, P = 0.00004), HER2 + GRB7 + MLN64 (OS, P < 0.0008; DFS, P < 0.0002), and HER2 + p21 (OS, P < 0.02; DFS, P < 0.04).

![Fig. 2. Comparison of HER2 expression and amplification in primary breast tumors (T) and corresponding lymph node metastases (N) of five patients determined with IHC, FISH, and quantitative RT-PCR. IHC, HER2 expression scored as 0 and 1+ (negative) or 2+ and 3+ (positive) is indicated. FISH analysis, red, HER2 signals; green, centromere 17 signals. +, specimens harboring a HER2 amplification; --, nonamplified specimens. Quantitative RT-PCR, red line, the cutoff between high (scored as HER2 positive) and low relative expressions of HER2.](image-url)
Thus, the sum of normalized HER2 and MLN64 expression levels separated the poor and the good prognosis groups even better than HER2 or MLN64 alone. Conversely, combinations of HER2 + GRB7 and HER2 + p21 did not provide a more powerful outcome predictor than HER2 alone. Likewise, the combination of HER2 + MLN64 + GRB7 did not further increase the prognostic power of the combination of HER2 + MLN64.

**Discussion**

Accurate determination of HER2 status is of central importance in the prognosis and therapy of breast cancer. Numerous studies indicate that HER2 overexpression correlates with a poor breast cancer prognosis (5–7). Here we compared different methods to analyze HER2 status at the DNA, mRNA, and protein levels and assessed whether quantitative RT-PCR is a useful alternative to the current “gold standard” IHC + FISH method. In addition, mRNA expression of GRB7, MLN64, and p21 was evaluated by quantitative RT-PCR and correlated with clinical outcome data. We show that HER2 status correlated equally well with OS and DFS when determined by quantitative RT-PCR or IHC. Moreover, the combined evaluation of HER2 and MLN64 by quantitative RT-PCR predicts the OS and DFS with a higher accuracy than the scoring of HER2 expression by IHC and HER2 amplification status by FISH. Fewer than 100 patients were analyzed in the present study; thus, the statistical power is limited. However, for the purpose of comparing the prognostic accuracies of different methods to analyze an already well-established molecular marker, this sample size is sufficient and is well in the range of previously reported comparative multimethodologic measurements of HER status in breast cancer (41, 42, 44). It is unlikely that the performance

![Fig. 3. Kaplan-Meier curves of the cumulative OS (A) and DFS (B) for six different methods of HER2 status determination: quantitative RT-PCR (Q-RT-PCR), IHC, FISH, IHC combined with FISH, quantitative RT-PCR combined with IHC, and quantitative RT-PCR combined with FISH. Solid lines, HER2-positive cases; dashed lines, HER2-negative cases, as defined in the text. Calculated \( P \) values and the number of patients \( (n) \) in each of the two study arms are indicated in each curve.](www.aacrjournals.org)
relative to each other of the various methods will be dramatically altered by a larger sample size. In fact, most of the markers analyzed correlated significantly with the OS and DFS in our study, including the newly assessed quantitative RT-PCR assays for **GRB7** and **MLN64**.

The current standard method for assessment of HER2 status is IHC (29, 30). However, 2+ IHC results are recommended to be further validated by FISH analysis (30, 38, 39). FISH analysis goes beyond the possibilities of many smaller histopathology laboratories, requiring expensive equipment, specialized expertise, and a fluorescence microscopy facility, and is therefore often omitted. Furthermore, many different IHC methods are applied in different laboratories, yielding marked discordance in the results for the HER2 status. This discrepancy might be due to technical differences such as the use of different antibodies, different fixatives, variations in the antibody titer or staining protocol, and variability in air humidity, temperature, the water used, or technical skills, let alone interobserver variability.

A number of studies have used quantitative real-time PCR for the assessment of HER2; however, most of them evaluated HER2 gene amplification at the DNA level, not the expression of HER2 at the mRNA level (41, 42, 44–48). These studies assessed the extent of concordance of IHC, FISH, quantitative PCR, and in some cases quantitative RT-PCR, but did not compare the prognostic power of each method (i.e., the correlation of HER2 positivity with OS or DFS). Ginestier et al. (42) considered FISH to be the gold standard and compared it with results obtained by IHC with five different antibodies, those by quantitative PCR (to determine amplification of the HER2 gene), and those by quantitative RT-PCR (to determine HER2 mRNA expression). These authors observed an overall concordance of 82% to 93%, 84%, and 88% with FISH, respectively. Another study assessed HER2 expression by IHC and quantitative RT-PCR as part of a multigene RT-PCR assay and found a sensitivity and specificity of the HER2 RT-PCR assay relative to IHC of 84% and 89%, respectively (47). Here we assessed HER2 status with FISH,

**Fig. 4.** A, relative expression levels of **GRB7**, **MLN64**, and **p21** determined by quantitative RT-PCR in 75 breast tumors, normalized to the mean expression level of 4 normal breast samples (arrowheads). Arrow, the cutoff discriminating between tumors scored as positive (high relative expression levels) or negative (low relative expression levels). B, visualization of the log-rank calculations used for determination of the cutoffs shown in (A). The highest peak in each curve depicts the statistically most significant cutoff discriminating best between patients with a good prognosis and patients with a poor prognosis, which was used for subsequent analyses.
quantitative RT-PCR, and IHC and correlated each result with the OS and DFS. Thus, we did not consider any individual method but instead the correlation of HER2 status with disease outcome as the gold standard, facilitating an unbiased comparison of the performance of each method. Here we show that HER2 analysis by quantitative RT-PCR seems to be clinically as useful for diagnosis as the current standard methods IHC and FISH because it exhibits a comparable prognostic power.

The primary goal in the development of an effective molecular assay should be a high level of sensitivity and specificity in detecting the molecular alteration in question. Allred and Swanson (29) consider an assay to have achieved technical validity when it is sensitive, specific, reproducible, and interpretable in a uniform manner from laboratory to laboratory, which would clearly be the case for quantitative RT-PCR. Quantitative RT-PCR is simple, cost-effective, and rapidly produces quantitative, numerical, and reproducible results. The most important advantage of quantitative RT-PCR is the fact that interpretation of the results is straightforward, easily amenable to standardization, insensitive to interobserver variability, and does not require experienced pathologists because these results are a simple number, which can be either above or below a predetermined threshold. In contrast, interpretation of IHC results is inherently difficult and time-consuming, requires experienced pathologists, and is influenced by several technical factors such as the use of different antibodies, fixatives, staining protocols, and interobserver variability. FISH is also quantitative and reproducible but results are considerably more difficult to interpret than those of quantitative RT-PCR. Moreover, FISH is expensive, time-consuming, and requires specialized expertise and equipment. The equipment for quantitative RT-PCR is also not available in all histopathology laboratories and quite expensive but less so than the equipment required for FISH. A major disadvantage of quantitative RT-PCR may be the specific requirements for handling of tissue specimens to preserve the integrity of RNA; however, because postgenomic methods such as DNA microarrays are more and more widely used, appropriate tissue handling procedures are being adopted in parallel.

Fig. 5. Kaplan-Meier curves of the cumulative OS (A) and DFS (B) for quantitative RT-PCR results of HER2, GRB7, MLN64, and p21 individually and in biologically relevant combinations. Solid lines, specimens expressing high levels of the indicated genes; dashed lines, specimens with weak expression of these genes. Calculated P values and the number of patients (n) in each of the two study arms are indicated in each curve. Note that strong expression of HER2, GRB7, and MLN64, but weak expression of p21, correlates with a poor prognosis (short OS and DFS).
HER2 and MLN64 are invariably coexpressed and coamplified in breast and ovarian cancer, and HER2, GRB7, and MLN64 are also closely associated in the mouse genome (24). Moreover, p21 is transcriptionally up-regulated by HER2 and seems to be a potential mediator of HER2 signaling (28). Here we report that HER2 expression closely correlates with GRB7, MLN64, and p21 expression and that these genes, like HER2, are powerful prognostic markers for breast cancer. This could simply be due to the close coexpression with HER2, GRB7, and MLN64 being “innocent bystanders” and mere surrogate markers for HER2 status, which would be the actual event triggering tumorogenesis. Alternatively, GRB7 and MLN64 might have oncogenic potential by themselves and cause cancer progression independently of or synergistically with HER2. Interestingly, two other genes of the genomic region containing HER2, DARPP32 and Telethonin, although coamplified, are not overexpressed in breast cancer cells, indicating that amplification is not sufficient to trigger overexpression (25). GRB7 is a signal transduction molecule homologous to RAS-GTPase activating protein, which binds to and is activated by a large number of receptor tyrosine kinases, including HER2 (17). Moreover, GRB7 is implicated in cell invasion and metastatic progression of esophageal carcinoma (19, 49). Recently, GRB7 has been shown to interact with calmodulin, which may contribute to the high motility observed in many cancer cells, their metastatic spread, and the neovascularization required for tumor progression (50). MLN64 is expressed preferentially in malignant tissues, specifically breast tumors and breast cancer cell lines, but not in normal cells (24). MLN64 has steroidogenic activity and may be involved in an intratumoral steroidogenic process leading to cancer progression (22). Thus, overexpression of both MLN64 and GRB7 seems to be causally involved in tumorogenesis. Moreover, HER2 and GRB7 are components of a common signal transduction cascade and MLN64 and HER2 are both regulated by members of the SP1 transcription factor family (25). Thus, HER2, GRB7, and MLN64 may be involved in a common biological pathway and the unbalanced activity of each of these genes may contribute to the poor prognosis of breast cancer patients.

In summary, using Kaplan-Meier analyses together with log-rank statistics for cutoff determination, we show that quantitative RT-PCR analysis of a combination of HER2 and MLN64 predicts the OS as well as the DFS more accurately than analysis of HER2 status with IHC, FISH, or a combination of IHC and FISH. Additional quantitative RT-PCR analysis of GRB7 or p21 does not enhance the prognostic power further and can therefore be omitted. The prognostic power and sensitivity was rather impressive for most of the methods compared, and the small differences observed do not convincingly argue that current, well-established methods should be replaced by quantitative RT-PCR. For example, the predictive power of quantitative RT-PCR of HER2 and MLN64 was higher than that of IHC whereas the sensitivity of IHC was higher than that of quantitative RT-PCR (29% versus 19%). However, quantitative RT-PCR is a simple, fast, and highly automated technology which, most importantly, produces quantitative and numerical results of which the interpretation does not require experienced pathologists, a key bottleneck with IHC. Moreover, design and validation of quantitative RT-PCR assays for novel marker genes such as MLN64 or GRB7 is fast and straightforward whereas production of new specific antibodies and establishment of IHC assays can be quite tricky. With the emergence of additional targeted therapies, which require accurate diagnostic assays, the demand for new assays is likely to increase in the future. Along these lines, quantitative RT-PCR of HER2 and MLN64 seems to be a very useful complementary or alternative method for assessment of HER2 status in breast cancer.

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