RESEARCH COMMUNICATION

HER-2/neu Amplification Determined by Real-Time Quantitative PCR and Its Association with Clinical Outcome of Breast Cancer in Thailand

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Abstract

    HER-2/neu has been found to be amplified or overexpressed in about 20-30% of breast cancers, in association with negative prognosticators and shortened survival. Determination of HER-2/neu status in breast-cancer patients, to select for adjuvant treatment with trastuzumab, is becoming standard breast-cancer clinical practice. This study aimed to investigate HER-2/neu status in breast-cancer by real-time quantitative polymerase chain reaction (PCR), allowing accurate and precise quantification of HER-2/neu amplification in tumor tissues. We evaluated 112 breast-cancer samples, of which 42 (37.5%) had HER-2/neu amplification. After a mean follow-up period of 71 months, HER-2/neu amplification was found to be significantly associated with increased risk of death (HR = 6.367, 95% CI = 1.787-22.684), even after adjusting for age, clinical stage, tumor size, lymph-node status, and histologic grade. These findings support a negative prognostic role for HER-2/neu in breast-cancer survival. We suggest that real-time quantitative PCR analysis of HER-2/neu amplification represents an alternative technique for establishing HER-2/neu status in routine clinical practice.

Keywords: HER-2/neu gene - real-time quantitative PCR - breast cancer

Introduction

The HER-2/neu oncogene (c-erbB-2) is a member of the epidermal growth factor receptor family and encodes a 185-kDa transmembrane glycoprotein with tyrosine kinase activity (Schechter et al., 1984; Akiyama et al., 1986). HER-2/neu is mapped to the long arm of chromosome 17 (17q21) (Muleris et al., 1997) and its protein product is involved in signal transduction (Yarden, 2001; Marmor et al., 2004). Amplification of the HER-2/neu gene, and overexpression of its receptor protein, are found in about 20-30% of breast cancers (Slamon et al., 1989; Owens et al., 2004; Ross et al., 2004).

A monoclonal antibody, trastuzumab, was developed to target HER-2/neu, as a novel therapy for breast-cancer patients. Several studies of human breast cancer have recently demonstrated the clinical benefits of trastuzumab after chemotherapy, with significant overall survival benefits over chemotherapy alone (Slamon et al., 2001; Piccart-Gebhart et al., 2005; Romond et al., 2005; Smith et al., 2007). HER-2/neu amplification has also been shown to correlate with a poor prognosis (Slamon et al., 1987; Borg et al., 1994; Press et al., 1997; Révillion et al., 1998; Carr et al., 2000; Riou et al., 2001), and with resistance to conventional adjuvant chemotherapy and tamoxifen (Borg et al., 1994; Carlomagno et al., 1996; Têtu et al.,

1998). Determination of HER-2/neu status has become of major importance in clinical practice for patients with breast cancer.

The most common determination techniques for HER-2/neu include detection of protein overexpression by immunohistochemistry and the evaluation of gene amplification by fluorescence in situ hybridization (FISH) and chromogenic in situ hybridization (CISH) techniques (van de Vijver, 2002; Yaziji et al., 2004; Peiró et al., 2007; Mayr et al., 2009). All were approved by the US Food and Drug Administration. Real-time quantitative PCR is a relatively new technique for measuring HER-2/neu gene amplification, with potentially high sensitivity, specificity, reliability, and low cost. The high sensitivity of quantitative real-time PCR means that even nanograms of low-quality DNA, extracted from formalin-fixed paraffin-embedded (FFPE) tissues, may be detectable and quantifiable (Lehmann and Kreipe, 2001; Gjerdrum et al., 2004), leading to the possibility of performing retrospective clinical and molecular studies on the large specimen archives stored in pathology departments across the world.

This study used real-time quantitative PCR analysis of DNA from FFPE archival breast-cancer tissues to examine HER-2/neu gene amplification and to correlate these findings with patients' clinical outcomes.

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Materials and Methods

Patients and tissue samples

Tissue samples from 112 patients with primary breast cancer were studied, Two paraffin blocks, one containing breast-cancer tissue and the other normal tissues, were selected from the National Cancer Institute archives from the years 2002-2003. All breast tissues had been fixed in 10% neutral buffered formalin and embedded in paraffin under standard conditions. Hematoxylin-eosin-stained sample sections from each tumor block were examined microscopically to confirm the presence of > 80% cancer cells. Paired normal tissues from the same patient were used as controls, and showed histologically normal features.

None of the patients had undergone radiation or chemotherapy before surgery. The maximal and mean periods of follow-up were 83 and 71 months, respectively. Clinical stage was classified according to the American Joint Committee on Cancer TNM staging system. This study was approved by the Ethics Committee of the National Cancer Institute, Thailand.

DNA preparation

FFPE breast samples (primary tumors and normal tissues) were cut by microtome. Three 10-μm-thick serial sections were placed in a 1.5-ml tube, 1.2 ml xylene added and the samples vortexed. The tissue was pelleted by centrifugation at room temperature for 5 min at 14,000 rpm. The supernatant was removed and the pellets were washed with 1.2 ml of 100% ethanol and vortexed. Again, the sample was centrifuged at room temperature for 5 min at 14,000 rpm. The ethanol wash step was repeated once more. The samples were then re-suspended in 600 μl of Cell Lysis Solution and 3 μl of Proteinase K Solution was added (Bio-Rad). Samples were incubated overnight at 55°C with gentle shaking. The next day, 3 μl of RNase A Solution (Bio-Rad) were added, and the sample was incubated for 15 min at room temperature. The DNA was isolated by AquaPure DNA Isolation Kit (Bio-Rad), as described by the manufacturer.

Real-time quantitative PCR

The relative quantification of the Her-2/neu gene and a reference gene (RNaseP) was performed by Bio-Rad IQ5 Real-Time PCR (Bio-Rad Laboratories, USA). Quantifications of the HER-2/neu gene and the reference gene were accomplished using two different hybridization probes, enabling dual color detection in the same PCR tube. The sequences, primer and the probe concentrations, are shown in Table 1. Hybridization probes, primers, Taq DNA polymerase and IQ Supermix (Bio-Rad Laboratories, USA), were mixed together with 3μl template DNA, resulting in a final volume of 25μl, according to the manufacturer's instructions. Activation of Taq DNA polymerase (hot-start) and denaturation of the template DNA was performed at 95°C for 10 minutes, followed by denaturation at 95°C for 15 seconds, and annealing at 60°C for 1 minute. This profile was repeated 50 times, after which the specimens were cooled to 40°C. The reaction was performed in triplicate. The relative

Table 1. Sequences and Concentration of Primers and Hydrolysis Probes used for Real-Time Quantitative PCR

Oligonucleotide (conc.)	Sequence
HER-2 FP (5)	5'-CCA GGA CCT GCT GAA CTG GT-3'
HER-2 RP (5)	5'-TGT ACG AGC CGC ACA TCC -3'
HER-2 Probe (5)	HEX-CAG ATT GCC AAG GGG ATG AGC TAC CTG-BHQ
RNaseP FP (2.5)	5'- AGA TTT GGA CCT GCG AGC G-3'
RNaseP RP (2.5)	5'- GAG CGG CTG TCT CCA CAA GT-3'
RNaseP Probe (2.5)	Cy5- TTC TGA CCT GAA GGC TCT GCG CG -BHQ

Conc,concentration, pmol; FP, forward primer; RP, reverse primer

amount of PCR product generated from each primer set was determined on the basis of the cycle threshold (Ct) value. The relative quantification was calculated by the [delta] [delta] Ct method (Livak and Schmitten, 2001). A more than two-fold increase in ratio (> 2) should be regarded as positive for Her-2/neu DNA amplification.

Statistical analysis

The Chi-square test was used to compare relationships between HER-2/neu status and clinic-pathological variables. Kaplan-Meier survival curves were established to estimate overall survival (OS), and the log-rank test was used to compare differences in survival between groups. The Cox regression proportional hazard model was used to evaluate the impact of HER-2/neu status on OS in both univariate and multivariate analyses. All P values were two sided, and results were considered statistically significant at $P < 0.05$. Analyses were done using the SPSS 18.0 statistical software package (SPSS Inc., Chicago, IL, USA)

Survival time was calculated from operation time to death, or date of last follow-up. Patients who did not experience an event were censored from the study at end of follow-up.

Results

For the 112 patients, the mean age at diagnosis was 50.8 years (range 26-76 years). No significant association was found between HER-2/neu status and age ($P = 0.694$),

Table 2. Clinical Features by HER-2/neu Status

Variables		Amplified	Not-Amplified	P value
Number of patients		42 (37.5)	70 (62.5)	
Age (years)	≤ 50	24 (40.7)	35 (59.3)	
	> 50	18 (35.3)	33 (64.7)	0.694
Stage	I + II	30 (36.6)	52 (63.4)	
	III + IV	12 (42.9)	16 (57.1)	0.653
Tumor size	≤ 3	29 (34.9)	54 (65.1)	
(cm)	> 3	12 (46.2)	14 (53.8)	0.356
Lymph node	Negative	18 (30.0)	42 (70.0)	
status	Positive	24 (46.2)	28 (53.8)	0.117
Histological Grade	1	4 (30.8)	9 (69.2)	
	2	18 (47.4)	20 (52.6)	
	3	18 (36.7)	31 (63.3)	0.463
Status	Alive	28 (29.8)	66 (70.2)	
	Deceased	14 (77.8)	4 (22.2)	<0.001

Table 3. Univariate and Multivariate Analyses of Overall Survival

Variables		Univariate			Multivariate		
		HR	(95% CI)	P-value	HR	(95% CI)	P-value
Age (years)	≤ 50 vs >50	0.851	(0.324-2.238)	0.744	0.820	(0.288-2.341)	0.711
Stage	I+II vs III+IV	3.370	(1.336-8.496)	0.010	1.561	(0.411-5.926)	0.513
Tumor size (cm)	≤ 3 vs > 3	3.397	(1.309-8.819)	0.012	1.948	(0.627-6.053)	0.249
Lymph node status	Negative vs positive	4.512	(1.484-13.71)	0.008	1.994	(0.488-8.154)	0.337
Histological	Grade1 vs 2	0.494	(0.061-4.018)	0.510	0.641	(0.075-5.481)	0.684
	Grade 1 vs 3	1.954	(0.744-5.133)	0.174	0.983	(0.344-2.812)	0.975
HER-2/neu	Negative vs positive	6.535	(2.149-19.89)	0.001	6.367	(1.787-22.68)	0.004

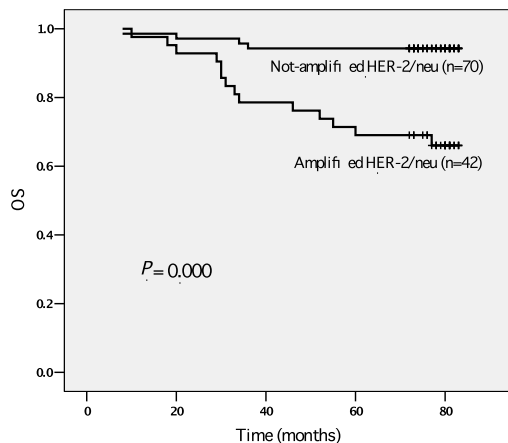HR, hazard ratio, CI, confidence interval

Figure 1. Kaplan-Meier curves for HER-2/neu status.
Overall survival (OS) curves showing survival differences between HER-2/neu amplified and not-amplified cases

stage (P = 0.653), size of primary tumor (P = 0.356), lymph-node status (P = 0.117), and histological grade (P = 0.463) (Table 2). During the follow-up time, 18 patients died from their cancers.

Kaplan-Meier curves for overall survival are shown in Figure 1. Patients with HER-2/neu amplification were more likely to die of the disease, giving OS rates of 66.7% compared with 94.3% for those without HER-2/neu amplification (P = 0.000). This significance was retained in Cox regression analysis when analyzed with patient age, clinical stage, tumor size, lymph node status, and histologic grade in both the univariate and multivariate Cox regression (P = 0.001 and 0.004, respectively). The hazard ratio for HER-2/neu amplification was 6.535 (95% CI = 2.149-19.868) in univariate analysis, and 6.367 (95% CI = 1.787-22.684) in multivariate analysis (Table 3).

Discussion

HER-2/neu gene amplification and protein overexpression have been found in about 20-30% of breast cancers, and associated with a poor prognosis (Slamon et al., 1989; Slamon et al., 1987; Borg et al., 1994; Press et al., 1997; Révillion et al., 1998; Carr et al., 2000; Riou et al., 2001; Owens et al., 2004; Ross et al., 2004). Amplification/overexpression of HER-2/neu is shown to correlate with a variety of negative prognostic factors (Gusterson et al., 1992; Quénel et al., 1995; Révillion et al., 1998; Ross and Fletcher, 1998) and is associated with shorter survival, higher recurrence rates, and lower response to chemotherapy and hormone therapy (Carr et

al., 2000). Recently, HER-2/neu has become clinically important as a target for antibody-based therapy with trastuzumab. Adjuvant treatment of primary HER-2/neu-positive breast cancers with trastuzumab has been shown to improve patients' outcomes markedly (Tuma, 2005). Detection of HER-2/neu status in patients with breast cancer, to select adjuvant treatment with trastuzumab, is therefore becoming the standard in breast cancer clinical practice worldwide.

The development of real-time PCR techniques makes it possible to perform more precise quantitative analysis of gene amplification. These techniques are easy and quick to perform, can be used to run multiple samples, and can be automated, making them potentially useful techniques for screening tumors for HER-2/neu amplification in a routine clinical setting (Kim et al., 2002; Königshoff et al., 2003; Kulka et al., 2006). This study used a novel real-time PCR technology, which utilizes the 5'exonuclease activity of Taq polymerase, in combination with a hybridization probe indicating PCR product accumulation, presenting the advantage of a very sensitive exact quantification of minute amounts of DNA or RNA in FFPE tissues (Gibson et al., 1996; Heid et al., 1996). Using this method in the present study, HER-2/neu amplification detected 37.05% of 112 breast cancer samples. Our finding is similar to another study which determined HER-2/neu amplification in 37% of breast cancers by quantitative real-time PCR (Kim et al., 2002).

Moreover, our study found that patients with HER-2/neu amplification were more likely to die (HR = 6.367, 95% CI = 1.787-22.684) than those without, even after adjusting for the effects of patient age, clinical stage, tumor size, lymph node status, and histological grade. In breast cancer, an accurate evaluation of HER-2/neu status is needed, due to its clinical utility as a prognostic factor, and predictor of response to treatment (Slamon et al., 1989; Ring et al., 2006). In our data, HER-2/neu amplification, detected by quantitative real-time PCR, was significantly associated with poor overall patient survival, suggesting that real-time quantitative PCR is a promising method for use in the routine detection of HER-2/neu amplification. No specialist is needed to interpret the results, so that it may serve as a feasible alternative to fluorescence in-situ hybridization (FISH).

Acknowledgements

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