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Original Article

Evaluation of HER2/*neu* Status by Real-Time Quantitative PCR in Breast Cancer

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Over-expression of the human epidermal growth factor receptor-2 (HER2/*neu*) has been observed in many cancers, and is associated with a poor prognosis. Recent adjuvant treatment with anti-HER2 monoclonal antibodies in breast cancer has increased the demand for an evaluation of the HER2/*neu* status in breast cancer. The aim of this study was to investigate the HER2/*neu* status in breast cancer by a real-time quantitative polymerase chain reaction (PCR) method using LightCycler (Roche Diagnostics, Mannheim, Germany). DNA samples from the fresh tumor tissues of 27 patients with breast cancer were analyzed in parallel using immunohistochemistry (IHC) and the other prognostic parameters including estrogen receptor, progesterone receptor, cytokeratin, and DNA ploidy. Ten (37%) out of 27 cases tested were positive for HER2/*neu*, while 16 (73%) out of 22 tested positive through an IHC study. The correlation between the DNA aneuploidy and the positive results for HER2/*neu* were only observed using the real-time PCR method ($p < 0.05$). There was no significant correlation between the HER2/*neu* status and the S-phase fractions of the DNA ploidy or other parameters. This study demonstrated that there is marked discordance in the results for the HER2/*neu* status according to the various methods used. Real-time quantitative PCR for HER2/*neu* appears to be clinically useful due to its simplicity and ability to produce rapid results.

Key Words: HER2/*neu*, breast cancer, prognosis, polymerase chain reaction

INTRODUCTION

The HER2/*neu* (ERBB2) gene is a proto-oncogene located on chromosome 17q21, which codes a 185-kd transmembrane oncoprotein with tyrosine kinase activity. It has some homology with the epidermal growth factor receptor. The *neu* gene was first discovered in the DNA of the rat neuroblastoma and the human equivalent (HER2) of *neu* was cloned independently from a complementary DNA library and from genomic DNA (c-erbB-2).[1] Since the first report[2] on the association between HER2/*neu* gene amplification and the disease prognosis, over-expression of this gene has been observed in 10 - 30% of patients with breast cancer. The over-expression correlated with a poor prognosis.[3]

In HER2/*neu* positive patients combined treatment of chemotherapy and anti-HER2 monoclonal antibodies, Trastuzumab (Herceptin; Genentech, San Francisco, CA) that down-regulate the erbB-2 receptor, has been known to improve the time to progression, the response rates and the survival rates, when compared with chemotherapy alone.[4,5] Recently, the American Society of Clinical Oncology (ASCO) included erbB-2 as a candidate for a clinically utilizable tumor marker,[6] which may increase the demand for the assays. HER2/*neu* is also known to be associated with a negativity for the estrogen/progesterone receptors, a high histological grade and proliferative index, and an increased number of metastatic lymph nodes.[7] Therefore, some discrepancy in the results due to the different methods used may be encountered. In this study, real-time quantitative PCR was performed, and the results were compared with those of IHC. In addition, the other prognostic parameters and the practical applicability of the real-time quantitative PCR method were evaluated.

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MATERIALS AND METHODS

Patients and specimens

Fresh mastectomy tissue specimens were obtained from 27 patients with breast cancer (21 infiltrating ductal carcinoma, 3 infiltrating lobular carcinoma. 1 malignant phyllodes tumor, 1 cribriform carcinoma, and 1 unclassified carcinoma). None of these patients had received Herceptin treatment. One patient had a metastasis to the bone marrow and lymph nodes, and 13 patients had lymph node involvement.

DNA isolation & quantitative PCR

Tumor tissues were scratched with a knife in PBS buffer and the DNA was extracted using an Easy-DNA^{TM} kit (Invitrogen Corp, Carlsbad, CA, USA). Quantitative real time PCR was performed using a LightCycler and HER2/*neu* DNA quantification kit (Roche Diagnostics, Mannheim, Germany).

A 112 base pair (bp) fragment of HER2/*neu* gene and a 133 bp fragment of a gastrin reference gene located on chromosome 17q21 were amplified by primer specific PCR according to manufacture's instructions.

Briefly, after adding the extracted DNAs as well as the calibrator DNA provided with the kit and PCR grade water as a negative control, PCR was performed as follows; after an initial 6 minute preincubation step at 95℃, 45 amplification cycles were run, each consisting of 95℃ for 10 seconds, 58℃ for 10 seconds and 72℃ for 10 seconds. Two sets of hybridization probes were used. One was labeled with LightCycler-Fluorescein (wavelength 530 nm) and LightCycler-Red 640 (wavelength 640 nm), and the other was labeled with LightCycler-Fluorescein and LightCycler-Red 705 (wavelength 710 nm). The fluorescence signals were measured after each annealing step. The relative amounts of HER2/*neu* DNA compared to the reference gene DNA were calculated with the LightCycler Relative Quantification Software provided by Roche Molecular Biochemicals. The final results were calculated as a ratio of HER2/*neu* to reference the gene copies in the test sample, which were normalized with a ratio of HER2/*neu* to the reference gene copies in the calibrator DNA. A ratio above 2.0 was regarded as being positive for HER2/*neu* amplification.

Immunohistochemical studies

Immunohistochemical (IHC) studies were performed on paraffin-embedded sections using the reagents (DAKO, Carpinteria, CA, USA), including the rabbit anti-human c-erbB-2 oncoprotein (dilution 1:200), the monoclonal anti-human estrogen receptor (dilution 1:100), the monoclonal anti-human progesterone receptor (dilution 1:10), and the monoclonal anti-human cytokeratin (dilution 1:100).

Flowcytometric study for DNA ploidy

The frozen tumor tissues were scratched with a knife in PBS buffer until white fibrins were noted and penetrated through the 100 μm pore mesh. The filtered fluid was centrifuged at 1200 rpm for 10 minutes and 500 μL of a citrate buffer was added to remove the upper layer fluid, which was followed by staining with Trypan blue (1 : 1) and cell counting. After adding solutions A (100 mL stock solution, 3 mg Trypsin), B (100 mL stock solution, 50 mg trypsin inhibitor, 10 mg RNase A), and C (100 mL stock solution, 41.6 mg Propidium Iodide, 116 mg Spermine tetrahydrochloride), the samples were filtered through a 40 μm pore mesh before the analysis with the Coulter EPICS XL (Coultronics France, Margency, France).

RESULTS

The ene amplification curves for the HER2/*neu* gene and the reference gene are shown in Fig. 1. Ten (37%) out of 27 specimens from the breast cancer patients tested positive for HER2/*neu* in real-time PCR, while 16 (73%) out of 22 specimens tested positive in the IHC studies using the rabbit anti-human c-erbB-2 oncoprotein (Table 1). There was no association of the HER2/*neu* status with metastasis. A correlation between the DNA aneuploidy and the positive results for HER2/*neu* were only observed using the real-time PCR method ($p < 0.05$). There was no correlation

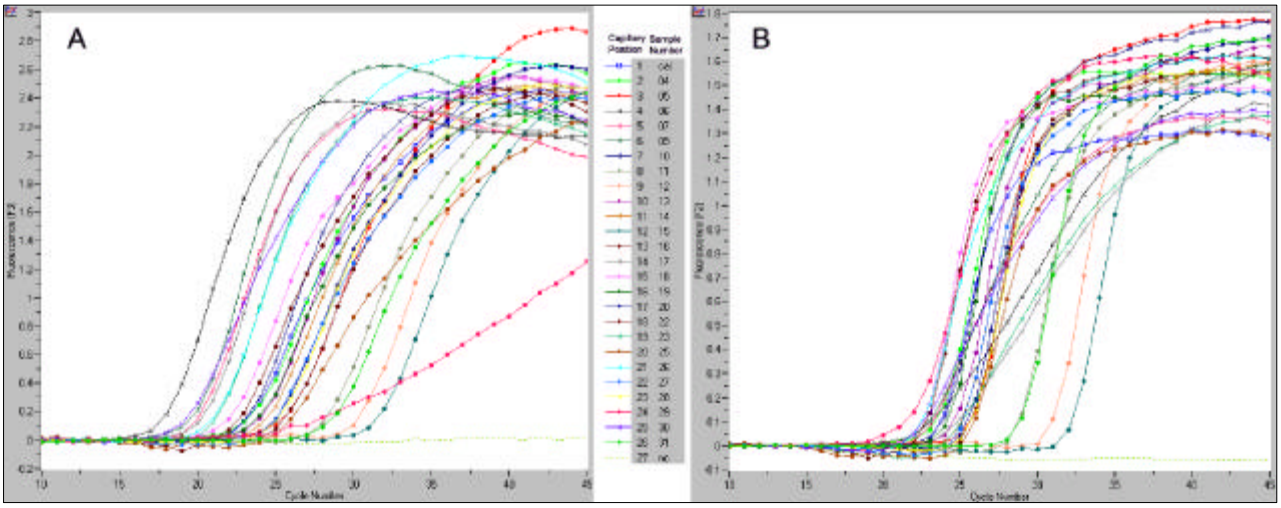

Fig. 1. Fluorescence data for HER2/neu is shown in channel 3 (A) and for the reference gene in channel 2 (B), respectively. Sample #4, #5, #11, #12, #13, #15, #16, #18, #19, #20, #18, #25, #27, #29, and #31) are negative for HER2/*neu* DNA over-amplification. Sample #6, #7, #9, #10, #14, #17, #23, #26, #28, and #30 are positive for HER2/*neu* DNA over-amplification. The LightCycler Relative Quantification Software version 1.0 was used to calculate the relative amount of HER2/*neu* DNA amplification. The final result is expressed as a ratio of T(test):R(reference) in the sample, relative to the ratio of T:R in the LightCycler-HER2/*neu* Calibrator DNA with a cut off value of 2.00.

Table 1. HER2/*neu* Status Using the IHC and Real-time PCR Methods

		IHC			total
		P	N	NT	
Real-time PCR	P	7	1	2	10
	N	9	5	3	17
total		16	6	5	27

IHC, immunohistochemistry; P, positive; N, negative; NT, not tested.

between the HER2/*neu* results and the S-phase fractions of the DNA ploidy as well as the other parameters including the estrogen receptor, progesterone receptor, and the cytokeratin levels (Table 2).

DISCUSSION

Current methods used to evaluate the HER2/*neu* gene status are immunohistochemistry (IHC), Southern blotting, fluorescence in-situ hybridization (FISH), and the polymerase chain reaction (PCR). Gene amplification (DNA level) is usually determined by FISH, whereas protein over-expression is determined by IHC.[8,9] Although a correlation between HER2/*neu* amplification by FISH and over-expression by IHC was good, a number of discordant cases have been reported, indicating the need for a standardized method for clinical applications.[10]

In this study, comparative data of HER2/*neu* status assessed by different methods is reported. The results demonstrated marked discordant results between the real time quantitative PCR and IHC methods. According to another study[1] on the HER2/*neu* status assessed by quantitative PCR, amplification of the HER2/*neu* gene was shown in 24.1% of breast cancers. This data is similar but slightly lower than result of 37% in this study. This difference may suggest that real time quantitative PCR using a LightCycler and a

Table 2. Association between the HER2/*neu* Status in the Real-time PCR Method and the Parameters

		HER2/*neu*		*p* value
		P	N	
BM metastasis	P	0	1	0.434
	N	10	16	
LN metastais	P	4	10	0.345
	N	6	7	
DNA	diploidy	1	10	0.013
	aneuploidy	9	7	
SPF	high	8	11	0.401
	low	2	6	
ER	P	5	12	0.455
	N	3	2	
	NT	2	3	
PR	P	6	11	0.971
	N	2	3	
	NT	2	3	
CK	P	0	1	0.53
	N	10	15	
	NT	0	1	

BM, bone marrow; LN, lymph node; SPF, s-phase fraction; ER, estrogen receptor; PR, progesteron receptor; CK, cytokeratin: P, positive; N, negative; NT, not tested.
p values are calculated by χ^2-test.

HER2/*neu* DNA quantification kit may be more sensitive than other quantitative PCR methods.

For immunohistochemistry (IHC), 16 (73%) out of 22 breast cancers were positive with the rabbit anti-human c-erb-2 oncoprotein (DAKO), which were higher than those with 36.6%1 in the HercepTest (DAKO). This discrepancy may be due to technical differences including the use of different antibodies, different fixatives, and variations in the antibody titers or interpreting the stain. A comparison of different antibodies was performed by Press et al.[11] who reported that several antibodies exhibited nonspecific cytoplasmic staining, which may have been scored as being positive in some studies, which possibly contributed to some of the discrepancies. This suggests that the IHC method may result in false positive or inconsistent results according to the antibodies used. Although the positivity (37%) for HER2/*neu* in real-time PCR was lower than that using the IHC method (73%), only real-time PCR results showed a significant correlation with the clinical parameters such as aneuploidy. This may suggest that real-time PCR has an advantage over the IHC method.

In addition, there are possible explanations for the discordances between the real time quantitative PCR and IHC results, including specimen differences. Fresh tumor tissue was used in the real-time PCR measurements and paraffin-embedded tissue was used in IHC. Another possible cause for discrepancy may be intra-observer error due to subjectivity not only in the IHC interpretation, but also in the real-time PCR amplification efficiencies, particularly in the first cycles, which depend not only on the melting temperature of

the amplicon, but also on the behavior of the amplicon's genomic vicinity.[12]

In contrast to other methods including conventional PCR, the gastrin gene was used as a reference gene, which is located on the same chromosome as the HER2/*neu* gene in order to decrease the error due to chromosome abnormalities. The gastrin gene is known to be overexpressed in the intestinal type of gastric cancer and it was reported to be amplified in the same nuclei as the HER2/*neu* gene, and the hybridization signals were localized to the same region of the nucleus.[13] This FISH study on 40 breast cancer cell lines demonstrated that gastrin gene amplification is unique in gastric cancer and simultaneous amplification of both genes is important for the tumorigenesis of intestinal gastric cancer.

With respect to a high throughput and flexible applicability of the sample status such as formalin-fixed archived materials, PCR could be the method of choice. However, the PCRs are semi-quantitative, and the reference gene is usually not located on chromosome 17, where the HER2/*neu* gene exists. This makes it difficult to determine if the amplification is due to a small region of the chromosome or due to the whole chromosome in the case of chromosome aneuploidy, with a loss or gain. Moreover, the PCR method is relatively expensive and labor-intensive.

Quantitative PCR methods need either an internal or external standard. The advantage of an internal standard is to be able control the PCR efficiency between the samples and the end-point analysis but internal standard systems have a dynamic range of 2 logs.[14] The advantages of external standards are a greater dynamic range and results can be obtained from a single tube per sample. However, the disadvantages include the need for a complete standard curve for each run, a separate standard curve for the target and reference genes or equal efficiencies of the two reactions. When using external standards, analysis is best performed during the log phase of the PCR.

Several advantages of fluorescent PCR over traditional PCR are the elimination of manual procedures such as sample tracking due to reactions performed in a single capillary tube thereby reducing the chance of PCR product contamination. Real-time quantitative PCR for HER2/*neu* appears to be clinically useful due to its simplicity and rapid results.

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