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# Quantitative Measurement of HER2/neu Oncogene Amplification and p53 Tumor Suppressor Gene Deletion by RT-PCR in Breast Cancer*

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## ABSTRACT

**Objective:** The aim of the study was to quantitatively evaluate HER2/neu oncogene amplification and p53 tumor suppressor gene deletion in breast cancer with real-time polymerase chain reaction (RT-PCR).

**Materials and Methods:** Sections obtained from the tumor tissues of 50 patients were paraffinized on slides, and DNA extraction was performed. HER2/neu amplification and p53 deletion were analyzed using RT-PCR with respect to immunohistochemistry (IHC).

**Results:** For 25 patients with breast cancer, we compared IHC and RT-PCR results for the quantitative measurement of HER2/neu expression. We found a significant correlation between the results obtained using IHC and RT-PCR ($p < 0.05$). Taking the results of IHC as reference, the sensitivity and specificity of the RT-PCR method were 57% and 83%, respectively. HER2/neu amplification and p53 deletion did not have a significant correlation with tumor size, histological grade, lymph node invasion, and status of estrogen receptor and progesterone receptor ($p>0.05$, for all).

**Conclusion:** RT-PCR measured gene levels reliably and accurately with high sensitivity and specificity, making it superior to IHC, which is subjective.

**Keywords:** Breast cancer, p53, HER2/neu, RT-PCR

## INTRODUCTION

Breast cancer is the most common malignant disease in women (1). Breast cancer has the second highest mortality rate, following lung cancer, in women, and therefore, is frequently researched (2). The HER2/neu protooncogene has key functions in regulating normal cell growth under physiological conditions, with oncogenic amplification in 25%–30% of the patients with breast cancer. Patients with this oncogene amplification have frequent relapses of cancer, a shorter life expectancy, and resistance to existing treatments, all indicating a poor prognosis. Clinicians have used anti-HER therapy to target this oncogene, modifying therapy protocols (3). p53 gene mutation has been reported in 30%–50% of breast cancer cases. p53 is a tumor suppressor gene and is called the protector of genomes. Normal functions of p53 include regulation of cell growth, gene transcription, DNA repair, and genomic stability. The presence of p53 mutations is critical to tumor growth, prognosis, and treatment response. Any alterations in the p53 gene and amplification of the HER2/neu oncogene jointly affect the pathogenesis of invasive ductal breast cancer (4). Therefore, researchers have investigated the co-existence of HER2/neu and p53 mutations in breast cancer, with controversial results (5, 6). The presence of both HER2/neu amplification and p53 tumor suppressor gene mutation is prognostically crucial for breast cancer. Researchers have detected a poor prognosis in patients with HER2/neu amplification and p53 mutations. However, the

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results concerning histopathological prognostic factors, such as lymph node status and tumor size, remain unclear. In this study, we aimed to compare immunohistochemistry (IHC) and real-time polymerase chain reaction (RT-PCR) RT-PCR results for the quantitative measurement of HER2/neu amplification in patients with breast cancer. Moreover, we aimed to examine the presence of a relationship between patients with p53 deletion and patients with HER2/neu amplification by quantitatively measuring the gene copy number with RT-PCR. We also examined the correlation between RT-PCR analysis of HER2/neu amplification, as well as p53 gene deletion, and histopathological prognostic factors in these patients.

## MATERIALS AND METHODS

### Patient Samples

This retrospective study was conducted with 50 patients, of which 46 had invasive breast cancer and 4 had benign breast disease. The histological type, grade, and size of the tumor; presence of lymph node invasion; and status of estrogen receptor (ER) and progesterone receptor (PR) were known for all patients, as assessed by IHC. Results for HER2/neu quantification by IHC were available for 25 patients. IHC is based on the principle of revealing specific proteins in tissues and cells using monoclonal and polyclonal antibodies. The cellular localization of the target protein is examined under light microscopy. Tumor tissues sections (10-µm thick and 50–100 mg in weight) were obtained, paraffinized on slides, and stored in a refrigerator at +4°C until DNA isolation was performed using High Pure PCR Template Preparation Kit (Roche Diagnostics Systems).

### RT-PCR Analysis

RT-PCR provides quantitative results in a short time by measuring the fluorescence signal, which increases simultaneously with nucleic acid amplification. Mutations in the target nucleic acid can be detected using fluorescence probes (7). HER2/neu was quantified using DNA isolated from tumor tissues using a LightCycler (LC) RT-PCR device from Roche Diagnostics Systems. The LC HER2/neu DNA Quantification Kit was used for the quantitative measurement of HER2/neu. The results of the samples run in the LC PCR program were exported to the quantification software. The ratio of HER2/neu copy number targeted for each sample to the reference gene copy number was calculated. HER2/neu amplification was considered negative or positive if this ratio was <2 or ≥2, respectively. DNA samples from the same patients were quantified with RT-PCR on the LC device to detect p53 deletion. Using the appropriate PCR program, a standard calibration curve was generated using DNA obtained from the blood of a healthy individual. Then, p53 was detected in the DNA sample of each patient. The PCR program provided the gene copy number and the insulin like growth factor 1 (IGF1) gene copy number, which served as the reference gene. These values were entered into the loss of heterozygosity (LOH) formula, as follows:

$$LOH = 2X \frac{n_T^{p53} / n_T^{IGF\text{-}1} - 1}{nNp53 / nNIGF - 1}$$

$n_T^{p53}$ = p53 gene copy number in tumor tissue

$n_T^{IGF\text{-}1}$ = IGF-1 gene copy number in tumor tissue

nNp53 = p53 gene copy number in control blood

nNIGF -1 = IGF-1 gene copy number in control blood

The LOH values were interpreted as follows:

LOH = 1 → No difference between the tumor tissue and healthy control in terms of allelic status

0 < LOH < 1 → Deletion in one p53 allele or IGF1 amplification

LOH > 1 → Amplification of one p53 allele or presence of one IGF-1 deletion

### Statistical Analyses

Statistical analyses were performed using SPSS 13.0 software. The correlation between the results yielded by IHC and RT-PCR for the quantification of HER2/neu in 25 patients with breast cancer was evaluated by the chi-square test. The sensitivity and specificity of RT-PCR were calculated with reference to IHC. The correlation between HER2/neu positivity and p53 gene deletion was investigated by the chi-square test. Tumor grade and size, presence of lymph node invasion, and ER and PR results assessed by IHC were known for 46 patients with invasive ductal breast cancer. The Mann–Whitney U test was

**Table 1.** Comparison of the RT-PCR and IHC methods.

| | | RT-PCR HER2/neu oncogene | | | | | | p |
|---|---|---|---|---|---|---|---|---|
| | | Positive | | Negative | | Total | | |
| | | n | % | n | % | n | % | |
| IHC HER2/neu oncogene | Positive | 4 | 57.1 | 3 | 16.7 | 7 | 28.0 | 0.043* |
| | Negative | 3 | 42.9 | 15 | 83.3 | 18 | 72.0 | |
| | Total | 7 | 100.0 | 18 | 100.0 | 25 | 100.0 | |

RT-PCR: Real-time polymerase chain reaction, IHC: Immunohistochemistry; *p < 0.05

used to compare quantitative data for HER2/neu amplification and p53 deletion, according to prognostic factors. The chi-square test and Fisher's exact chi-square test were used for the comparison of qualitative data. The results were assessed at 95% confidence interval and $p < 0.05$ significance level.

## RESULTS

The ratio of HER2/neu measured quantitatively by RT-PCR to the reference gene ranged between 0.31 and 24.3 (average = 2.76 ± 4.29). According to RT-PCR analysis, 24% of the

**Table 2.** Correlation between HER2/neu oncogene amplification and p53 gene deletion.

| | | HER2/neu oncogene | | | | | | p |
|---|---|---|---|---|---|---|---|---|
| | | Positive | | Negative | | Total | | |
| | | n | % | n | % | n | % | |
| p53 | Positive | 6 | 50.0 | 14 | 40.0 | 20 | 42.6 | 0.545 |
| | Negative | 6 | 50.0 | 21 | 60.0 | 27 | 57.4 | |
| | Total | 12 | 100.0 | 35 | 100.0 | 47 | 100.0 | |

**Table 3.** Correlation between HER2/neu oncogene amplification and prognostic factors.

| Prognostic factors | | HER2/neu oncogene | | | | | | p |
|---|---|---|---|---|---|---|---|---|
| | | Positive | | Negative | | Total | | |
| | | n | % | n | % | n | % | |
| Grade | 2 | 8 | 66.7 | 24 | 70.6 | 32 | 69.6 | 0.800 |
| | 3 | 4 | 33.3 | 10 | 29.4 | 14 | 30.4 | |
| Lymph node | Positive | 10 | 83.3 | 29 | 85.3 | 39 | 84.8 | 0.871 |
| | Negative | 2 | 16.7 | 5 | 14.7 | 7 | 15.2 | |
| ER | – | 3 | 25.0 | 7 | 20.6 | 10 | 21.7 | 0.804 |
| | + | - | - | 2 | 5.9 | 2 | 4.3 | |
| | ++ | 4 | 33.3 | 9 | 26.5 | 13 | 28.3 | |
| | +++ | 5 | 41.7 | 16 | 47.1 | 21 | 45.7 | |
| ER | Positive | 9 | 75.0 | 27 | 79.4 | 36 | 78.3 | 0.750 |
| | Negative | 3 | 25.0 | 7 | 20.6 | 10 | 21.7 | |
| PR | – | 2 | 16.7 | 6 | 17.6 | 8 | 17.4 | 0.804 |
| | + | 4 | 33.3 | 5 | 14.7 | 9 | 19.6 | |
| | ++ | 1 | 8.3 | 11 | 32.4 | 12 | 26.1 | |
| | +++ | 5 | 41.7 | 12 | 35.3 | 17 | 37.0 | |
| PR | Positive | 10 | 83.3 | 28 | 82.4 | 38 | 82.6 | 0.939 |
| | Negative | 2 | 16.7 | 6 | 17.6 | 8 | 17.4 | |
| Tumor size | ≥2 cm | 5 | 41.7 | 19 | 55.9 | 24 | 52.2 | 0.939 |
| | <2 cm | 7 | 58.3 | 15 | 44.1 | 22 | 47.8 | |
| Tumor size (Mean ± SD) | | 1.93 ± 0.73 | | 2.72 ± 2.04 | | 2.52 ± 1.82 | | 0.373 |

ER, estrogen receptor; PR, progesterone receptor

**Table 4.** Correlation between the presence of p53 gene deletion and prognostic factors.

| Prognostic factors | | p53 | | | | | | p |
|---|---|---|---|---|---|---|---|---|
| | | Positive | | Normal | | Total | | |
| | | n | % | n | % | n | % | |
| **Grade** | **2** | 11 | 55.0 | 18 | 78.3 | 29 | 67.4 | 0.104 |
| | **3** | 9 | 45.0 | 5 | 21.7 | 14 | 32.6 | |
| **Lymph node** | **Positive** | 18 | 90.0 | 18 | 78.3 | 36 | 83.7 | 0.420 |
| | **Negative** | 2 | 10.0 | 5 | 21.7 | 7 | 16.3 | |
| **ER** | **–** | 4 | 20.0 | 6 | 26.1 | 10 | 23.3 | 0.232 |
| | **+** | 1 | 5.0 | 1 | 4.3 | 2 | 4.7 | |
| | **++** | 8 | 40.0 | 3 | 13.0 | 11 | 25.6 | |
| | **+++** | 7 | 35.0 | 13 | 56.5 | 20 | 46.5 | |
| **ER** | **Positive** | 16 | 80.0 | 17 | 73.9 | 33 | 76.7 | 0.637 |
| | **Negative** | 4 | 20.0 | 6 | 26.1 | 10 | 23.3 | |
| **PR** | **–** | 3 | 15.0 | 5 | 21.7 | 8 | 18.6 | 0.613 |
| | **+** | 5 | 25.0 | 4 | 17.4 | 9 | 20.9 | |
| | **++** | 6 | 30.0 | 4 | 17.4 | 10 | 23.3 | |
| | **+++** | 6 | 30.0 | 10 | 43.5 | 16 | 37.2 | |
| **PR** | **Positive** | 17 | 85.0 | 18 | 78.3 | 35 | 81.4 | 0.704 |
| | **Negative** | 3 | 15.0 | 5 | 21.7 | 8 | 18.6 | |
| **Tumor size, cm** | **≥2 cm** | 13 | 65.0 | 10 | 43.5 | 23 | 53.5 | 0.158 |
| | **<2 cm** | 7 | 35.0 | 13 | 56.5 | 20 | 46.5 | |
| **Tumor size, cm (Mean ± SD)** | | 2.21 ± 0.70 | | 2.85 ± 2.46 | | 2.52 ± 1.82 | | 0.616 |

ER, estrogen receptor; PR, progesterone receptor

cases were positive and 76% were negative for HER2/neu amplification. The LOH values calculated for the detection of p53 gene deletion by RT-PCR varied between 0.01 and 9.0, (average = 2.07 ± 2.60). For the p53 deletion, 43% of the cases were positive and 57% were negative. A statistically significant correlation was found between the HER2/neu results obtained using RT-PCR and IHC ($p < 0.05$). In addition, 4/7 HER2-positive cases detected by RT-PCR tested positive with IHC, whereas 15/18 HER2-negative cases tested by RT-PCR tested negative with IHC. When evaluated with respect to IHC, RT-PCR had a sensitivity of 57.1% and specificity of 83.3% (Table 1).

Chi-square test analysis showed no significant correlation between the HER2/neu results for 46 patients with invasive ductal breast cancer and 4 patients with benign breast disease by RT-PCR and the results for p53 quantified using RT-PCR ($p = 0.545$; Table 2).

No statistically significant correlation was found between HER2/neu amplification and tumor grade, lymph node invasion, status of ER and PR, or tumor size ($p > 0.05$ for all; Table 3).

No statistically significant correlation was found between the presence of p53 deletion and tumor grade, lymph node invasion, status of ER and PR, or tumor size ($p > 0.05$ for all; Table 4).

## DISCUSSION

In breast cancer, neoplastic transformation and tumor growth occur through multistep, complex genetic alterations. Oncogenic activation and inactivation of tumor suppressor genes result in cancer development. HER2/neu amplification and p53 mutations play an important role in the development of invasive ductal breast cancer (8). Alterations in these two genes are not only crucial for tumorigenesis, but also have prognostic significance. RT-PCR is a quantitative method that

provides rapid results by measuring fluorescence signals that increase with nucleic acid amplification (9). Gene level measurements provide insights into cancer development, progression, and response or resistance to treatment or tumor behavior (10).

Königshoff et al. (4) quantitatively measured HER2/neu in DNA samples from breast tumor tissues with RT-PCR and compared the results obtained with IHC. The authors detected a good correlation between the results obtained with these two methods ($p = 0.029$). Murad et al. (11) reported that the concordance rate between IHC and RT-PCR was 79.3%. In our study, DNA isolation was performed on paraffinized tumor tissues of patients with breast cancer, and the quantitative measurement of HER2/neu amplification was performed using RT-PCR, which is the most commonly used method for measurement. We found a statistically significant correlation between the results obtained by RT-PCR and IHC ($p = 0.043$). Studies have reported that HER2/neu is present in 25%–30% of the patients with breast cancer, who experienced frequent relapse and had a shorter life expectancy (12, 13). In our study, HER2/neu was detected in 24% of the patients with breast cancer by RT-PCR. In a study investigating the correlation between HER2/neu and other prognostic factors, Borg et al. (14) reported a negative correlation between HER2/neu positivity and steroid receptors. The authors noted that HER2/neu amplification was accompanied by a high histological grade; however, the correlation of HER2/neu amplification with lymph node status and tumor size was unclear. In our study, we did not observe any significant correlation between HER2/neu amplification with tumor size, histological grade, lymph node invasion, ER status, or PR status ($p > 0.05$). Consistent with our findings, tumor size and lymph node status are not associated with HER2/neu amplification (15). Although HER2/neu amplification is expected to be associated with high histological grade, the retrospective study yielded no statistically significant result, probably because most of the randomly selected cases were grade 2. Although a negative correlation was reported between HER2/neu amplification and steroid receptors (15), we detected no such correlation in our study, because many of our patients were positive for ER and PR.

Gentile et al. (16) detected LOH in p53 deletion in 43% of the patients with breast cancer. In our study, LOH in p53 gene deletion was calculated using RT-PCR, and p53 gene deletion was detected in 43% of the patients with breast cancer. Dimitrakakis et al. (6) reported a correlation between p53 gene expression and the presence of the HER2/neu oncogene ($p = 0.005$). Fedorava et al. (17) reported an indirect association of p53 gene deletion and HER2/neu expression. Tsutsui et al. (5) determined that the p53 protein and the HER2/neu oncogene were independent prognostic factors. In this study, there was no significant correlation between HER2/neu amplification quantified using RT-PCR and p53 gene deletion ($p = 0.545$).

High-grade tumors, ER negativity, and PR negativity are common in the presence of changes in p53 gene (18, 19). Our study lacked a significant relationship probably because most of our randomly selected patients had grade 2 tumors and many had ER and PR positivity.

Sadia et al. (20) determined an abnormal gene expression of p53 in all grades of breast tumors, but no significant difference in the down-regulation or up-regulation of p53 across different grades of breast tumor ($p > 0.05$). Similarly, we detected no significant correlation between p53 gene deletion and histological grade ($p > 0.05$).

In conclusion, RT-PCR can be successfully used for quantifying gene expression due to its high sensitivity and specificity. In this study, we quantified HER2/neu amplification and p53 deletion using RT-PCR. With respect to IHC, RT-PCR had a sensitivity of 57% and specificity of 83%. Therefore, it can be confidently used to replace IHC, which is a subjective method. Although HER2/neu amplification and p53 deletion play a joint role in the pathogenesis of breast cancer, the results of our study suggest that they are two independent prognostic factors.

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