Quantitative real-time PCR assay with immunohistochemical evaluation of HER2/neu oncogene in breast cancer patients and its correlation with clinicopathological findings

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ABSTRACT

Human epidermal growth factor receptor HER2/neu status is an important prognostic factor for breast cancer as it is crucial in stimulating growth and cellular motility. Overexpression of HER2/neu is observed in 10%−35% of the human breast cancer and is associated with prognosis and response to treatment. The magnitude of amplification must be determined to facilitate better prognosis and personalized therapy in the affected patient. This study aims to investigate the HER2/neu status in breast cancer by concurrent HER2/neu protein overexpression immunohistochemically with HER2/neu DNA amplification by quantitative real-time polymerase chain reaction (PCR), allowing accurate and precise quantification of HER2/neu amplification after a follow-up period. A total of 54 paired tissue samples from formalin-fixed paraffin-embedded (FFPE) breast cancer patients enrolled in this study were collected to evaluate tumor and normal tissues. Only cases with 80% and more tumor cells were included. For confirmation of immunohistochemistry (IHC) results, qPCR was used to determine the HER2/neu amplification. The association between clinicopathological variables like age, tumor size, histological grade, stage, lymph node status, hormone receptor status, family history, recurrence rate, and vital status was evaluated. We observed that 11/54 (20.4%) of the tumor tissues are positive for HER2/neu protein overexpression by IHC. A total of 8 out of these 11 cases (72.7%), which presented a score of 3+, showed gene amplification of HER2/neu. The concordance rate between IHC and qPCR was 94.4%. HER2/neu gene amplification was found to be significantly associated with recurrence, increased risk of death, and progesterone receptor status, supporting a negative prognostic role of HER2/neu in breast cancer survival. In conclusion, IHC can be used as an initial screening test to detect HER2/neu protein overexpression, and the use of qPCR can verify the IHC results and establish HER2/neu status in routine clinical practice.

KEY WORDS: Breast cancer, HER2/neu, immunohistochemistry, quantitative real-time PCR

INTRODUCTION

It is estimated that there were more than 3.5 million women living in the US with a history of invasive breast cancer as of January 1, 2016, and an additional 246,660 will be newly diagnosed in 2016. The median age at diagnosis is 61. About 19% of breast cancers occur among women younger than 50, while 44% occur in those older than 65. Breast cancer is a heterogeneous disease with a variety of morphological and molecular characteristics and response to therapy and clinical outcome. It is divided into five molecular subtypes: luminal A, luminal B, human epidermal growth factor receptor 2 (HER2) positive, basal-like, and normal-like. This categorization assists in understanding the mechanisms that regulate differentiation and cell proliferation, thus allowing a better prognosis, and providing important tools for the choice of therapy. Breast cancer with HER2 overexpression currently comprises 10%−35% of all cases of human breast cancer, and amplification of the gene is associated with more than 96% of these cases.
The HER2/neu oncogene encodes for a 185 kDa transmembrane tyrosine kinase growth factor receptor that belongs to the epidermal growth factor receptor family.[7] The gene is located at the long arm of chromosome 17(q21) and is expressed in the epithelial cells.[8] In breast cancer, its amplification is strongly associated with poor prognosis in terms of shorter periods without relapse and shorter survival.[6,8-11] More interestingly, HER2/neu status affects the response and resistance to therapies.[12] It has been shown that amplification of HER2/neu is related to tumor size, lymph node metastasis, a high S-phase fraction, aneuploidy, and low level of steroid hormone receptors. These factors might increase the rate of proliferation of tumor cells.[13] It has also been reported that angiogenesis and expression of vascular endothelial growth factor increase when HER2/neu is amplified.[14] It has been observed that the degree of HER2/neu overexpression is higher in early forms of breast cancer than that in more advanced invasive carcinomas.[13] This suggests that alterations in HER2/neu alone cannot lead to progression from relatively benign to a more malignant phenotype in breast tumors. It has been stated that HER2/neu status determines whether certain anti HER2 drugs (e.g., trastuzumab, lapatinib, pertuzumab, and T-DM1) are recommended. The HER2 gene is important in tumor cell growth and tumors that have increased levels of HER2 (as measured by HER2 gene amplification or HER2 protein overexpression) and usually have a higher growth rate and more aggressive clinical behavior.[15] The most common detection methods of HER2/neu include measurement of protein overexpression by immunohistochemical assay and detection of gene amplification by the fluorescence in situ hybridization (FISH) technique.[16] Both of these methods are approved by the US Food and Drug Administration. PCR based methods have become important in clinical analyses, especially, the quantitative PCR methods such as real-time polymerase chain reaction (PCR), which is based on detection of DNA amplification. The HER2/neu gene is amplified in parallel with a reference gene that has a low risk of copy number variation in breast cancer, and then the copy number ratio between HER2/neu and the reference gene is determined.

Real-time PCR is cost effective, and many samples can be analyzed at the same time.[16] The HER2 overexpression is currently defined according to the 2014 ASCO/CAP guideline scoring system to achieve reproducible assay performance.

The aim of the present study was to investigate the usefulness of real-time PCR in evaluating HER2 status in breast cancer and to compare the results with the corresponding findings obtained from immunohistochemistry analysis and clinicopathological data.

**METHODS AND MATERIALS**

A total of 54 breast cancer cases were enrolled in this study. A prior consent was obtained from the patients. Paired tissue samples comprising normal and tumor tissues were selected from the department of pathology and anatomical science, School of Medicine, University of Missouri, Colombia, from January 2010 to January 2011. All the breast tissues were fixed in 10% neutral buffered formalin and embedded in paraffin under standard conditions. Hematoxylin and 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 patients were used as controls and showed histologically normal features. Clinical data including age, stage, histological grade, lymph nodes status, recurrence, family history, and vital status were collected (Table 1). None of the patients had undergone radiation or chemotherapy before surgery. Clinical stage was classified according to the American Joint Committee on Cancer’s TNM Staging System. This study had an Institutional Review Board (IRB) approval obtained from the Department.

**Table 1: Clinicopathological features by HER2/neu status**

| Variables                  | Amplified | Nonamplified | P     |
|----------------------------|-----------|--------------|-------|
| Number of patients         |           |              | 0.539 |
| Age (year)                 |           |              |       |
| <50                        | 8 (14.8%) | 46 (85.2%)   |       |
| ≥50                        | 2 (12.5%) | 14 (87.5%)   |       |
| Stage                      |           |              | 0.725 |
| I+II                       | 8 (20%)   | 34 (80%)     |       |
| III+IV                     | 0         | 12 (100%)    |       |
| Tumor size                 |           |              | 0.927 |
| ≤2                         | 4 (14.8%) | 23 (85.2%)   |       |
| (cm) >2                    | 4 (14.8%) | 23 (85.2%)   |       |
| Lymph node status          |           |              | 0.445 |
| Positive                   | 0         | 20 (100%)    |       |
| Negative                   | 8 (23.5%) | 26 (76.5%)   |       |
| Histologic grade           |           |              | 0.423 |
| I/II                       | 4 (12.9%) | 27 (87.1%)   |       |
| III                        | 4 (17.4%) | 19 (82.6%)   |       |
| ER status                  |           |              |       |
| Positive                   | 7 (18.9%) | 30 (81%)     | 0.121 |
| Negative                   | 1 (10%)   | 9 (90%)      |       |
| PR status                  |           |              |       |
| Positive                   | 5 (15.2%) | 28 (84.8%)   | 0.047 |
| Negative                   | 3 (23.1%) | 10 (76.9%)   |       |
| Family history of cancer   |           |              |       |
| Positive                   | 1 (5.3%)  | 18 (94.7%)   | 0.250 |
| Negative                   | 7 (20%)   | 28 (80%)     |       |
| Recurrence                 |           |              |       |
| Positive                   | 6 (60%)   | 4 (40%)      | 0.041 |
| Negative                   | 2 (4.5%)  | 42 (95.5%)   |       |
| Vital status               |           |              |       |
| Alive                      | 4 (8.9%)  | 41 (91.1%)   | 0.040 |
| Deceased                   | 4 (44.4%) | 5 (55.6%)    |       |

**Table 2: Sequences and concentrations of primers and hybridization probes used for real-time quantitative PCR**

| Oligonucleotide | Conc. | Sequence            |
|-----------------|-------|---------------------|
| HER2 forward    | 5     | 5'-CCA GTA CCT GCT GAA CTG CTG GT-3' |
| HER2 reverse    | 5     | 5'-TGT AGC AGC CGC ACA TCC -3' |
| HER2 probe      | 5     | 5'-HEX CAG ATT GGC ZEN AAG GGG ATG AGC TAC CTG 3' ABKFQ |
| RNaseP forward  | 2.5   | 5'-AGA TTT GGA CCT GGC AGC G-3' |
| RNaseP reverse  | 2.5   | 5'-GAG CGG CTG TCT CCA CAA GT-3' |
| RNaseP probe    | 2.5   | 5'-6FAM TTC TGA CCT GAC GGC TCT GGC CG 3' 6TAMSp |
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**DNA extraction**

FFPE breast samples (tumor and normal tissues) were cut using Leica microtome. Sections measuring 2 × 10 μm were placed in a 1.7 ml microcentrifuge tube; then 1 ml xylene was added to the sample, vortexed, and centrifuged at full speed for 2 min. After the supernatant was removed, 1 ml of ethanol (200 proof) was added, vortexed for 10 s, and then centrifuged at full speed for 2 min at room temperature. The samples were air-dried for 15 min until no residual ethanol could be seen. The samples were resuspended in 180 μl A tissue lysis (ALT) buffer with the addition of 20 μl proteinase K and then vortexed. Incubation occurred at 56°C for 3 hr and 90°C for 1 hr. Samples were then cooled at room temperature. Then we added 1 μl Rase A. 200 μl buffer AL was added and vortexed; then 200 μl ethanol was added and vortexed. The entire lysate was transferred to the MinElute column and centrifuged. The DNA was isolated by QIAamp™ DNA FFPE tissue kit (QIAGEN) as described by the manufacturer. A NanoDrop 2000 spectrophotometer was used to read the concentrations.

**Immunohistochemistry**

The rabbit monoclonal antibody SP3 for HER2/neu (Cell Marque Corporation, Rocklin, CA, USA) was immunostained on the Benchmark ULTRA automated stainer (Ventana Medical Systems, Tucson, AZ, USA). The antigen retrieval method using the ultraView DAB Detection Kit CC2 on a preheated slide at 91°C for 68 min (standard). CC2 is the equivalent of a citrate buffer antigen retrieval buffer. The dilution used for HER2 SP3 was RTU (prediluted) with an immunoglobulin concentration of 1.18 μg/ml with primary antibody incubation time of 32 min. Negative and positive control slides were included in each assay. Samples were interpreted according to the American Society of Clinical Oncology/College of American Pathology (ASCO/CAP) guidelines: Negative (0), weakly positive (2+), and strongly positive (3+), with a threshold of more than 10% of the tumor cells that must show homogenous, dark circumferential (chicken wire) pattern to call results 3+, HER2 positive.[19,10] The estrogen receptor (ER) and progesterone receptor (PR) results were interpreted according to the ASCO/CAP recommended guidelines.[15,18]

SP3 is a rabbit monoclonal antibody that recognizes the extracellular domains of HER2/neu receptors and is a reliable candidate to evaluate the expressions of HER2 in breast cancer cases.[19,20]

**Real-time quantitative PCR**

Quantification of the HER2/neu gene was determined using an Mx300P QPCR System (Agilent Technologies, Santa Clara, CA, USA) with RNase P as the reference gene. The hybridization probes used for quantification are based on primers and probes described by Chariyalertsak et al.[21] The sequence, primer, and probe concentrations are listed in Table 2. Primers, probes, and prime time master mix were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA). Primer/probe mixes and master mix were combined with 3 μl template DNA in a final volume of 20 μl. Amplification conditions were denatured at 95°C for 10 min, followed by denaturation at 95°C for 15 s and annealing at 60°C for 1 min. The cycle was repeated 50 times and samples were cooled to 40°C. Triplicate reactions were performed

| Description, n (%) |
|--------------------|
| Estrogen receptor status | Negative | 15 (27.8%) |
| Progesterone receptor status | Positive | 38 (70.4%) |
| HER2/neu status | Positive | 11 (20.4%) |
| Negative | 43 (79.6%) |

**Table 3: Estrogen, progesterone, and HER2/neu status in breast cancer patients studied**

| Description, n (%) |
|--------------------|
| Negative | 43 (79.6%) |
| Positive | 3 (5.6%) |

**Table 4: Comparison between IHC and real-time PCR HER2 protein expression and DNA amplification status in 54 cases of breast cancer**

| IHC | Nonamplified (%) | Amplified (%) |
|-----|-----------------|---------------|
| Negative | 43 (79.6%) | 0 |
| Positive | 3 (5.6%) | 8 (14.8%) |

| IHC score | n (%) | HER2/neu ratio Mean (range) | Ratio <2 n % | Ratio ≥2 n % |
|-----------|-------|-----------------------------|-------------|-------------|
| 0 or 1+  | 30 (55.6%) | 1.28 (1.03-1.53) | 30 (55.6%) | - |
| 2+       | 13 (24.0%) | 1.29 (0.79-1.52) | 13 (24.0%) | - |
| 3+       | 11 (20.4%) | 1.77 (1.12-2.02) | 3 (5.6%) | 8 (14.8%) |

**Table 5: Comparison between IHC and real-time HER2 protein expression and DNA amplification status in 54 cases of breast cancer according to IHC classification**

**Figure 1:** Hematoxylin and eosin (H and E) staining of breast cancer tissues OHB1080E, OHB1080AA, and OHB1080iii. IHC of tumor tissues with HER2/neu and estrogen (ER) and progesterone receptors (PR).
and the mean cycle threshold value (Ct) was used to determine the relative amount of PCR product.

**Statistical analysis used**

Pearson’s Chi-squared test was performed to evaluate the association between clinicopathological variables and HER2/neu amplification.

All P values were two-sided, and results were considered statistically significant at P < 0.05. All calculations were performed with IBM statistical analysis SPSS 23.

**RESULTS**

A total of 54 breast cancer patients were included in this study, with ages ranging between 32 and 90 years old (mean age = 56.06 years). No statistical correlation was found between HER2/status and age (P = 0.539), stage (P = 0.725), tumor size (P = 0.927), lymph node status (P = 0.445), ER receptor status (P = 0.121), and family history (P = 0.250). Patients with amplified HER2/neu were more likely to have higher rate of recurrence [X2 = 4.193, P = 0.041] compared to the patients with unamplified tumors. Comparison of individual HER2/neu quantification values showed a significant correlation with the vital status of the patients (P = 0.040). Also, a marginal significant correlation was observed with PR receptor status (P = 0.047), as shown in Table 1.

**Estrogen, progesterone, and HER2/neu status in breast cancer using immunohistochemistry**

FFPE sections were performed in all breast cancer cases to determine the representative samples with >80% tumor cells [Figure 1 and Table 1]. Estrogen receptors showed positive results in 39 (72.2%) samples, and 15 (27.8%) cases were negative. A total of 38 (70.4%) cases proved to be positive for progesterone receptors and 16 (29.6%) were negative. The tumor tissue with positive results for HER2/neu was identified in 11 (20.4%) cases of breast cancer, and negative results were identified in 43 cases [Table 3].

**q RT-PCR compared to IHC**

Table 4 shows the comparison between HER2/neu identification using IHC compared to q real-time PCR. HER2 amplification levels were significantly lower in cases presenting with low protein expression (0 or 1+) than those in cases with high expression (3+). None of the low protein cases (score 0 or 1+) showed gene amplification by q RT-PCR, and none of the cases comprising 2+ immunostaining showed gene amplification by q RT-PCR, as shown in Table 5.

A total of 8 out of 11 cases (72.7%) that presented a score of 3+ showed gene amplification of HER2, as shown in Table 5. Concordance between q RT-PCR and IHC was observed in eight cases (concordance rate 94.4%).

**Clinicopathological data in comparison with HER2 status**

HER2 data were also compared to the clinicopathological features as shown in Table 1. No statistical correlation was observed between age, tumor size, clinical stage, lymph node status, histological grade, ER receptor status, and family history of cancer.

A marginally significant correlation between tumor recurrence, vital status, and PR receptor status was detected.

**DISCUSSION**

The HER2/neu gene has been extensively studied as a prognostic and predictive marker in clinical breast cancer, making this receptor a valuable target for the treatment of human breast cancer. HER2/neu overexpression has been observed in 10%–35% of human breast cancers. HER2 status is predominantly evaluated by immunohistochemical staining, because it is easy to perform and has relatively low cost.

However, a wide range of sensitivity and specificity was observed among various commercially available antibodies. Quantitative real-time PCR has the potential to become standard in terms of its performance, accuracy, sensitivity, broad dynamic range, and high throughput capacity. A high concordance rate was observed especially between 3+ score by IHC and gene amplification by q RT-PCR. A total of 8 out of 11 cases with a score 3+ by IHC showed gene amplification. Three cases with 3+ status and HER2 amplification showed transcript downregulated expression. Concordance between IHC and q RT-PCR results was 94.4%. The discordance between these methodologies could include interobserver errors, due to subjectivity of IHC interpretation and q RT-PCR analysis, which can cause discrepancies particularly in the initial cycles, which depend not only on the melting temperature of the amplicon, but also on the behavior of the amplicon. Using PCR-based methods, the expression of a tumor- or tissue-specific gene and the presence of genetic abnormalities can be detected in a clinical specimen with higher sensitivity (one malignant cell out of 106_107 normal cells) than that of other techniques such as light microscopy (one malignant cell out of 102_103 normal cells). Using RT-PCR, the nucleic acid molecules can be amplified 1010-fold.

In the present study, a good correlation rate between IHC and q RT-PCR was observed. No correlation was found between HER2/neu gene levels by IHC, gene copy number, or clinicopathological data, including the age, stage, tumor size, histological grade, lymph node status, family history, and ER receptor status. Similarly, the absence of correlation between HER2 status and clinical and pathological features has been reported in other studies. A correlation was observed for HER2 status by IHC and RT-PCR with tumor recurrence and vital status. Marginal correlation with PR receptor status (P = 0.047) is in agreement
with a study by Ji et al. in which tumors with HER2 amplification were more likely to be PR-negative.[30]

Several methods can be used to determine the HER2/neu DNA amplification and protein overexpression including fluorescence in situ hybridization (FISH), IHC, and chromogenic in situ hybridization (CISH), which are semiquantitative.[30] Quantitative real-time PCR can be used for quantitative measurements of HER2/neu DNA.[5] In the current study, we use two different methods to confirm HER2/neu protein overexpression and DNA amplification in breast cancer patients. The standard method to identify HER2/neu status is the IHC technique, where HER2/neu protein expression is detected with different antibodies, different binding affinities, and different epitope specificities, thereby creating differences in HER2/neu overexpression rates[5] in addition to the scoring system, which relies on the subjective measures of staining intensity and pattern.[5] Therefore, IHC can only be the initial screening strategy to distinguish between positive and negative cases of HER2/neu amplification in breast cancer patients.[31] Although FISH is the gold standard for detection of the HER2/neu amplification, this method does not assess the gene expression and cannot identify cases in which the gene product is overexpressed in the absence of gene amplification. The only quantitative technique is quantitative real-time PCR, which offers a more accurate, reliable, and simple method in detecting HER2/neu amplification.[32] However, only 8 out of 54 cases (15%) of the tumor tissue demonstrated positive HER2/neu DNA amplification.

The current study revealed that 94.4% cases were in concordance with IHC and q RT-PCR. Two other studies were performed by Rosa et al.[33] and Olsson et al.[34] The concordance between IHC and q RT-PCR was observed in 59 cases out of 75 cases (78.7%). In the second, there was an 86% concordance rate between real-time PCR and IHC. Although several techniques can be used in order to determine the HER2/neu gene expression, q RT-PCR is more convenient, easier, and rapid compared to IHC, FISH, and CISH. Nistor et al.[35] conclude from their results, obtained from gene amplification of HER2/neu, that real-time PCR combined with the IHC approach for determination of HER2/neu status in breast cancer patients may be an effective and efficient strategy, but HER2/neu detection using qPCR was more accurate and reproducible compared to IHC. False-positive and false-negative results are still seen, despite intensive effort to establish standard methods for detection of HER2/neu amplification by IHC, FISH, and CISH. This is related to fixed tumor tissue in which formalin fixation may cause damage to HER2/neu epitopes. In IHC, conditions like tissue processing, reagent variability, antigen retrieval methods, scoring interpretation, tumor heterogeneity, and case selection or study IHC showed that 3/54 cases (5.6%) were false positives, while qPCR showed unamplified HER2/neu DNA. These three false-positive cases were of 3+ score by IHC.

CONCLUSION

The results obtained by IHC for HER2/neu gene expression were comparable with the results obtained by q RT-PCR for the HER2 amplification and suggest that q RT-PCR is a viable alternative to FISH in evaluating tumors that are uncertain by IHC. IHC should be used as a screening method in the laboratories and methods including q RT-PCR, FISH, and CISH should be performed to confirm the results. It is also recommended that laboratories should establish a high concordance rate between IHC and PCR.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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