HER-2/neu gene amplification assessment in breast cancer patients in Isfahan province by real time PCR, differential PCR and immunohistochemistry

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A B S T R A C T

Introduction: The amplification status of proto oncogene HER-2/neu is one of the major molecular prognosis markers in breast cancer and recent adjuvant treatment with Trastuzumab has increased a request for the evaluation of HER-2/neu status in breast cancer. The aim of our study was the evaluation of HER-2/neu amplification status in malignant breast cancer by PCR techniques such as differential PCR and real time PCR and comparison of results of two methods together and with IHC results in some specimens.

Methods: 86 malignant breast cancer tissue specimens were analysed initially by differential PCR and then by real time PCR. Sections from paraffin-embedded or fresh tissue samples were homogenized by squash and then DNA extraction was performed on cell suspension. A standard curve was initially plotted using BioEasy SYBR Green I for using 2−ddct method. A 98 bp fragment of HER-2/neu gene was co-amplified in the same reaction tube with a 150 bp fragment of INFγ gene for differential PCR.

Results: The IHC results existed only for 27 of 83 assessed samples by dPCR and for 30 of 86 assessed samples by real time PCR. 29 out of 83 (35%) samples tested by dPCR and 28 out of 86 (32.5%) samples tested by real time PCR have HER-2/neu gene amplification.

Conclusion: There was concordance between the results of real time PCR and differential PCR in 61 of 83 specimens (73.5%) tested by both method. Furthermore, in comparison of IHC results with these two methods, 70% concordance between IHC and differential PCR, 63% concordance between IHC and real time PCR and 55.5% concordance between three methods were observed.

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1. Introduction

Breast cancer is the most common cancer and one of the major causes of cancer-related death among women worldwide (Gunnarsson et al., 2003; Yarden, 2001). Breast cancer occurs in Iranian women at least one decade earlier than their Western counterparts (Ebrahimi et al., 2002) and the incidence rate is 120 cases per 100,000 women in Iran country (Mousavi et al., 2007). Chromosomal structural aberration includes amplification of genomic regions containing oncogenes is frequently observed in breast cancer and plays an important role in breast tumor initiation and progression (Friedrichs et al., 1993; Kuwahara et al., 2004). Chromosomal segment 17q11–q12 is a commonly amplified region in human breast cancer and HER-2/neu (on 17q12–21) is the most important amplified oncogene in breast tumor (Bieche et al., 1996). The HER-2/neu (also known as ErbB2, NEU, NGL, TKP1, CD340, HER2) protooncogene encodes a 185 kDa transmembrane phosphoglycoprotein with tyrosine kinase activity and belongs to a family of four similar epidermal growth factor receptors involved in normal cell growth and differentiation (Burstein, 2005; Eccles, 2001; Tapia et al., 2007; Yarden, 2001). Gene amplification and protein over expression of HER-2/neu oncogene have been observed in 10–34% of breast cancer (Ross and Fletcher, 1999) and are associated with parameters indicative of tumor aggressive such as large tumor size, high-grade tumor, advanced tumor stage, young age at diagnosis, lymph node involvement and absence of steroid hormone receptor expression (Burstein, 2005; Kamali-Sarvestani et al., 2004). Therefore HER2-positive breast cancers have a poor prognosis, greater risk of recurrence, reduced overall survival (OS), shorter disease-free survival (DFS) and reduced response to chemotherapy and hormone therapy, especially Tamoxifen antiestrogen therapy (Burstein, 2005; Rubin and Yarden, 2001). Trastuzumab drug (Herceptin®) is a new treatment option for targeted therapy of HER-2 positive breast tumors (Nitta et al., 2008; Slamon et al., 2001). Trastuzumab is a humanized monoclonal antibody directed against the extracellular domain of HER-2/neu protein (Beauclair et al., 2007; Burstein, 2005; Susini et al., 2004).
2010) and is effective in improving patient survival, reduced tumor pro-
gression and increment of response rate to other chemotherapy regi-
mens (Barberis et al., 2008; Kim et al., 2002), only in breast cancer pa-
ients with HER-2/neu gene amplification and/or over expression. Thus, the assessment of HER-2/neu status is necessary in selecting pa-
ients eligible for this therapy and the appropriate method for the deter-
mination of HER-2/neu status should be simple, sensitive, precise, rapid, reproducible and inexpensive (Merkelbach-Bruse et al., 2003). The most common detection methods for HER-2/neu status are IHC for de-
tection gene expression at the protein level and FISH for detection of gene amplification (Bieche et al., 1999; Rosa et al., 2009). IHC is the first and the most frequently used method for assessing HER-2/neu status in most pathology laboratories (Gjerdrum et al., 2004; Shousha et al., 2009). It is relatively rapid, easy and low cost but has low accuracy and reproducibility and gives a high percentage of false-positive results among the cases with HER-2/neu week over expression (+2 score) (Merkelbach-Bruse et al., 2003). To increase the accuracy and reproduc-
ibility of results of HER-2/neu assessment, samples with an IHC score of moderate should be retested by another method (Ellis et al., 2004). FISH technique is a precise method to measurement of HER-2/neu gene copy number in breast cancer cells but it is time consuming, expensive and difficult, thus its routine use is not feasible (Merkelbach-Bruse et al., 2003; Shousha et al., 2009). Molecular techniques in comparison to mi-
oscopic techniques are faster, cheaper and with more accuracy (Barberis et al., 2008). Polymerase chain reaction techniques are able to estimate HER-2/neu gene copy number from small amounts of tumor tissues (Gjerdrum et al., 2004). Assessment of HER-2/neu amplifi-
cation by PCR can be performed by measuring the amount of product either after a given number of cycles in the linear and/or plateau phase (end point PCR) or after a varying number of cycles during exponential phase (quantitative PCR) (Bieche et al., 1996). Differential PCR is the end point PCR assay and do via co-amplification of a single copy refer-
ce gene and HER-2/neu gene within the same reaction tube and the level of HER-2/neu amplification is calculated by the ratio between the intensity of two PCR product band visualised on a gel of tumor sample in comparison with this ratio in normal sample (Zadroznj et al., 2002). Real time PCR is a quantitative, sensitive, accurate and cost efective method for analysing a large number of frozen and formalin fixed paraffin embedded breast cancer specimens particularly when starting quantities of tumor tissue are minimal (Barberis et al., 2008). The aim of this study was to assess HER-2/neu status in malignant breast cancer tissues by differential PCR and real time PCR and assess agreement be-
tween results of two methods. The comparison has been made between the results of these two methods with IHC.

2. Materials and methods

2.1. Tumor samples

This study was approved by the University of Isfahan. This study is a fundamental survey and all the samples were collected from the patients by their permission. Detailed questionnaires, including clini-
cal and family history, were initially collected. Written consent was then given to collect blood samples. In this study, 86 malignant breast cancer tissues, contain 67 fresh mastectomy tumor specimens or core biopsy specimens and 19 paraffin tumor blocks, were analysed. Five specimens of adjacent healthy breast tissue from breast cancer pa-
ients were used as normal samples without HER-2/neu gene amplifi-
cation. Two 10μm sections were cut from any block of paraffin-
embedded tumor tissue for DNA extraction.

2.2. DNA isolation

DNA extraction was carried out by the method previously de-
scribed by Shi et al. in 2004. In this method 10μm thick sections from paraffin-embedded tissue samples and 50–100 mg sections from fresh tumor tissue samples were cut and homogenized by squash in mortar and boiled in a lysis buffer. Then extraction process-
es were performed on cell suspension.

2.3. Real time PCR

2.3.1. Theoretical basis

Real time PCR exploits the reality that if amplification efficiency be perfect (100%), PCR products during the exponential phase and after each cycle ideally doubles. Thus, the quantity of PCR product in this phase is in proportion to the quantity of initial template. This is ac-
complished by using fluorescent technology. The fluorescence of DNA dye or probe is monitored for each cycle during PCR. At a cycle during exponential phase, PCR products accumulate enough thus fluorescence increase above background. This cycle is known as Ct and demonstrator initial template gene copy number. As the number of initial template copy’s increases, fluorescence appears sooner and Ct is lower. The relative copy number between two samples is determined by 2^-/ddCt method. Because the total amount of DNA present in different samples is variable thus results of the target gene are nor-
malized to a reference gene. Reference gene is invariant in various tis-
ues. In this study reference disomic, gene was UBC gene, which maps to a chromosome region in which no genetic alterations have been found in breast cancer.

2.3.2. Primer

Genetic sequence database at the National Center for Biotechnical Information (NCBI) was used to pull out the sequences [GenBank ID: NG_007503.1 and Gl:171906587 for HER-2/neu and NG_027722.1, Gl:307133773 for UBC gene]. Primers for HER-2/neu gene (target) and UBC gene (reference) were designed with the use of Allel IID software and were checked with the program Oligo 5.0 for the ab-
sence of false priming sites, formation of primer−dimer and hairpin structures. Primers were purchased from BIONEER. A 100 bp frag-
ment of HER-2/neu gene and a 153 bp fragment of UBC gene were amplified by PCR with specific primers.

2.3.3. Standard curve

When 2^-/ddCt method can be used that the amplification efficiency of HER-2/neu and UBC be approximately identical and perfect. Five se-
rial dilutions of genomic DNA were prepared. Each dilution was am-
plified using HER-2/neu and UBC primers. Ct values were calculated for both HER-2/neu and UBC and each gene standard curve is plotted. In a standard curve, Ct values from each dilution are plotted versus the log DNA concentration.

2.3.4. Real time quantitative PCR

Real time PCR was performed using a chromo4 (Bio Rad) device and BioEasy SYBR Green I real time PCR kit. For each PCR run, a master mix was prepared on ice. PCR reactions were carried out in a total vol-
ume of 25 μl. Two PCR reaction tubes were set up for each sample, one for HER-2/neu gene and one for UBC gene. The reaction mix contained 2× SYBR Mix (12.5 μl) from the kit, 0.4 pmol/μl from each primer and 4 U Taq DNA polymerase, 15 ng/μl genomic DNA. 2× SYBR Mix included PCR buffer, MgCl2, dNTP mixture, SYBR Green®. Thermal cycling condition included an initial denaturation for 10 min at 95 °C and 45 cycles that consisted of a denaturation step at 95 °C for 20 s, an annealing step at 69 °C for 10 s, an elongation step for 13 s at 70 °C. Fluorescent measurements were taken after the elongation step. Each sample was run in two replicates and mean Ct values was used for further calculation.

2.3.5. Calculation of the amounts of HER-2/neu DNA

Relative quantification was given by the ratio between the Ct value of HER-2/neu and UBC gene in the tumor sample relative to this ratio in the normal sample by 2^-/ddCt method. If 2^-/ddCt
value ≥ 2, it is considered positive for HER-2/neu gene amplification. A value < 2 is regarded as negative for HER-2/neu amplification.

2.4. Differential PCR

2.4.1. Assessment of the quantitative accuracy of differential PCR

A dilution series consisting of four two-fold dilution of HER-2/neu positive tumor sample DNA and normal sample DNA was prepared. Each dilution was used for DPCR. The ratio of HER-2/neu gene to INFγ gene in HER-2/neu positive tumor sample relative to this ratio in a normal sample determined to test the linearity of the measurement achieved by DPCR with the number of copies of HER-2/neu in each dilution.

2.4.2. Primers

Primers for target gene HER-2/neu and for reference gene INFγ were selected from Goebel et al. (2002) paper and checked with the program Oligo5. The primers were then purchased from Bioneer.

2.4.3. Differential PCR condition

A 98 bp fragment of HER-2/neu gene was co-amplified in the same reaction tube with a 150 bp fragment of INFγ gene. Final reaction volume was 50 μl containing 1.4 PCR buffer, 3.5 mM MgCl2, 0.8 pmol/l of each primer, 0.4 mM of each dNTP and 4 U Taq polymerase and 10 ng/μl of genomic DNA. The thermal cycling conditions comprised a primary denaturation at 95 °C for 10 min then 35 cycles at 94 °C for 1 min, 58.4 °C for 1 min and 70 °C for 1 min followed by incubation at 72 °C for 10 min.

2.4.4. Determination of HER-2/neu gene amplification

After performance of dPCR, products were separated by electrophoresis using 2% agarose gel containing 1 mg/μl of ethidium bromide. A 50 bp DNA size marker has been used here (Fig. 1). The intensity of HER-2/neu band and INFG band was determined for each sample by means of Image J software. The measure of HER-2/neu gene amplification was given by the ratio between HER-2/neu band intensity / INFγ band intensity for tumor samples in comparison with this ratio for normal sample. A ratio < 2 is regarded as negative for HER-2/neu amplification and a ratio ≥ 2 is regarded as positive for amplification (Fig. 2).

3. Results

3.1. Validate standard curve

The standard curve was constructed from the genomic DNA extracted from a normal breast sample that was serially diluted five-fold (four points: 250, 50, 10, 2). The standard curve genes HER-2/neu and UBC were demonstrated a standard linear relationship between the Ct of each gene and log of the starting copy number of that gene. The r² value of HER-2/neu and UBC genes was approximately similar and between 0.96 and 1. The amplification efficiencies of both genes were perfect and thus the 2⁻ddCt calculation is valid and precise (Fig. 3).

3.2. Quantitative accuracy of differential PCR

The ratio of HER-2/neu/INFγ in HER-2/neu positive tumor sample relative to this ratio in a normal sample in each dilution showed a linear relation between the measurements determined by dPCR with HER-2/neu copy’s number in each dilution.

3.3. HER-2/neu gene status in breast cancer by differential PCR

Among the 83 malignant breast tumor tissue tested by dPCR, 29 samples (35%) showed HER-2/neu gene amplification (Table 1). In HER-2/neu positive samples, extra copies of HER-2/neu ranged from 2 to 12 fold. In HER-2/neu negative samples by dPCR, the ratio HER-2/neu to INFγ in a tumor sample relative to HER-2/neu to INFγ intensity in a normal sample ranged from 0.8 to 1/87.

3.4. HER-2/neu gene status assessment in breast cancer by real time PCR

Real time PCR was performed on 86 malignant breast tumor tissue, and 28 samples (32.5) showed HER-2/neu gene amplification (Table 1). In HER-2/neu positive sample by real time PCR (RQ ≥ 2), 2 to 21 fold increment of HER-2/neu copy was observed (Fig. 4).

3.5. Comparison of HER-2/neu status assessed by dPCR and IHC

Among the 83 malignant breast tumor samples, there were IHC results only for 27 cases (15 cases with a score of 0 or +1, six cases with a +2 score and 6 with a +3 score). Comparison of dPCR results with IHC results showed concordance between two methods in 19 of 27 cases. 10 cases were positive by both methods, and nine cases were negative by both methods. Thus, the concordance rate between dPCR and IHC was 70%. The discrepancies were six cases with the negative score by IHC but with HER-2/neu gene amplification by dPCR and 2 another cases with weak over expression of HER-2/neu (+2 score) by IHC but not amplified by dPCR (Table 2).

3.6. Comparison of HER-2/neu status assessed by real time PCR and IHC

From the 86 malignant breast tumor samples, only 30 cases had IHC results (15 cases with a score of 0 or +1, eight cases with a +2 score and seven cases with a +3 score). The comparison between results of real time PCR and IHC methods revealed agreement in 19 of 30 cases. Seven cases were positive and 12 cases were negative by both method. These results showed concordance of 63% between
HIC and real time PCR. In 11 discordant samples, three cases were scored as 0 or +1 while had HER-2/neu gene amplification by real time PCR and 8 other discordant cases had HER-2/neu overexpression by IHC but not HER-2/neu amplified by real time PCR (Table 3).

3.7. Comparison of dPCR and real time PCR

83 breast tumor specimens were assessed by both real time PCR and dPCR methods. Of these, 61 cases were concordant. The concordance rate between dPCR and real time PCR was 73.5%. 44 cases were positive and 17 cases were negative by both methods. In 22 discordant samples, 12 cases were positive by dPCR but without HER-2/neu amplification by real time PCR while 10 cases were negative by dPCR but had HER-2/neu amplification by real time PCR (Table 4).

3.8. Comparison of IHC, dPCR and real time PCR assays of HER-2/neu status

In this study, 27 breast tumor specimens with IHC results were assessed by real time PCR and dPCR. The concordance observed between three methods was 55.5% (15 cases of 27 samples). Six cases were positive and nine cases were negative by three methods. Three of the 12 discordant samples demonstrated HER-2/neu over expression by IHC while had not HER-2/neu amplification by both PCR methods. Three cases of the 12 discordant samples were negative by IHC and real time PCR but positive by dPCR and 4 other discordant cases were positive by IHC and dPCR but not amplified by real time PCR.

Table 1

HER2 gene amplification status in breast tumor tissues in assessment by real time PCR (A) and by differential PCR (B).

<table>
<thead>
<tr>
<th>HER2 gene amplification</th>
<th>Fresh tumor size</th>
<th>Paraffin tumor size</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>23</td>
<td>5</td>
<td>28</td>
</tr>
<tr>
<td>Negative</td>
<td>44</td>
<td>14</td>
<td>58</td>
</tr>
<tr>
<td>Total</td>
<td>67</td>
<td>19</td>
<td>86</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>19</td>
<td>10</td>
<td>29</td>
</tr>
<tr>
<td>Negative</td>
<td>48</td>
<td>6</td>
<td>54</td>
</tr>
<tr>
<td>Total</td>
<td>67</td>
<td>16</td>
<td>83</td>
</tr>
</tbody>
</table>

3.9. Determination of HER-2/neu status by dPCR in the samples with and without HER-2/neu protein over expression as indicated by IHC

3.9.1. Determination of HER-2/neu status by real time PCR in the samples with and without HER-2/neu protein over expression as indicated by IHC

15 samples with HER-2/neu protein over expression indicated by IHC (seven samples with +3 score and eight samples with +2 score) were evaluated by real time PCR and seven cases of them (47%) revealed discordance and had normal copies of HER-2/neu. Also in the assessment of 15 samples with normal expression of HER-2/neu (IHC scored 0 or +1) by real time PCR, 12 cases were negative for HER-2/neu gene amplification and only three cases (20%) had HER-2/neu gene amplification. The levels of HER-2/neu amplifications in three samples were 2.9, 4.2, and 12.

4. Discussion

Treatment type selection in breast cancer patients depends on several factors such as the stage of tumor, age of patients, the size of tumor, the grade and receptor expression status such as estrogen, progesterone and HER-2/neu receptors in the level of tumor cells (Rosa et al., 2009). Breast cancer cells generally express the estrogen receptor and are responsive to tamoxifen (anti estrogen) therapy (Lewis et al., 2004). But has been demonstrated, in HER-2/neu positive breast cancer cells, the levels of steroid hormone receptors are lower than HER-2/neu negative breast cancer tumors. Thus are typically resistant to tamoxifen (Burstein, 2005; Kamali-Sarvestani et al., 2004; Menard et al., 2000). Trastuzumab therapy along with chemotherapy is only effective for improving HER-2/neu positive breast cancer patient’s survival and allows breast conservation in patients who initially require a mastectomy (Eccles, 2001; Susini et al., 2010). Furthermore, the cost of trastuzumab therapy in the United States is approximately 759 million dollars per annum (Barberis et al., 2008). Due to high drug costs and side effects such as cardiac toxicity, the clinical use of trastuzumab requires the identification of HER-2/neu positive breast tumors. Detection of HER-2/neu gene amplification is more important than the detection of HER-2/neu protein over expression. Because the tumors that have HER-2/neu gene amplification in comparison with the tumors that have HER-2/neu over expression in absence of gene amplification are more aggressive and associated with worse survival (Kuo et al., 2007). In the current study, two different techniques have been performed, a semi-quantitative differential PCR approach and a full quantitative real time PCR method. Twenty-nine samples of 83 malignant breast tumor tissue (33%) by dPCR and 28 samples of 86 breast tumor tissues (32.5%) by real time PCR...
real time PCR showed HER-2/neu gene amplification. These results are approximately in agreement with results of Kim et al. (2002) and Venter et al. (1987) that by a differential method have been indicated HER-2/neu amplification in 37% and 33% of cases respectively. There were IHC results only for 27 of 83 samples analysed by dPCR and 30 of 86 samples analysed by real time PCR. The concordance rate between IHC and dPCR was 70%, between IHC and real time PCR was 63% and between dPCR and real time PCR was 73.5%. The concordance observed between three methods was 55.5% (15 of 27 cases). Three discordant samples were scored negative (0 or +1) by IHC while had HER-2/neu amplification by both PCR methods. Thus IHC results for these three samples are not valid. It is possible that result from errors that have occurred during IHC performance. 3 other discordant cases involved 3 cases with +3 score and 5 cases with +2 score by real time PCR showed normal copies of HER-2/neu gene. This study like many other studies indicated breast cancer specimens were positive by IHC but not HER-2/neu amplified. In breast cancer, 92–95% of cases of HER-2/neu over expression result from HER-2/neu gene amplification (Pauletti et al., 1996). But over expression of HER-2/neu protein may not be solely owing to gene amplification. But many other mechanisms explain discrepancies between protein expression and gene amplification. One of these mechanisms is chromosome 17 polysomy. Polysomy of ch17 has been reported in a significant proportion of breast cancers with moderate over expression of HER-2/neu (+2 score) in the absence of HER-2/neu gene amplification (Bose et al., 2001; Salido et al., 2005; Watters et al., 2003). Ch17 polysomy is a key prognosis index for breast carcinoma patients. Because patients with ch17 polysomy in the absence of HER-2/neu gene amplification have better prognosis compared to patients with HER-2/neu amplification (Torrini et al., 2007). The other mechanisms are mutations in the HER-2/neu gene itself or mutations in regulator genes of HER-2/neu expression. The one disadvantage of the PCR techniques is genomic DNA extraction of heterogenous tumor tissues that have tumor cells in a background of non-tumor cells. The contamination of tumor tissues by normal cell with 2 copies of HER-2/neu gene can result in the dilution of HER-2/neu positive tumor cells and thus may hide the presence of HER-2/neu amplification and false negative results is presented (Lewis et al., 2004; Merkelbach-Bruse et al., 2003). For removing this problem, the cell suspension of each sample should be evaluated in order to selection cases with sufficient proportion of tumor cells, otherwise non tumor cells could be deleted using laser assisted microdissection. (Kim et al., 2002; Merkelbach-Bruse et al., 2003; Omalley et al., 2001; Susini et al., 2010). The other agent can be, low accuracy of IHC test in detection of cases with the weak over expression of HER-2/neu gene. In IHC test, scoring is based on an evaluation of the intensity and completeness of membrane staining. But several antibodies exhibited nonspecific cytoplasmic staining which may result in the presentation of false-positive results (Kim et al., 2002; Lewis et al., 2004). At present, it is recommended that more than one method be used for the evaluation of HER-2/neu status. Real time PCR in comparison to other methods such as differential PCR is a rapid sensitive, reliable, accurate, cost effective and reproducible method especially when using laser.

Table 2
Comparison of HER2 test results by IHC and Differential PCR in 28 breast tumor tissues.

<table>
<thead>
<tr>
<th>Immunohistochemistry</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+3</td>
</tr>
<tr>
<td>Differential PCR</td>
<td>6</td>
</tr>
<tr>
<td>Positive</td>
<td>4</td>
</tr>
<tr>
<td>Negative</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
</tr>
</tbody>
</table>

Table 3
Comparison of HER2 test results by IHC and real time PCR in 30 breast tumor tissues.

<table>
<thead>
<tr>
<th>Immunohistochemistry</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+3</td>
</tr>
<tr>
<td>Real time PCR</td>
<td>7</td>
</tr>
<tr>
<td>Positive</td>
<td>4</td>
</tr>
<tr>
<td>Negative</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
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</tbody>
</table>

Table 4
Comparison of HER2 test results by differential PCR and real time PCR in 30 breast tumor tissues.

<table>
<thead>
<tr>
<th>Real time PCR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Differential PCR</td>
<td>17</td>
</tr>
<tr>
<td>Positive</td>
<td>17</td>
</tr>
<tr>
<td>Negative</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
</tr>
</tbody>
</table>

Fig. 4. The amplification curve and Ct value of HER-2/neu and UBC genes. A: Normal sample without HER-2/neu amplification, B: Tumor sample with HER-2/neu amplification.
microdissection. This method can be applied as a powerful tool in routine clinical laboratories especially to confirm the IHC results and able to fertilizer a large number of samples such as small tumor tissues and FFPE tissues in a short time and detect a two-fold difference in HER-2/neu gene initial copies. It is recommended to distinguish whether extra HER-2/neu copies owing to a small region of chromosome 17 amplification or due to chromosome 17 polymyosy, is used from reference gene which is on chromosome 17 such as gastrin gene.

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