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Original Article

Quantitative real-time polymerase chain reaction is an alternative method for the detection of HER-2 amplification in formalin-fixed paraffin-embedded breast cancer samples

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Abstract: Fluorescent in situ hybridization (FISH) and immunohistochemistry (IHC) are the most common methods that are used to quantify HER-2 gene and protein levels, respectively, in human breast cancer. However, due to bad sample quality, some samples are unable to be subjected to a FISH assay. We evaluated 71 formalin-fixed paraffin-embedded (FFPE) breast carcinoma specimens by quantitative real-time polymerase chain reaction (qPCR), IHC, and FISH. We also performed qPCR and FISH assays on delayed formalin-fixed (DDF) samples. The qPCR results were in complete concordance with the results of IHC and FISH. In regards to the DDF samples, the HER-2 fluorescent signal seemed decayed compared with that of the DDF samples after 1 h. However, the qPCR method still works well up to 12 hours. Our results indicated that qPCR was obviously superior to FISH in cases that were not fixed in a reasonable amount of time. However, qPCR can be an alternative method by which to perform HER2 amplification assays in breast cancer.

Keywords: HER-2 copy number variation, breast neoplasm, quantitative real-time polymerase chain reaction, IHC, FISH, delayed formalin-fixed

Introduction

Breast cancer ranks first in the incidence of female cancers and is one of the major causes of death among women. The over-expression of HER-2 has been observed in 10-30% of patients with breast cancer. HER-2 gene amplification or protein over-expression in breast cancer cells is closely associated with aggressive clinical behavior [1-3]. A large number of patients with HER-2 positive breast cancer benefit from Trastuzumab-based therapy [4, 5].

The HER-2 gene is a proto-oncogene located on chromosome 17q21, which codes for an 185 KDa transmembrane protein with tyrosine kinase activity [6]. It is an important member of the ErbB pathway and plays essential roles in ErbB signaling. According to the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) guidelines, immunohistochemistry (IHC), in-situ hybridization techniques, and other effective detection methods can be used for the detection of HER-2 overexpression or amplification [7]. In regards to immunostaining assays, the HER-2 protein is mainly detected on the membrane and is evaluated by a semiquantitative scoring system that ranges from 0 (no expression) to 3+ (high expression). Despite the economic convenience and its widely available for most labs, interobserver variability is a weakness of IHC [8, 9]. FISH is also typically used to detect HER-2 gene amplification and has a high specificity and sensitivity [10]. FISH offers quantitative results on gene copy number variation, and therefore, it can provide more detailed information on the genetic alterations in cancer cells [11]. However, FISH is a time-consuming and expensive method compared with IHC. In addition, the results of FISH may also be ambiguous (e.g., dual-probe HER-2/CEP17 ratio < 2.0 with

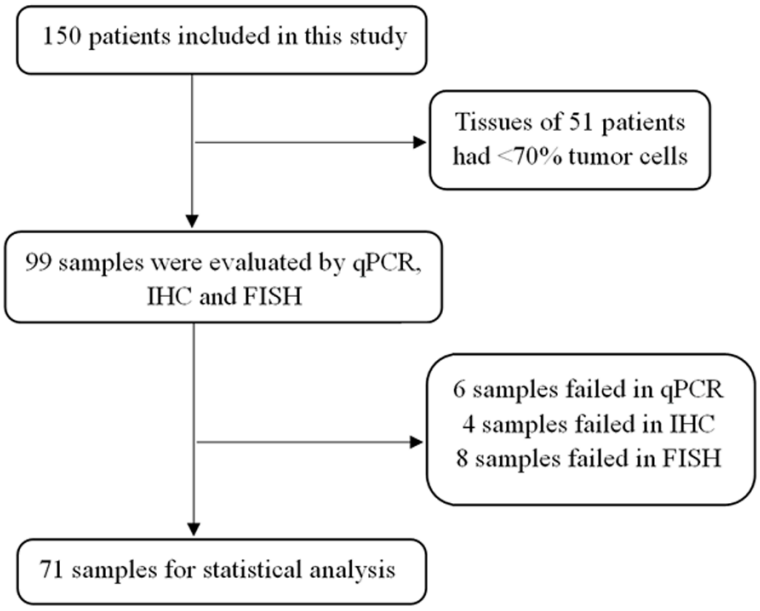

Figure 1. Flow diagram of this study.

an average HER-2 copy number ≥ 4.0 and < 6.0 signals/cell, or dual-probe HER-2/CEP17 ratio ≥ 2.0 with an average HER-2 copy number < 4.0). These two situations are considered to be equivocal, and thus additional nuclei must be counted [7].

Quantitative real-time polymerase chain reaction (qPCR) is commonly used to assess copy number variation (CNV). It has been reported that qPCR may be a highly sensitive, accurate, and reliable method for the assessment of CNV in other types of cancer cells (e.g., colorectal cancer, ovarian cancer, and melanoma), especially in formalin-fixed paraffin-embedded (FFPE) tissues [12-16]. Because the genomic DNA in FFPE samples is always fragmented, the quantification of the HER-2 gene CNV by qPCR, which targets short sequences, may result in more sensitive and accurate results.

The ASCO/CAP advocated the prompt placement of breast specimens into a fixative to minimize the ischemic time to within 1 hour after resection [7]. A small proportion of FFPE samples always fails to provide a result after FISH for CNV evaluation due to poor sample quality in routine biopsy work. We therefore also wondered whether the qPCR assay might be performed as an alternative for those samples that were not promptly placed in fixative and that failed to produce a result by FISH.

In this study, we compared three methods in the evaluation of the HER-2 status in 71 breast tumor specimens. The aim of this study was to explore the concordance among qPCR, FISH and IHC in the assessment of HER-2 CNV in breast cancer. Our results suggested that qPCR can be a stable and reliable alternative method for the evaluation of CNV of HER-2 status in breast cancer especially for samples that were not promptly placed in fixative.

Materials and methods

Tissue sampling

We collected paraffin-embedded tumor tissues from 150 breast cancer patients who underwent breast surgery in 2012 at West China Hospital. Samples where tumor cells comprised ≥ 70% of the sample were selected. After specimens with a small sample size and those where the DNA was of poor quality were excluded, 71 samples remained (**Figure 1**).

Cell lines and cell culture

The human breast carcinoma cell lines SK-BR-3 and BT-474 (over-expressing HER-2) were used as positive controls, while MDA-MB-231, MCF-7 (no-expressing HER-2) were used as negative controls. BT-474 cells were cultured in RPMI-1640 supplemented with 10% FBS at 37°C and 5% CO_2, while the others were cultured in DMEM supplemented with 10% FBS at 37°C and 5% CO_2.

DNA extraction

Sections 4-μm thick was obtained from FFPE blocks, and an area of invasive carcinoma was marked on the corresponding H&E-stained slide. The tissues were deparaffinized and rehydrated in xylene and consecutively increasing dilutions of ethanol, respectively. The DNA was extracted using the phenol chloroform method. The purity and integrity of the DNA were evaluated by calculating the O.D. 260:280 ratio.

Table 1. Sequences of housekeeping genes and target gene in this study

Gene name	Forward primer sequence	Reverse primer sequence
Actin	5'TCCACGAAACTACCTTCAACTC 3'	5'GGCAATGCCAGGGTACAT 3'
TFRC	5'ACTTCCTCTCTCCCTACGTATC 3'	5'GCAGTTTCAAGTTCTCCAGTAAAG 3'
GAPDH	5'CCTCAAGATCATCAGCAATGCCTC 3'	5'GTGGTCATGAGTCCTTCCACGATA 3'
HER-2	5'ATGAGCTACCTGGAGGATGT 3'	5'CCAGCCCGAAGTCTGTAATTT 3'

QPCR

The qPCR mixes were prepared according to the Bio-Rad SsoFast Evagreen protocol #172-5204AP (Singapore). Amplifications were performed in a Bio-Rad CFX Connect Real-Time System (California, USA) under the following conditions: 98°C for 2 minutes followed by 39 amplification cycles at 98°C for 15 s and 60°C for 15 s. Data were analyzed by the CFX manager 3.0 software. Three housekeeping genes were used for the quantification of HER-2 CNV status in this study (**Table 1**).

The CNV of HER-2 was calculated using a comparative threshold cycle (Ct) with the following formula: CNV = $2^{-\Delta\Delta CT}$, where $\Delta\Delta Ct = (Ct,_{target}-Ct,_{reference})_{sample}-(Ct,_{target}-Ct,_{reference})_{normal}$. The target represents HER-2 and the reference represents given housekeeping genes [17].

Immunohistochemical methods

IHC for HER-2 was performed on 4-μm thick paraffin sections. After deparaffinization and blocking for endogenous nonspecific proteins, the slides were further incubated overnight at 4°C with the following primary antibodies: rabbit anti-human c-erbB-2 (1:400; Dako, Copenhagen, Denmark). After 5 washes in phosphate-buffered saline, the slides were incubated with horseradish peroxidase-labeled secondary antibody for 30 minutes at room temperature. Then, the slides were developed using the Dako REALTM EnVisionTM Detection System (DAKO Code K5007; Dako, Glostrup, Denmark)

Immunohistochemical scoring.

The IHC results were reported using the ASCO/CAP 2013 HER-2 Test Guideline. The scoring system used was as follows: IHC 3+ denotes circumferential membrane staining that was complete and intense; IHC 2+ denotes circumferential membrane staining that was incomplete and/or weak/moderate and within > 10% of the invasive tumor cells or complete and circumferential membrane staining that was intense and within ≤ 10% of the invasive tumor cells; IHC 1+ denotes incomplete membrane staining that was faint/barely perceptible and within > 10% of the invasive tumor cells; IHC 0 was defined as no staining or membrane staining that was incomplete and that was faint/barely perceptible and within ≤ 10% of the invasive tumor cells. IHC 0 or 1+ were designated as "negative" while IHC 3+ was designated as "positive" for HER-2 expression. Tumors with a score of IHC 2+ were considered equivocal cases, which were further recommended for FISH analysis. Cases with an IHC score of 2+ and FISH positivity were considered to be positive [7].

FISH

The sections were dried at 60°C overnight before deparaffinization in xylene and rehydration in ethanol. The specimens were heated in a pretreatment solution for 20 minutes. Then, the slides were submitted to proteolytic digestion by pepsin at 37°C for 10 minutes. Hybridization was performed in a hybridizer (Thermo Brite, USA) at 85°C for 5 minutes, followed by 16 hours at 45°C. The probes (Abbott/Vysis, USA) were a mixture of Texas-red labeled HER-2 sequence and fluorescein (fluorescein isothiocyanate)-labeled nucleic acid probe that targets the centromeric region of chromosome 17. After a stringent wash at 65°C for 10 minutes, the slides were mounted with a fluorescence mounting medium and cover slipped for further evaluation.

Test on delayed formalin fixed samples

A total of 5 patients with invasive breast carcinoma were included in this study (collected between July 2014 and August 2014). The fresh samples in this study were tissues that remained after a pathological biopsy was performed. The cold ischemia time ranged from 0 h to 96 h. Fresh tumor was divided into 9 parts, each with a diameter of 3 mm. The delayed fixation times were set as follows: 0 h, 1 h, 2 h, 3 h,

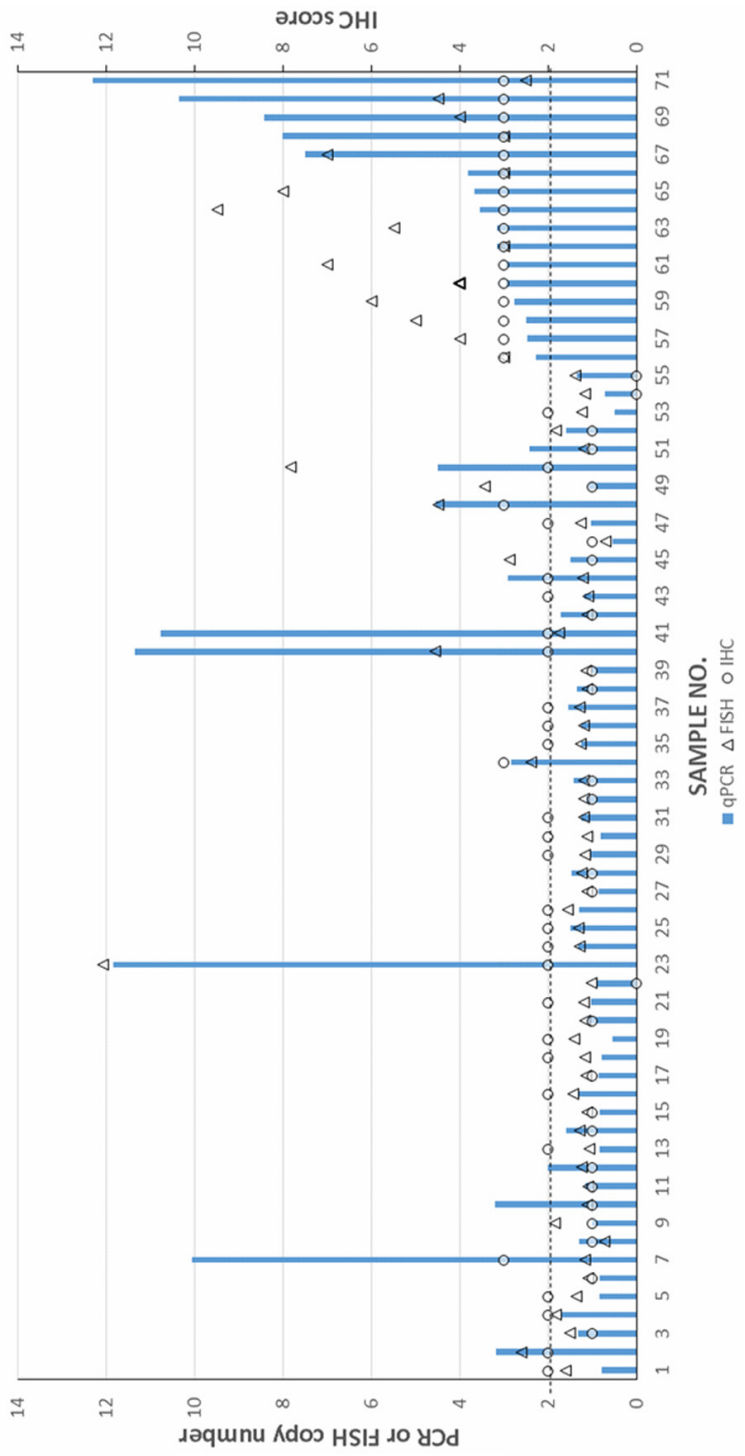

Figure 2. Summary of qPCR, IHC, and FISH results for FFPE tissue samples from 71 breast carcinomas. Each qPCR copy number value represents the HER-2 status for each sample. The dashed horizontal line indicates the qPCR and FISH threshold cutoff of 2 copies in order for a sample to be deemed HER-2 positive. The FISH values were expressed as the copy number (HER-2/CEP17 ratio). The dashed horizontal line also demarcates the IHC-positive (IHC 3+) samples from those that are IHC-equivocal (2+), and IHC-negative (0, 1+). Sample NO.37 (negative by qPCR at 1.56, equivocal by FISH at 1.3 copies), sample NO.41 (positive by qPCR at 10.78, negative by FISH at 1.76 copies), and sample No. 44 (positive by qPCR at 2.91, negative by FISH at 1.21) were shown.

4 h, 12 h, 24 h, 48 h, and 96 h. The total fixation time for all samples was 24 hours. After fixation in neutral-buffered formalin (10% vol/formalin in water; pH 7.4), all samples were subjected to dehydration, paraffin immersion and embedding using a standard procedure.

Statistical analysis

Statistical analyses were performed with IBM SPSS (version 22).

Results

Establishment of the PCR assay

Housekeeping genes were widely used to detect the gene copy number variation [7, 12-14, 16, 18, 19]. In this study, we used multiple housekeeping genes (ACTIN, TFRC, GAPDH) for the precise detection of the target gene copy number. The Ct value variation of housekeeping genes was small and the variation coefficient was less than 5%. The statistical Ct value per sample was the average value for three parallel samples, and the ΔCt values of three parallel samples were less than 0.5.

To define the normal HER-2 gene copy number relative to the three reference genes in our test, we used 50 non-tumor samples. We used the following formula: $\Delta Ct_{normal} = (Ct_{target} - Ct_{reference})_{normal}$. The gene copy number ratio of the unknown sample ≥ 2 were defined as amplified. The gene copy number ratio of the unknown sample < 2 were defined as non-amplified [20, 21].

To confirm the results of our qPCR assay, we tested this method on four cell lines in which the HER-2 gene copy number was already known. Based on

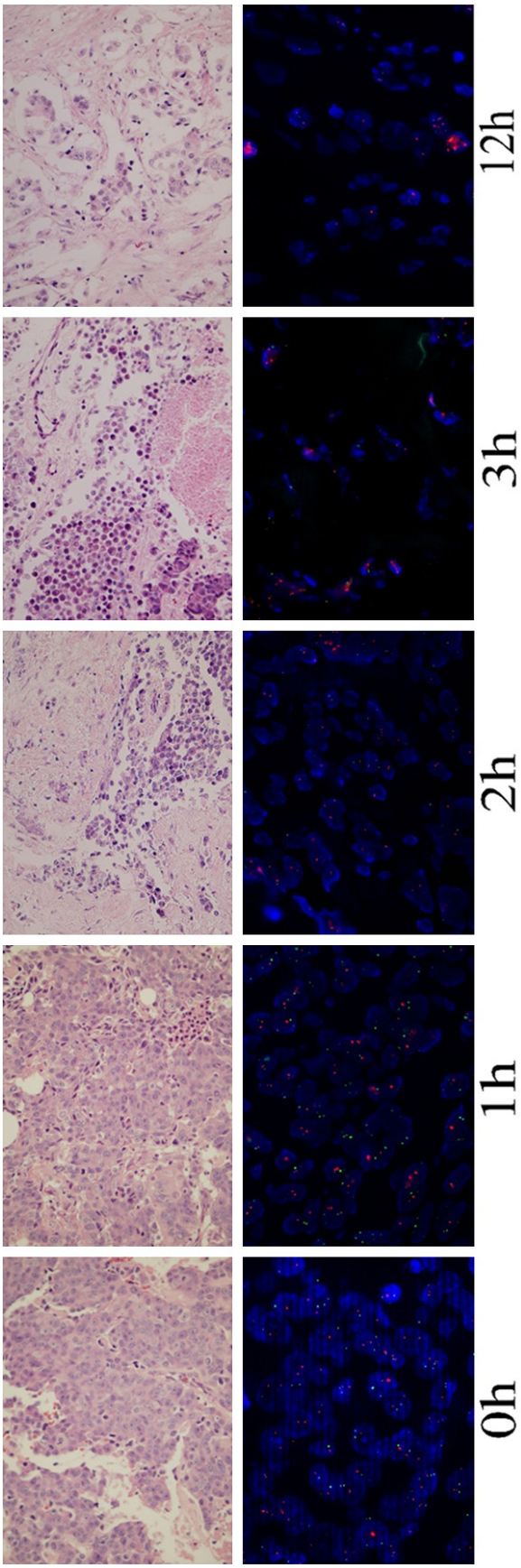

Figure 3. Representative H&E and fluorescence in-situ hybridization (FISH) images at four typical cold ischemia time points for case 4. There was a dim or absent HER-2 signal rather than the centromere signal; the tissue morphology and nuclei become blurred (2 h). Over time, background nonspecific staining was increased. We also found that nuclear bubbling and background became hazy with a dusty appearance. Original magnification, × 40.

our qPCR method, the ratios of SK-BR-3 and BT-474 were 22.06 and 2.51, respectively, which were considered amplified with respect to HER-2. The ratios of MCF-7 and MDA-MB-231 were 0.176 and 0.096, respectively, which were considered non-amplified with respect to HER-2. These results were consisted with those of other reports [22-25]. Similarly to previous studies, we used 2 as the cutoff point for the PCR results [20, 21, 26].

Comparison of qPCR, IHC and FISH

Out of the 71 samples, 45 samples were determined to be negative for HER-2 by qPCR and were distinguishable from the 26 remaining samples that tested positive for HER-2. IHC and FISH were also performed on these 71 samples. Of all the 45 cases that were negative by qPCR, IHC showed that 25 samples were negative and 20 samples were equivocal (2+). Of the 20 equivocal samples, 19 samples were determined to be negative for HER-2 by FISH, and 1 sample (No. 37) was also equivocal by FISH (HER-2 = 4.49, CEP17 = 3.46, HER-2/CEP17 = 1.3) according to 2013 ASCO/CAP HER2 testing guideline. Of the 26 samples that were positive by qPCR, 19 samples were deemed to be positive by IHC, and 7 cases were determined to be equivocal. Of the 7 equivocal samples according to IHC, 5 cases were positive by FISH. Another 2 cases (No. 41 and No. 44) were negative by FISH. We specifically analyzed these two cases. The detailed FISH result of case No. 41 showed that the average of HER-2 signal was 4.98 and that the average CEP17 signal was 2.83. We considered case NO.41 to be multiploid, which means that the HER-2/CEP17 ratio was below 2 (HER-2/CEP17 = 1.76).

For the other case (No. 44), we verified the morphological heterogeneity in a FISH assay. In the region of the invasive carcinoma, 12% of the tumor cells had a HER-2/CEP17 ratio above 2. Thus, qPCR correctly identified the 71 breast

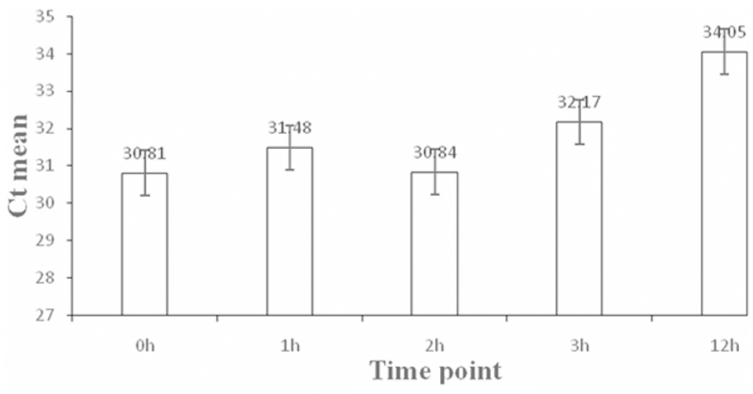

Figure 4. QPCR was performed for case 4 at four typical cold ischemia time points. The Ct values at 0 h, 1 h, 2 h and 3 h were 30.81, 31.48, 30.84 and 32.17, respectively. The Ct value at 12 h was 34.05. No difference was observed in the Ct values with regard to the cold ischemia time ($P > 0.05$).

carcinoma cases as follows: 44 HER-2 negative (61.98%), 24 HER-2 positive (33.80%), and 3 HER-2 equivocal (4.22%) cases for a 100% concordance with the results of IHC and FISH (**Figure 2**).

QPCR and FISH assay on samples that were not immediately fixed

Because a small proportion of the samples is always unsuccessful with respect to HER-2 evaluation by FISH due to bad sample quality in routine biopsy, we wondered whether qPCR might be a superior assay over FISH on samples that were not fixed within 1 hour because our qPCR method was designed to detect short target sequences. The cold ischemia time for the fresh samples were set as follows: 0 min, 1 h, 2 h, 3 h, 4 h, 12 h, 24 h, 48 h and 96 h. qPCR and FISH were performed for the samples at each time point.

In regards to the FISH assay, we detected that the fluorescent signal of all of the cases remained unchanged within 1 hour. A significant degradation in signal intensity was observed after 2 hours cold ischemia time (**Figure 3**). After 2 hours, the changes in the fluorescent signal of these specimens demonstrated at least one of the following: vague cellular outline, non-uniform weak signal (> 25% unscorable due to weak signal), poor nuclear resolution, or a background-obscuring signal. Interestingly, we found that the HER-2 signal (red) but not the chromosome CEP17 signal (green) was faded with delayed fixation times. **Figure 2C** and **2D** illustrates this finding very well. These data were in accordance with the ASCO-CAP criteria, which recommend up to 1 hour for the cold ischemia time. Nevertheless, the PCR results did not change over the cold ischemia time (**Figure 4**).

Discussion

As HER-2 now plays a critical role in the management of patients with invasive breast carcinoma, several methods including fluorescence in situ hybridization (FISH), immunohistochemistry (IHC) [27] and chromogenic in situ hybridization (CISH) have been used to determine HER-2 gene amplification and protein overexpression [28, 29]. FISH and IHC are the most common methods that are used and the only ones that have been approved by the US Food and Drug Administration (FDA) [30]. Although IHC staining is easy to perform and relatively inexpensive, IHC results are likely to be ambiguous and subjective [31]. Some have mentioned that variable IHC results are related to tissue quality, antibodies, fixatives, and the technical assessment [32, 33]. Therefore, IHC cannot always provide us with reliable results. Several studies have proposed that IHC be used as the first line screening method for the determination of HER-2 status. FISH is considered to be the second-line method for the detection of HER-2, especially for samples with an IHC score of 2+ [7, 34]. Although FISH is a sensitive, specific and accurate method [31], its disadvantages are that it is a time-consuming procedure that requires several hours for the hybridization step (~4 h), the washing and the quantification of the fluorescence signal in individual cells. Moreover, FISH results must be read in time. As time passes, the fluorescence signal weakens. Many other problems can also arise during the FISH procedure including prolonged storage, tissue loss, tissue autofluorescence and poor tissue morphology [35], FISH for HER-2 is not routinely performed in small hospitals due to the high cost of the assay and the need for necessary experimental equipment, which may be the primary reason why FISH is not widely used. Although IHC and FISH

involve the same microscopic techniques, it should be noted that the ASCO/CAP guideline and other studies have cautioned that approximately 20-26% of current HER-2 test results might be inaccurate [7, 36].

Quantitative real-time polymerase chain reaction (qPCR) has several advantages over FISH for the assessment of HER-2 amplification. First of all, qPCR is quick and simple, effective, credible and accurate, all of which make it possible to screen multiple samples in a short period of time. Furthermore, qPCR amplification and quantification are performed in the same reaction tube, which reduces the systematic errors to some extent. The study by Andrulis at al [37] was the largest study that has evaluated HER-2 amplification in 580 frozen tumor tissues. The result strongly suggested that qPCR could be a viable option because of its superior performance, fast speed, high accuracy, sensitivity, and considerable potential for extensive applications.

In our study, the qPCR results were in perfect concordance with those of IHC and FISH. Chromosome multi-body and morphological heterogeneity have been some of the difficulties in the diagnosis of breast cancer, such as in cases No. 41 and No. 44. In our opinion, PCR could also be used to identify the HER-2 gene amplification level in these intractable and equivocal cases. Clinicians should consider all factors to make better treatment decisions for patients. The only disadvantage of this technique is that the regions of tumor and normal tissues must be identified microscopically prior to qPCR analysis. This limitation could be abolished by the use of the laser capture microdissection (LCM) method. Because we did not have the capability for LCM, only FFPE samples that contained more than 70% tumor were included.

In this study, we also studied the effect of cold ischemia time on HER-2 testing. We found that, in cases that were not fixed within a certain amount of time, qPCR was obviously superior to FISH. As is well known, FFPE samples have been the standard means for pathologic tissue examination. Additionally, poorly preserved tissues might lead to morphologic changes.The ASCO/CAP advocated the prompt placement of specimens into fixative to minimize ischemic time to 1 hour or less, and fixation for 6 h-48 h in only 10% neutral-buffered formalin (NBF) [7, 34]. However, others have advocated different ideas, and have thought that the delay to formalin fixation (DFF) or the ischemic time could be longer [35, 38]. Portier, B. P. showed that a cold ischemia time of up to 3 h did not alter the detection of HER-2 by FISH [35].

Our study was not the first one to evaluate the effect of DDF on FISH, but was the first study that has attempted to solve this problem. Similar to the study by Khoury, T., we found that HER-2 signals decayed with time, especially after 1-2 h. DFF seemed to be the reason for the signal loss [39]. In addition, we found that HER-2 signals weaken more rapidly than CEP17 signals, which could lead to false negative results in some cases. The reason for this phenomenon is not yet clear, but in our opinion, DNA autolysis is likely responsible for it. Nevertheless, the qPCR results did not change with a cold ischemia time of up to 12 hours. Thus, when we encountered DFF issues, qPCR might be a surrogate for the HER-2 test. However, a study that includes a larger cohort is needed to verify this observation.

Based on our observations in this study, qPCR showed a significant correlation with existing standard diagnostic methods. In conclusion, we suggest that qPCR could serve as a useful and complementary screening tool for the detection of the HER-2 gene level in FFPE tissue samples. Sometimes, qPCR can compensate for the inadequacy of IHC and FISH and produce specific quantitative results. We believed that HER-2 detection with qPCR may be able to provide decisive information for the evaluation of the effects of Trastuzumab therapy in breast cancer patients.

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Disclosure of conflict of interest

None.

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