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Original Article

Evaluation of quantitative real-time polymerase chain reaction method in detecting *Her-2* gene status of immunohistochemically scored 2+ invasive breast carcinoma patients in Yunnan province of China

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Abstract: Human epidermal growth factor receptor-2 (*Her-2*) is a significant prognostic factor and the most important protein in breast carcinoma target therapy. We aimed to evaluate *Her-2* gene amplification status detected by quantitative real-time polymerase chain reaction (Q-PCR) with fluorescent in situ hybridization (FISH) as a golden standard, and searched the optimum threshold of Q-PCR when it can be equivalent to FISH. A total of 108 immunohistochemistry (IHC) 2+ invasive breast carcinoma cases from Yunnan province of China were enrolled in this study, assessed *Her-2* gene status by FISH and Q-PCR and investigated some clinicopathological variables association with *Her-2* amplification results of these two methods individually. A significant correlation of *Her-2* FISH results and Q-PCR amplification status with differentiation of lymphatic metastasis ($P = 0.001$, $P = 0.005$) was observed and metastasis ratio increased with the rising of mRNA expression. When the cutoff value set at 2.60, compared with FISH, Q-PCR had the same great sensitivity (96.59%), specificity (75%), negative predictive value (94.44%) and positive predictive value (83.33%). This study showed that Q-PCR (cutoff = 2.60) had an extremely good consistency (*kappa* = 0.739) with FISH and could assess *Her-2* status instead of FISH in some cases.

Keywords: Invasive breast carcinoma, *Her-2* gene amplification assessment, Q-PCR, FISH, Yunnan province of China

Introduction

Breast carcinoma is the most common malignant tumor (accounting for 15% of total female tumors) and ranks first in morbidity and mortality among Chinese female malignant tumors on one time. Per the current incidence trend of breast carcinoma, by 2030, the incidence cases and death toll will reach to 2.64 and 1.7 million, respectively [1, 2]. Recently, the morbidity of breast carcinoma remains high (first), but the mortality is declining (fifth) [2, 3]. Between 2005 and 2011, the 5-year relative survival was found to be 89% [4]. The increasing of survival is not only benefited from the establishment of the female population-wide screening and early detection system but also profited from the development of molecular biology technology in recent years and the improvement of comprehensive diagnosis and treatment standardization.

The evolution of breast carcinoma involves a variety of genetic material changes, including complex process of suppressor genes/oncogenes amplification or inactivation. Tyrosine kinase-human epidermal growth factor receptor 2 (*Her-2*, HER2/neu, c-erbB-2) is a proto-oncogene, amplifies and (or) protein over-expresses in 25% to 30% breast carcinoma patients [5-7]. The HER-2 positive patients are with tumor infiltration, poor prognosis, short disease-free survival period, early prone to relapse, and relatively insensitive to endocrine

therapy and chemotherapy, but can benefit from the treatment of the targeted drugs-recombinant DNA derived humanized monoclonal antibodies-Trastuzumab (Herceptin) [8, 9], lapatinib (Tykerb), and pertuzumab (Perjeta) [10]. At present, IHC and FISH are performed to detect *Her-2* status per ASCO/CAPs, predicting prognosis and guiding the breast carcinoma targeted drugs (especially Herceptin) [11].

As the first line screening method to assess *Her-2* protein expression, IHC is widely applied by clinic pathological laboratory since its low cost and simple operation. IHC membrane specificity staining was semi quantitatively scored as 0/1+ (negative), 2+ (equivocal) and 3+ (positive), led to subjective differences in interpretation [12, 13]. The Chinese Human Epidermal Growth Factor Receptor 2 Testing Guideline in Breast carcinoma indicated that IHC score of 2+ cases should perform ISH to ensure *Her-2* gene status [14-16]. Although being regarded as the golden standard [17], FISH is operational complexity, time-consuming and costliness [11]. The staining slide should be timely read; otherwise fluorescence quenching may lead to inaccurate interpretation. The Guideline also point out that whether the cases with dual-probe *Her-2*/CEP17 ratio ≥ 2.0 but average *Her-2* copy number/cell < 4.0 should be regarded as FISH positive is controversial, whether the cases with dual-probe *Her-2*/CEP17 ratio < 2.0 but average *Her-2* copy number/cell ≥ 4.0 and < 6.0 should be regarded as FISH positive is indeterminate. In addition, some samples fixed not in time also could not obtain reliable results by FISH [11, 18].

On account of high accuracy, sensitivity, reliability and stability, quantitative real-time polymerase chain reaction (Q-PCR) is widely applied to assess copy number variation (CNV) in many other types of carcinoma cells including colorectal carcinoma, ovarian carcinoma, and melanoma, especially in formalin-fixed paraffin-embedded (FFPE) tissues and fine needle aspiration cytology (FNAC) samples [19-24]. The quantification of *Her-2* gene CNV by Q-PCR is always targeting at short fragmented sequences which exists in FFPE samples.

Based on these, take the FISH as a golden standard, we screened 108 patients of breast invasive ductal carcinoma from Yunnan Province of China to evaluate Q-PCR when it applied to test *Her-2* gene amplification. Under certain conditions, due to its low cost and simple operation, Q-PCR is designed to be as an alternative method when FISH is unable to interpret. When the expression value is bigger than cutoff value, the result is objectively judged as positive and conversely as negative.

Materials and methods

Patient material

Tissue samples of 108 invasive breast carcinoma females aged 27-78 (47.4±11.5) who underwent modified radical mastectomy were screened during October 2014 to June 2016 at the Department of Pathology in the First People's Hospital of Yunnan Province of China. Anonymous use of redundant tissue for research purposes is part of the treatment agreement with patients. And the study was approved by the Ethics Committee of the First People's Hospital of Yunnan Province. All patients were confirmed for invasive breast carcinoma first time and had not received any treatment like chemotherapy, radiotherapy or biotherapy before surgery. All specimens were fixed in 10% formalin, embedded in paraffin, and then sliced into 2-3 microns' slides which in the following with IHC stain scores at 2+.

Immunohistochemistry (IHC)

Anti-*HER-2* monoclonal antibodies and MaxVision III secondary antibodies detection Kits were purchased from MaiXin Biotechnology development co., LTD. Each IHC stain was run with a small tissue array containing 0, 2+ and 3+ breast tumor sample taken along on the same slide as control. Three microns' thick slides were baking at 65°C and dewaxed to hydration. Antigens were recovered under high pressure in citrate buffer (pH = 6.0) solution for 3 min and endogenous peroxidases activity were blocked by 3% hydrogen peroxide solution in the dark for 10 min. Slides were incubated in primary antibody working solution at 27°C for 55 min, ready-to-use MaxVision III reagent at 27°C for 25 min, and then developed coloration with 3,3-diaminobenzidine (DAB). The slides should be washed sufficiently in PBS (pH 7.4) between every two steps and at last stained in hematoxylin, dehydrated in gradient alcohol, transparent in xylene and sealed slides with resinene.

Table 1. Primers for *Her-2* and *β-actin*

Gene	Sense primer sequence	Anti-sense primer sequence
β-actin	GCGCGGCTACAGCTTCA	CTTAATGTCACGCACGATTTCC
Her-2	GAAGGACATCTTCCACAAGAACAA	CGAGAGCGGTTGGTGTCTATC

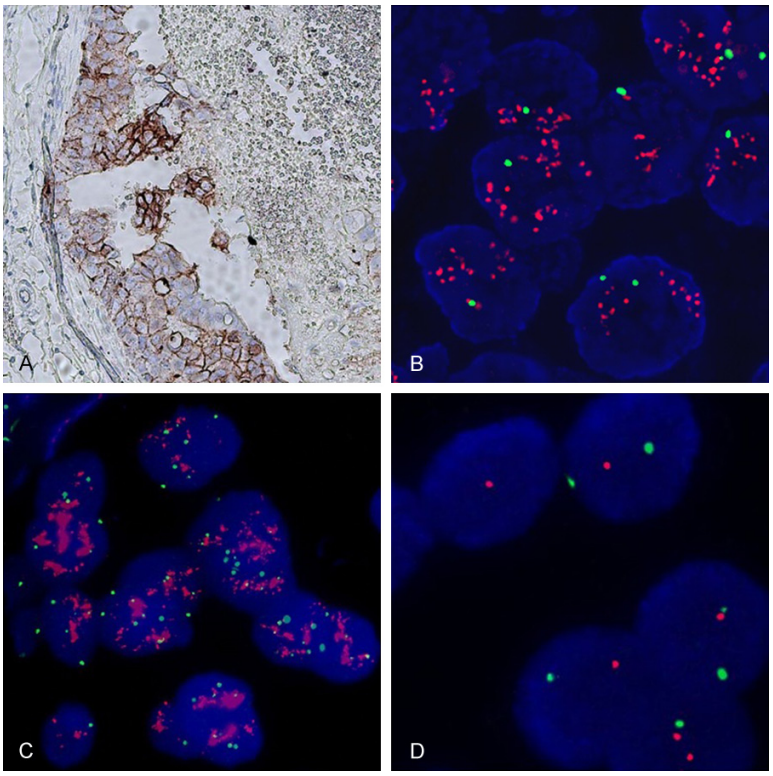

Figure 1. Example specimen of breast infiltrating ductal carcinoma IHC 2+ (A, 20 ×) results and FISH results. There were 38 cases (36.1%) with *Her-2* gene dot amplification (B, 100 ×) and 49 cases (45.4%) with *Her-2* gene cluster amplification (C, 100 ×); and there were 20 cases (18.5%) with no *Her-2* gene amplification (D, 100 ×).

IHC result is interpreted per the percentage of positive cells whose membrane presented clear brown or tan. We selected IHC ambiguity cases scored 2+ referred to The Chinese Human Epidermal Growth Factor Receptor 2 Testing Guideline in Breast carcinoma: more than 10% of the invasive carcinoma cells presented incomplete and/or weak to moderate membrane staining or 10% or less invasive carcinoma cells presented strong and circumferential membrane staining. Every slide was interpreted by two independent pathologists.

Fluorescent in situ hybridization (FISH)

Three microns' thickness slides were baked overnight, dewaxed, and boiled for 23 min at 100±5°C. Digested with pepsin for 13 to 15 min after airing and sufficiently washed in 2 × SSC. Dehydrated in 70%, 90%, 100% ethanol gradient solutions and added probe after air-dried (the following steps need to be operated away from light). Hybridization was performed in a hybridizer (Thermo Brite, USA) at 85°C for 5 min, followed by 16 hours at 37°C. After washing in 2 × SSC and 0.1% in NP-40/2 × SSC at 37°C, respectively, the slides were dried in the dark, dehydrated in 70% ethanol, and added DAPI solution for the further evaluation under a fluorescence microscope after sealing coverslip.

In distinct invasion tumor region, total GSP *Her-2* signals (red) and CEP17 signals (green) were counted respectively within 60 tumor cells nuclear to calculated the ratio of red signals to green signals. The samples with red/green ratios ≥ 2.0 or with red/green ratios < 2.0 but average *Her-2* copy number/cell ≥ 6.0 were treated as *Her-2* gene amplification; the samples with red/green ratios < 2.0 and average *Her-2* copy number/cell < 4.0 were treated as none *Her-2* gene amplification; the samples with red/green ratios < 2.0 and average *Her-2* copy number/cell ≥ 4 but < 6.0 or with red/green ratios ≥ 2.0 but average *Her-2* copy number/cell < 4.0 were indeterminate; the samples with many clustered red signals were directly treated as *Her-2* gene amplification without calculation.

Quantitative real-time polymerase chain reaction (Q-PCR)

DNA was extracted from paraffin-embedded tissue (PPFE) with DNA Rapid Extraction Kit (centrifugal column type) (TIANGEN, China). The tissue block was sliced up into 5-8 10-μM-thick pieces, lysis and dissolved, and kept the middle layer of water phase into a new centrifuge tube

Table 2. Correlation of *Her-2* FISH results with age, location, tumor size and lymphatic metastasis in 108 breast carcinoma patients

Clinicopathological parameters	Patients	*Her-2*		
		Positive (%)	Negative (%)	χ^2, P
Age				
< 40 years	22	16 (72.7)	6 (27.3%)	χ^2 = 0.769
≥ 40 years	86	72 (83.7)	14 (16.3%)	P = 0.38
Location				
Left	33	27 (81.8%)	6 (18.2%)	χ^2 = 0
Right	75	61 (81.3%)	14 (18.7%)	P = 1
Tumor size				
< 5 cm	96	82 (85.4%)	14 (14.6%)	χ^2 = 6.675
≥ 5 cm	12	6 (50%)	6 (50%)	***P = 0.01**
Lymphatic metastasis				
Positive	60	56 (93.3%)	4 (6.7%)	χ^2 = 10.862
Negative	48	32 (66.7%)	16 (33.3%)	****P = 0.001**

* and **significant *p* values are shown bold.

Table 3. Correlation of her-2 Q-PCR results with age, location, tumor size and lymphatic metastasis in 108 breast carcinoma patients

Clinicopathological parameters	Patients	*Her-2* CNV		
		≥ 2 (%)	< 2 (%)	χ^2, P
Age				
< 40 years	22	18 (81.8%)	4 (18.2%)	χ^2 = 0.718
≥ 40 years	86	78 (90.7%)	8 (9.3%)	P = 0.474
Location				
Left	33	31 (93.9%)	2 (6.1%)	χ^2 = 0.511
Right	75	65 (86.7%)	10 (13.3%)	P = 0.610
Tumor size				
< 5 cm	96	86 (89.5%)	10 (10.4%)	χ^2 = 0.494
≥ 5 cm	12	10 (83.3%)	2 (16.7%)	P = 0.630
Lymphatic metastasis				
Positive	60	60 (100%)	0 (0%)	χ^2 = 2.897
Negative	48	36 (75%)	12 (25%)	****P = 0.005**

**significant *p* values are shown bold.

after centrifugal. Added 2 times volume of anhydrous ethanol, 3 min standing after incorporating thoroughly, the liquid was injected into an adsorption column CR2. After centrifugal, it was washed with buffer GD, rinsed with buffer PW, idling centrifugal for 2 min, dried at room temperature for 2-5 min. Put the CR2 adsorption column into a clean centrifuge tube and add 30 μl elution buffer or ddH_2O preheated to 65°C TE, placed for 2-5 min at room temperature, and then centrifuged for 2 min at 12,000 rpm (~13,400 × g) to collect DNA. The purity and integrity of the DNA were evaluated by calculating the O.D. 260:280 ratios.

The Q-PCR mixtures were prepared per the Bio-Rad SsoFast Evagreen protocol (Singapore). The primers for *Her-2* and β-actin are shown in **Table 1**. Amplifications were performed in a Bio-Rad CFX Connect Real-Time System (California, USA) under the following procedures: 95°C for 10 min and followed by 39 amplification cycles at 95°C for 10 sec and 57°C for 30 sec. Then 65°C for 31 sec and followed by 60 amplification cycles at 65°C for 5 sec with +0.5°C/cycle, rising 0.5°C/s. Data were analyzed by the CFX manager 3.0 software. The housekeeping gene β-actin was used for the quantification of *Her-2* CNV status in this study. *Her-2* CNV was calculated using a comparative threshold cycle (Ct) with the following formula: CNV = $2^{-\Delta\Delta CT}$, where ΔΔCt = (Ct*Her-2*-Ctβ-actin) sample-(Ct*Her-2*-Ctβ-actin) normal. The gene copy number ratio of the unknown sample ≥ 2 was defined as amplified. The gene copy number ratio of the unknown sample < 2 was defined as non-amplified [23, 25, 26].

Statistics

Results obtained in this study were compared by cross tables and the concordance percentages and correlations (Spearman's rho) by Chi square test with GraphPad Prism5 statistical software and PASW statistics software. For Q-PCR, sensitivity, specificity, positive (PPV) and negative predictive value (NPV) were calculated with FISH as a golden standard.

Results

The mean age of the enrolled patients is 47.4-years old, which is similar to the relevant study

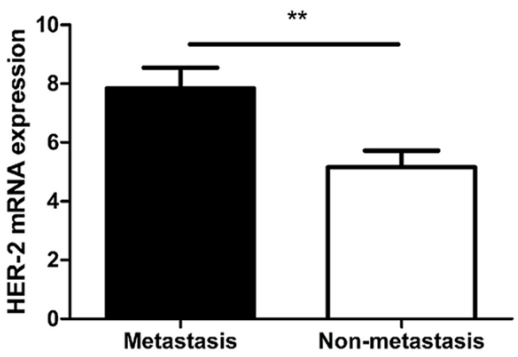

Figure 2. There was a significant statistical difference (**P < 0.01) of mRNA expression between lymphatic metastasis positive and negative specimen.

[2], much younger than western countries. There are 22 patients younger than 40 years old and 86 patients older than or equal to the age of 40. The tumors of 75 cases located in the right, and 33 cases located in the left. There were 60 cases with lymph node metastasis, and 48 cases without lymph node metastasis. Nighty-six cases tumors were smaller than 5 cm, and 12 cases were bigger than 5 cm.

All samples immunohistochemical results were scored at 2+ (**Figure 1A**) and then were analyzed for FISH and Q-PCR to determine *Her-2* gene amplification. In total 108 cases, there are 88 cases of FISH positive shown in **Figure 1B** and **1C** with positive rate of 81.5% and 20 cases of FISH negative shown in **Figure 1D** with negative rate of 18.5%.

There was no significant statistical difference of *Her-2* FISH amplification respect to different age groups ($P = 0.38$) and tumor location ($P = 1$). But, there was significant statistical difference ($\chi^2 = 6.675$, $P = 0.01$) for *Her-2* FISH amplification between different tumor sized. The positive rate of bigger size was 4.2 times (95% CI 2.084~8.466) of the smaller group. There was also a significant statistical difference ($\chi^2 = 10.862$, $P = 0.001$) for *Her-2* FISH amplification between lymphatic metastasis group and non-lymphatic metastasis group. The former positive rate was 7 times (95% CI 2.154~22.749) of the latter (**Table 2**).

The gene copies number ratios of the samples ≥ 2 were defined as amplified, and < 2 were defined as non-amplified. There was no significant statistical difference for *Her-2* Q-PCR amplified and non-amplified respect to age ($P = 0.474$), tumor location ($P = 0.610$) and tumor size ($P = 0.630$). However, there was a significant statistical difference ($P = 0.005$) for *Her-2* Q-PCR amplified and non-amplified between lymphatic metastasis positive group and negative group. The former positive rate was 1.333 times (95% CI 1.132~1.57) of the latter (**Table 3**).

It is worth to noting that there was a significant statistical difference (P < 0.01) of mRNA expression between lymphatic metastasis specimen and non-lymphatic metastasis specimen (**Figure 2**). And the more the amount of mRNA expression, the higher the metastasis cases proportion. When expressing quantity reached 16 times of the control, metastasis proportion reached 100% (**Figure 3**).

There were 60 cases in lymphatic metastasis group of which included 1 case with FISH and Q-PCR double-negative, 55 cases with double-positive and 4 cases with FISH negative but Q-PCR positive. Both results coincidence rate was 93.3%. There were 48 cases in non-lymphatic metastasis group of which included 12 cases with FISH and Q-PCR double-negative, 32 cases with double-positive and 4 cases with FISH negative but Q-PCR positive. Both results coincidence rate was 91.7% (**Figure 4**).

To find the correlation between Q-PCR and FISH results, after repeated testing, we setup *Her-2* Q-PCR cutoff value at 2.60. An mRNA ratio greater than or equal to 2.60 was taken as positive, and less than 2.60 was taken as negative. With FISH method as the gold standard, the sensitivity of Q-PCR was 96.59%, specificity was 75%, positive predictive value was 94.44%, and negative predictive value was 83.33%. So, concluded that the Q-PCR method is highly consistent with the FISH (kappa = 0.739) (**Figure 5**).

Discussion

Human epidermal growth factor receptor-2 (*Her-2*) gene amplification and/or mRNA and/or protein over-expression cause excessive proliferated cells forming tumors. Some research claimed that *Her-2* molecular positive expression ratio in breast carcinoma is between 20% and 30%, which is 3-4 times higher than the other carcinomas, especially higher in infiltrat-

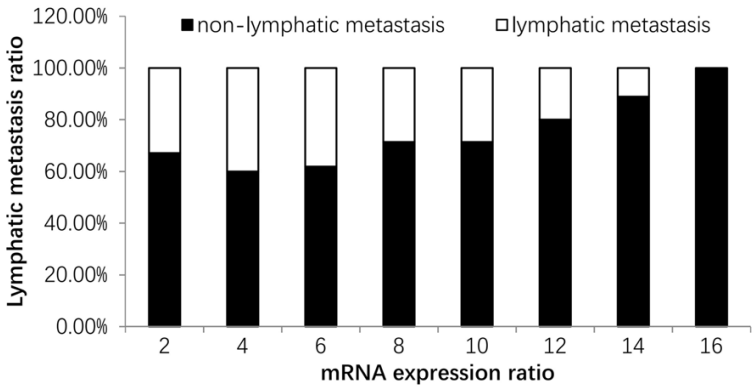

Figure 3. Nighty-four cases of mRNA expression quantity were 2 times in the control group, the lymphatic metastasis for 58 cases (61.7%). Sixty cases of mRNA expression quantity were 4 times in the control group, the lymphatic metastasis for 36 cases (60%). Forty-two cases of mRNA expression quantity were 6 times in the control group, the lymphatic metastasis for 26 cases (61.9%). Twenty-eight cases of mRNA expression quantity were 8 times in the control group, the lymphatic metastasis for 20 cases (71.4%). Twenty-eight cases of mRNA expression quantity were 10 times in the control group, the lymphatic metastasis for 20 cases (71.4%). Twenty cases of mRNA expression quantity were 12 times in the control group, the lymphatic metastasis for 16 cases (80%). Eighteen cases of mRNA expression quantity were 14 times in the control group, the lymphatic metastasis for 16 cases (88.9%). Six cases of mRNA expression quantity were 16 times in the control group, the lymphatic metastasis for 6 cases (100%).

ing ductal carcinoma with high malignancy. The target drugs such as Herceptin can precisely kill malignant cells with *Her-2* positive status instead of affecting normal cells survival. Breast carcinoma cells with HER-2 protein overexpression and/or *Her-2* gene amplification are sensitive to Herceptin. Moreover, *Her-2* molecular status is a significant prognosticator of breast carcinoma in clinic. IHC and FISH are universally accepted for these two molecular states [27-29]. Because of the efficient-cost and simple operation, IHC is much preferred by many hospital laboratories, meanwhile, its results might be more subjective per the artificially interpretation system. Besides, the inappropriate tissues processing can lead to ambiguous results sometimes [30]. As a protein targeted detection technology, IHC cannot report the true amplification status of *Her-2* gene precisely. Nucleic acid as a target detection technology, FISH is used to assess *Her-2* gene CNV, especially for indeterminate specimens with an IHC score of 2+ according to National Comprehensive Carcinoma Network (NCCN) Guidelines. Contrast with IHC, FISH result is much closer to the true amplification status of *Her-2* gene status in breast carcinoma cells with better stability, accuracy, sensitivity and repeatability. However, fluorescence signals must be observed and counted within 20 or even more invasive carcinoma cells in time which is time-consuming. Fluorescence quenching caused by delay or exposure in white light for a long time, this could ultimately lead to erroneous interpretation. For this reason, together with costly apparatus, FISH is not widely used in many primary hospitals. It is worth to 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 when detected by FISH [31].

Target short genes were amplified by Q-PCR method which has advantages in sensitivity, accuracy and reliability, especially the fragmented DNA in the delay fixed specimens [32]. Tianjie Pu (2015) et al. showed that the Q-PCR results did not change with a cold ischemia time of up to 12 hours. Thus, to the DFF (the delay to formalin fixation) issues, Q-PCR might be a surrogate for the *Her-2* detection. As a result, some researchers suggested that Q-PCR could be a stable and reliable alternative method for the evaluation of *Her-2* gene CNV in breast carcinoma especially for samples that were not promptly placed in fixative. In summary, Q-PCR has advantages in sensitivity, accuracy and reliability, easy operating, high throughput time-saving. Meanwhile, the only disadvantage of Q-PCR utilization in breast infiltrating ductal carcinoma is that it cannot locate the target area in situ, especially in some heterogeneous tumors and can be solved by laser capture micro dissection method [32].

Previous investigations showed that breast carcinoma in different regions, different races have differences. For ethnic minorities, economically and traffic underdeveloped and geographical restricted in Yunnan province, can lead to the particularity of the incidence of

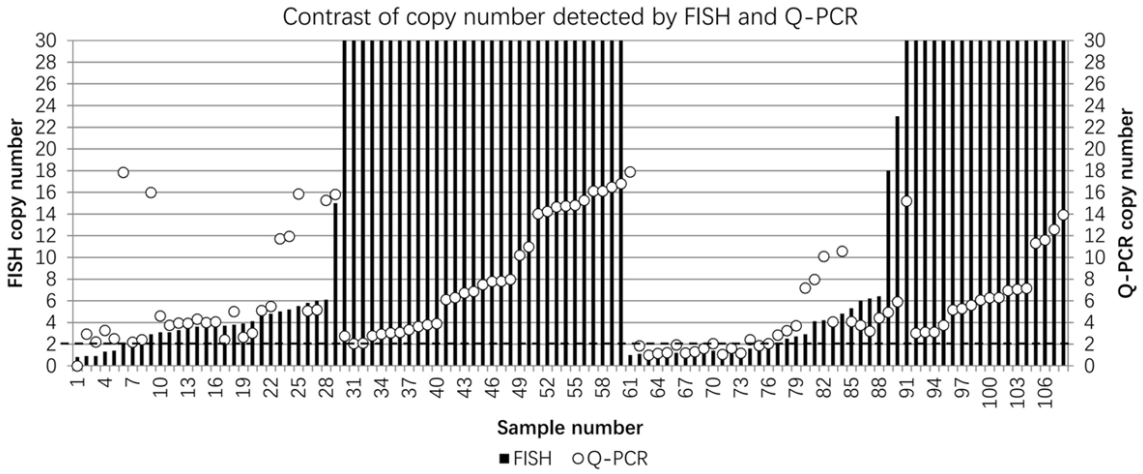

Figure 4. Summary of FISH and Q-PCR results for FFPE tissue samples from 108 breast carcinomas. Samples numbered 1 to 60 are lymphatic metastasis cases and samples numbered 61 to number 108 are non-lymphatic metastasis cases. Ratios of samples clustered amplification of FISH were determined at 30. Each Q-PCR copy number value represents the *Her-2* status for each sample. The dashed horizontal line indicates the Q-PCR and FISH threshold cutoff of 2 copies for a sample to be deemed *Her-2* positive. The FISH values were expressed as the copy number (*Her-2*/CEP17 ratio). There were 7 cases numbered 2, 3, 4, 5 (metastasis group), 61, 70, 74, and 76 (non-metastasis group) were positive by Q-PCR but negative by FISH.

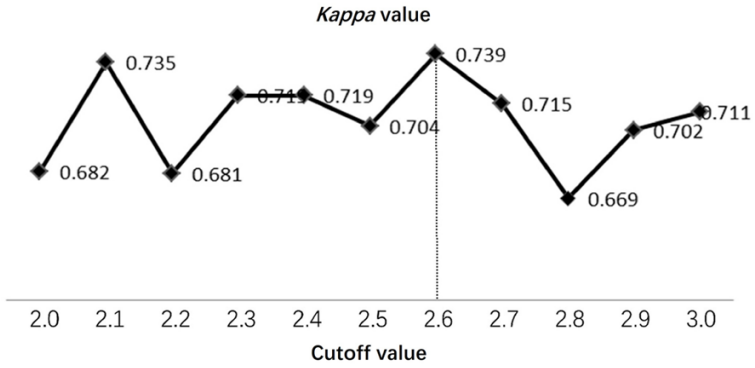

Figure 5. When the threshold value was set to 2.60, *kappa* value reached maximum (kappa = 0.739), which meant that Q-PCR method was highly consistent with the FISH method.

breast carcinoma. In conclusion, Undergoing the repeated tests, we set the reasonable cutoff value at 2.60 (*kappa* = 0.739) within the Q-PCR detection data of 108 females. Take the FISH as golden standard, when interpret the result per this value, Q-PCR method has a great sensitivity (96.59%), specificity (75%), negative predictive value (94.44%) and positive predictive value (83.33%), which can be used as a potential alternative of FISH to test *Her-2* gene status. Moreover, the correlation of *Her-2* FISH results and Q-PCR results are respectively compared with clinic pathological variables in 108 breast carcinoma patients in our study. And we found that just like FISH, Q-PCR result is significantly associated with lymphatic metastases as a prognostic indicator in clinic. The eight cases which are negative for FISH but positive for Q-PCR indicated that Q-PCR has bigger positive predictive value. In addition, when detected by Q-PCR, the higher the amount of mRNA expressed, the higher the risk of lymphatic metastases. With the expressing quantity reached 16 times, the metastases proportion is up to 100%. Our results indicated that in some cases, Q-PCR can be a reliable and stable alternative method for the evaluation of *Her-2* status in immunohistochemical scored 2+ breast carcinoma in Yunnan Province of China, even wider area.

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Disclosure of conflict of interest

None.

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