Review Article
Genetic heterogeneity of HER2/Neu in breast carcinoma: a meta analysis

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Abstract: Both immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) are accepted methods fordetermining clinical HER2 (or ERBB2) status. However, the reliability of IHC and FISH is questionable. The genetic heterogeneity (GH) of breast carcinoma is a major cause that makes its diagnosis and treatment far from being optimal. To clarify the impact of FISH and IHC testing on HER2 GH, a meta-analysis was performed in this study. A total of eight studies with 11478 patients retrieved from PubMed and Embase were included. A random-effects meta-analysis of all studies suggested GH was existed in FISH equivocal test (OR=30.98, 95% CI 21.19-45.30) and IHC equivocal test (OR=1.56, 95% CI 1.40-1.74), respectively. Further analysis indicated that the significant differences presented between GH and different HER2 expression by FISH test (x^2=864.23, P<0.001), and between GH and different HER2 expression using IHC test (x^2=206.52, P<0.001), respectively. Networks revealed that HER2 regulates different downstream proteins to coordinate numerous processes. Our results suggested that GH of HER2 differentially presented in a subset of HER2 amplified breast carcinomas, especially in cases with HER2 expression (FISH equivocal and IHC equivocal). There is a substantial difference in the frequency of GH among different ethnicities. HER2 GH is more likely to exist in FISH equivocal test than IHC equivocal test, and it is more convenient to document GH status by FISH test. We suggest FISH as the primary HER2 testing modality with breast carcinomas who are candidate for HER2 targeted therapy. FISH equivocal test is necessary to be further standardized in order to better define HER2 status and it is important to incorporate GH into the management of breast patients.

Keywords: Breast carcinoma, HER2, genetic heterogeneity, IHC, FISH

Introduction

Human epidermal growth factor receptor 2 (HER2, or ERBB2) has been shown to be amplified in approximately 20% of human breast carcinomas, and is a significant predictor of both overall survival and time to relapse in patients with breast cancer [1, 2]. *In vitro* and *in vivo* studies demonstrated HER2 is a potent oncogene that promotes tumor growth, angiogenesis and metastasis [3-5]. It leads to the development of trastuzumab (Herceptin; Genetech, San Francisco, CA), an anti-HER2 humanized recombinant monoclonal antibody (mAb), which has showed considerable clinical utility in patients with HER2-overexpressing breast tumors in both metastatic [6, 7] and adjuvant settings [8-10]. HER2 overexpression is also associated with tamoxifen-resistance of breast carcinoma,

and suppression of HER2 expression enhances the tamoxifen activity [11]. Therefore, the accurate assessment of HER2 status is important for breast carcinoma therapy. Immunohistochemistry (IHC) for detecting HER2 protein overexpression and fluorescence in situ hybridization (FISH) assays for quantifying *HER2* amplification were approved by the United States Food and Drug Administration [12]. However, less than half of the patients with HER2-positive cancers will be respond to trastuzumab therapy [7]. The reliability of IHC is questionable, since discrepant results may be obtained in different laboratories, possibly due to the subjective aspect of the IHC scoring system [13]. Although FISH is considered to be more reliable than IHC, it requires more pathologist's interpretation time and is more expensive [14]. Prospective studies have showed that approximately 20% of
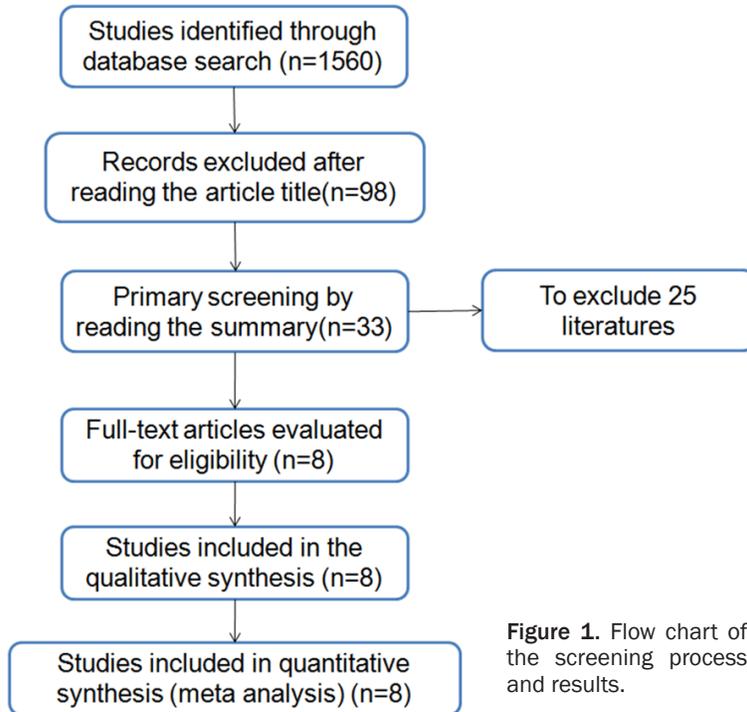
Figure 1. Flow chart of the screening process and results.

HER2 testing maybe inaccurate, and available data do not clearly demonstrate the superiority of either IHC or FISH as a predictor of benefit from anti-HER2 therapy [15]. The heterogeneity of breast carcinoma is a major cause that makes its diagnosis and treatment far from being optimal. Increased chromosome enumeration probe 17 (CEP17) is frequently reported in breast carcinoma [16] and might account for trastuzumab response in tumors. However, there are numerous discrepancies between CEP-17 count and accurately defining HER2 status [17-19]. HER2 genetic heterogeneity (GH) would affect the selection of patients for trastuzumab according to the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) [20]. GH is present if there are more than 5% but less than 50% of infiltrating tumor cells with a HER2/CEP17 signal ratio higher than 2.2 [21]. It was clear that a subpopulation of breast carcinomas examined for *HER2* gene amplification by FISH displayed intratumoral heterogeneity, and such cases could give rise to discrepant results between IHC and FISH assays for HER2 status [21, 22]. Gene expression profiling analysis has provided us insights into the complexity of breast tumors. Although there are abundant evidences that HER2-overexpressing tumors manifest distinct patterns of gene expression [23, 24], the basic

biology of this tumor subtype is not well understood.

It is important to provide practice guidelines for examining and reporting breast tumors with genetic heterogeneity for improvement of HER2 testing in breast cancer. New guidelines were established by ASCO and CAP for definition of these HER2 genetic heterogeneity (GH) as a first step towards evaluation of its clinical significance and impact on treatment [21]. HER2 GH implied partial amplification of HER2 gene and it might lead to some tumor cells gaining higher HER2 expression levels [25]. However, it remains unclear about the frequency and clinical significant of HER2 GH. To derive a more precise estimation of association between HER2 status and FISH or IHC testing, a meta-analysis was performed to help us to better understand the possible risk for GH in routine testing.

Materials and methods

Search strategy and data extraction

We browsed the databases of PubMed, Embase and Web of science using the combinations of the following search terms: "genetic heterogeneity", or "HER2 heterogeneity", and "breast cancer". The references in the studies were reviewed to check if additional studies exist.

Inclusion criteria and data extraction

The following criteria were used for the inclusion of eligible articles for our meta-analysis: (1) evaluation of genetic heterogeneity (GH) in breast carcinoma; (2) it was original data; (3) FISH-GH or IHC-GH study; (4) sufficient published data for estimating an odds ratio (OR) with 95% confidence interval (95% CI); (5) published before December 2013. We excluded the investigations with the absence of usable data that are essential for FISH-GH or IHC-GH analysis in this meta-analysis. For each study, the following data were recorded: first author's name, year of publication, nationality, total

Table 1. Genetic heterogeneity (GH) of individual studies in this meta-analysis

| No.	Studies	Country	Year	Number		PMID
				Cases	GH	
1	Shafi [36]	America	2013	251	71 (28.2%)	23539740
2	Chang [34]	Canada	2012	2522	666 (26.4%)	22282306
3	Seol [35]	Korea	2012	96	11 (11.5%)	22388760
4	Yang [25]	China	2012	617	94 (15.2%)	22476857
5	Ohlschlegel [33]	Switzerland	2011	530	160 (30.2%)	22011446
6	Lee [32]	Korea	2011	971	24 (2.5%)	21860549
7	Bartlett [31]	Scotland	2011	6461	2166 (33.5%)	21757600
8	Brunelli [20]	Italy	2009	30	4 (13.3%)	22476857
Total				11478	3196 (27.8%)	
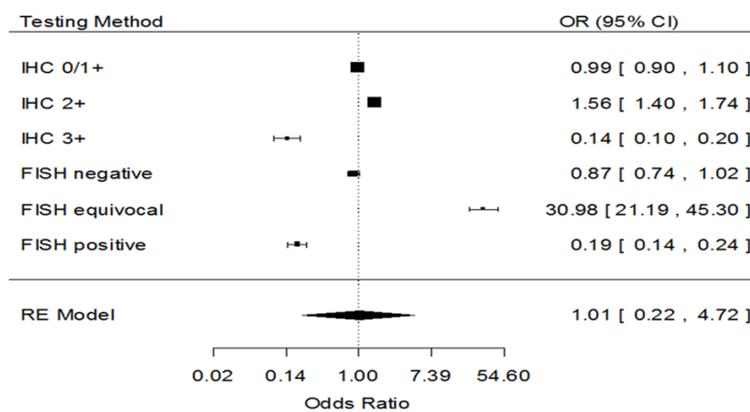
Figure 2. Forest plots of overall breast carcinoma HER2 Genetic heterogeneity (GH) with different HER2 status testing. The square and horizontal lines correspond to the testing-specific odds ratio (OR) and 95% CI. Diamonds represent the summary odds ratios, with 95% confidence intervals.

appropriate. Correlation analyses were carried out using Pearson's method. All statistical analyses were performed in the R environment, using several CRAN packages (http://cran.r-project.org/). Publication bias was evaluated using Begg's test [28] and Egger's test [29]. GeneSense [30] was used to build ERBB2 protein-protein interaction (PPI) networks based on literature and experimental data.

Results

Study characteristics

According to the inclusion criteria defined for the study available for this meta-analysis in **Figure 1**, we identified eight publications [20, 25, 31-36], consisting of 11478 cases from seven different countries in America, Canada, Korea, China, Switzerland, Scotland and Italy. Of the included studies, the sample sizes ranged from the smallest of 30 to the largest 6461 and the GH percentage ranged from 2.5% to 33.5%. The discrepancy of GH frequency exists in different ethnicities, such as Chinese and Scottish (15.2 *vs.* 33.5%). The summary characteristics of the studies are listed in **Table 1**.

Overall, the random-effects meta-analysis of GH suggested no significant GH risk in the combined results (OR=1.01, 95% CI=0.22-4.72) (**Figure 2**). FISH equivocal testing and IHC 2+ testing showed increased GH risk and were notably higher than the estimated effect for the

number of GH cases and FISH or IHC testing method.

Statistical analysis

The strength of association between the genetic heterogeneity (GH) and breast carcinoma was assessed by odds ratios (ORs) with corresponding 95% confidence intervals (CIs). The pooled OR was calculated for IHC negative (IHC 0/+1) *vs.* GH, IHC equivocal (IHC 2+) *vs.* GH, IHC positive (IHC 3+) *vs.* GH, FISH negative *vs.* GH, FISH equivocal *vs.* GH, FISH positive *vs.* GH, respectively. The combined OR was calculated by the random-effects model (Mantel-Haenszel) [26, 27]. The forest plots were used to describe an estimate of the overall results and variation between the results based on the individual studies that were included in the meta-analysis. Associations between the different parameters were assessed using the x^2 test when

Table 2. Meta-analysis of HER2 Genetic heterogeneity (GH) and HER2 FISH test

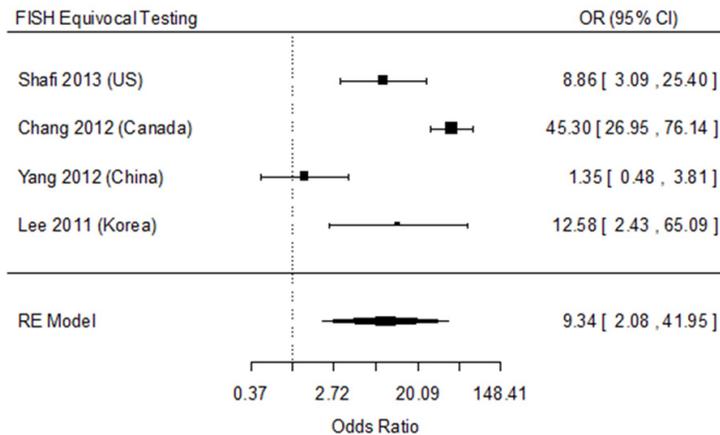| Variable	HER2 GH positive	HER2 GH negative	Total
FISH (x^2=864.23, P<0.001)			
FISH negative/Non-Amplified (<1.8)	551 (63.6%)	2419	2970
FISH equivocal/Equivocal (1.8-2.2)	236 (27.3%)	45	281
FISH positive/Amplified (>2.2)	79 (9.1%)	1091	1170
Total	866	3555	4421

Figure 3. Forest plot showing the results of 4 studies examining Genetic heterogeneity (GH) of breast carcinoma by FISH equivocal testing.

other testing methods. And the point estimates of the odds ratio by FISH equivocal testing were nominally greater than the odds ratio observed by IHC 2+ testing.

Impact of GH on HER2 tested by FISH

HER2 GH was found in 551 (63.6%) non-amplified (FISH negative) tumors and in 236 (27.3%) equivocal HER2 amplified (FISH equivocal) tumors, while only 79 (9.1%) amplified tumors (FISH positive) presented with HER2 GH (**Table 2**). HER2 GH was found in 6.8% FISH positive test and 24.2% non-positive test. Among the FISH non-positive test, HER2 GH cases were existed in 18.6% FISH negative test and 84.0% FISH equivocal test, respectively. Significant statistical difference for GH was observed between different HER2 expression by FISH testing (x^2=864.23, P<0.001). In the total cohort, there was significant association between FISH equivocal testing and GH risk under the random-effects model (OR=9.34, 95% CI=2.08-41.95) (**Figure 3**). The random-effects meta-analysis of GH yielded significant differ-

ences in the odds ratios between ethnic groups, such as Canadian and American (45.30 *vs*. 8.86). The odds ratios ranged from 1.35 to 45.30. Considering random effects model might give undue weight to individuals in small studies, we explained the high heterogeneity observed among studies based on the evaluation of publication bias. Begg's test (P=1) and Egger's test (P=0.578) showed no evidence of significant publication bias related to the FISH equivocal testing and GH risk.

Impact of GH on HER2 tested by IHC

Compared with FISH test, HER2 GH was identified in 1270 (60.0%) tumors that showed on HER2 negative expression (IHC 0/1+) and in 802 (37.9%) tumors demonstrated equivocal results of HER2 equivocal expression (IHC 2+), while only 46 (2.2%) positive expression (IHC 3+) presented with HER2 GH (**Table 3**). HER2 GH was found in 5.8% IHC positive (IHC 3+) test and 29.5% IHC non-positive test. Among the IHC non-positive test, the HER2 GH cases existed in 28.7% IHC negative (IHC 0/1+) test, 30.8% IHC equivocal (IHC 2+) test and 5.8% IHC positive (IHC 3+) test, respectively. Significant statistical difference for GH was observed between different HER2 expression by IHC testing (x^2=206.52, P<0.001). In the total cohort, there was significant association between IHC 2+ testing and GH risk under the random-effects model (OR=2.14, 95% CI=0.95-4.82) (**Figure 4**). Begg's test (P=0.233) and Egger's test (P=0.053) showed no evidence of significant publication bias related to the IHC equivocal testing and GH risk. The GH difference existed in the different ethnic group.

Network analysis of ERBB2 protein

The PPI network visualization was used to evaluate the regulatory relationship between ER-BB2 and associated proteins base on Gene-Sense [30]. The node net model showed that ERBB2 interacts with 15 downstream proteins (**Figure 5A**), such as epidermal growth factor

Table 3. Meta-analysis of Genetic heterogeneity (GH) and HER2 IHC test

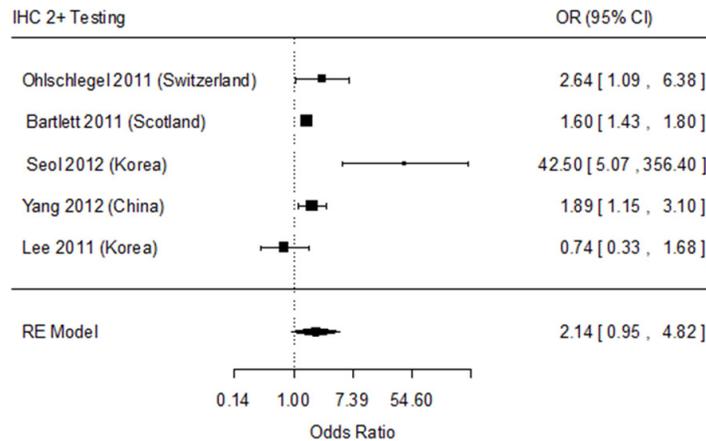| Variable	HER2 GH positive	HER2 GH negative	Total
IHC (χ^2=206.52, P<0.001)			
Negative (IHC 0/1+)	1270 (60.0%)	3149	4419
Equivocal (IHC 2+)	802 (37.9%)	1798	2600
Positive (IHC 3+)	46 (2.2%)	747	793
Total	2118	5694	7812

Figure 4. Forest plot showing the results of 4 studies examining Genetic heterogeneity (GH) of breast carcinoma by IHC equivocal (IHC 2+) test.

receptor EGFR and cell division cycle 2 CDC2, which couple with signal to further downstream signaling processes. The leaf net model was further used to evaluate the interactions of downstream proteins in **Figure 5B**. It shows that some downstream proteins such as EGFR and GRB2 have many interactions with other downstream proteins, while some downstream proteins, such as CDC2, do not show much interaction with other downstream proteins.

Discussion

HER2 inhibition is an effective therapeutic strategy for the treatment of HER2 overexpression tumors [37-39], and an accurate assessment of HER2 status is therefore critical to optimize clinical outcomes in patients with breast carcinoma and gives them the opportunity to receive therapy [40]. However, approximately 20% of current HER2 testing may be inaccurate base on FISH and IHC testing method [15, 41]. The genetic heterogeneity of breast carcinoma is a major cause that makes its diagnosis and treatment far from being optimal, and it has

been considered one crucial factor in assessing a patient's initial response to treatment and reflects the cellular complexity and dynamics within a tumor [42]. To clarify the impact of FISH and IHC testing on HER2 genetic heterogeneity, our study evaluated genetic heterogeneity status in different HER2 testing. The discrepancy of genetic heterogeneity frequency existed in different studies. HER2 genetic heterogeneity was noted in 27.8% of breast carcinomas, and the percentage ranged from 2.5% to 33.5%. It showed that genetic heterogeneity is present in a wide range of breast carcinomas might potentially alter response to treatment. Genetically heterogeneous carcinomas harboring *HER2*-amplified subclones might be sensitive to HER2-targeted therapy [33, 43]. Paik and coworkers' research suggested that patients with HER2 negative disease appeared to benefit from trastuzumab, and about 10% of patients with HER2 negative tumors responded to trastuzumb [44]. Finding the optimal protein regulatory factors is crucial to the understanding of the molecular events [30]. Protein-protein interactions of ERBB2 (or HER2) (**Figure 5**) may guide the formulation of meaningful hypotheses with regard to signaling pathways critical to tumorigenesis following ERBB2 deficiency. The leaf net model helps to identify specific proteins that regulate the genes or proteins of interest by the leaf networks. The leaf net (**Figure 5B**) showed that some downstream proteins such as EGFR and GRB2 having many interactions with other downstream proteins may be involved in important yet complex mechanisms in ERBB2 related signaling pathways; Some downstream proteins, such as CDC2 that do not show much interactions with other downstream proteins, may have a simple yet unique function with ERBB2. Current guidelines need to provide more clarity on the determination of the optimal cutoff percentage of HER2 genetic heterogeneity based on Trastuzumab responsiveness. Understanding the complex ways ERBB2 interacts with its downstream proteins at the
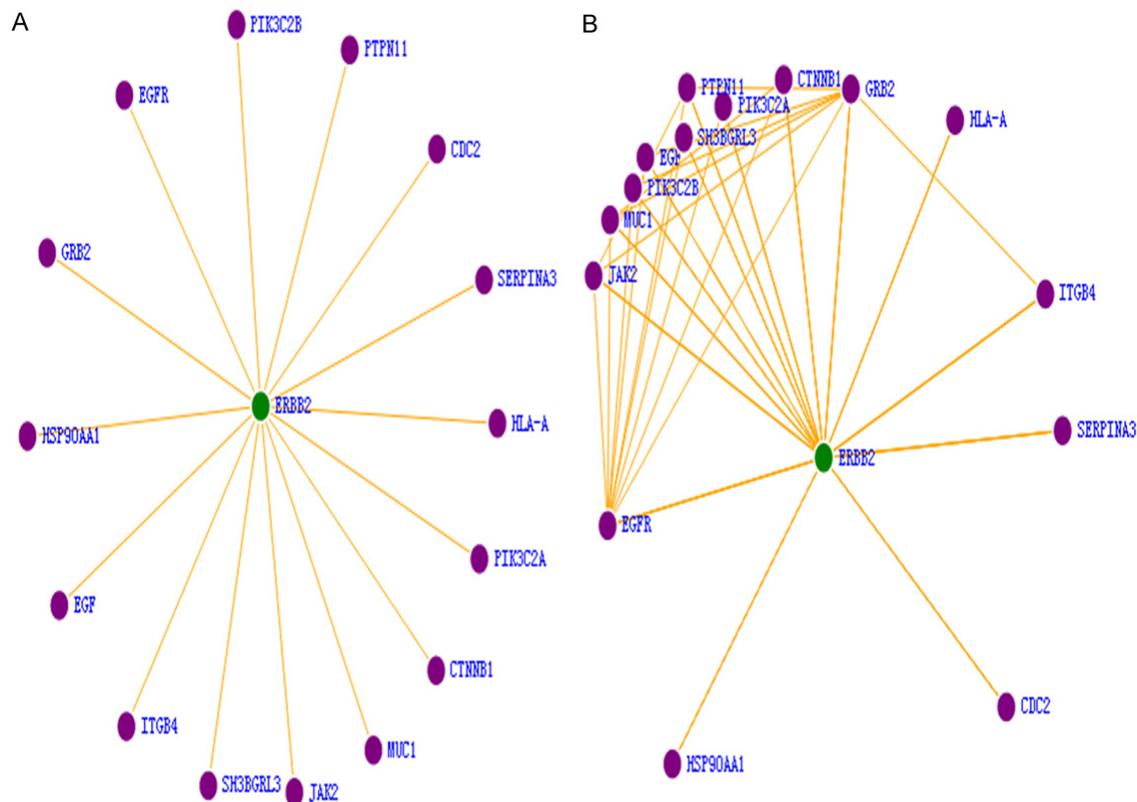
Figure 5. PPI network analysis for ERBB2 (HER2) protein. The green circle indicates the node protein. The purple circles indicate leaf proteins, and the orange lines indicate interactions. A. The node network of ERBB2 protein. A shows the interactions of ERBB2 and 15 downstream proteins. B. The leaf network of ERBB2 protein. The leafnet model in B is used to evaluate the interactions of downstream proteins.

different genetic heterogeneity level maybe crucial for discovering and analyzing mechanisms involved in trastuzumab resistance. Evaluation of the HER2 status in breast carcinoma with genetic heterogeneity may be beneficial before treatment selection. Yang [25] and Seol [35] observed smaller size, lower grade and greater incidence of hormone receptor positivity in the cases with genetic heterogeneity compared with HER2 amplified cases, whereas Ohlschlegel [33] noted a higher tumor grade in cases with genetic heterogeneity. Shafi showed that Genetic heterogeneity for HER2 is present in a significant proportion of breast carcinomas that would otherwise be classified as HER2 FISH negative [36]. Genetic heterogeneity has been well documented and represents subclonal diversity within the tumor and its presence may increase subjectivity in HER2 interpretation by the pathologist [21].

Our results further demonstrated that HER2 genetic heterogeneity were mainly existed in

FISH equivocal test (84.0%) and IHC equivocal test (30.8%), indicating that HER2 genetic heterogeneity is a substantial cause of equivocal HER2 testing results in breast carcinoma by FISH and IHC. The higher incidence of genetic heterogeneity in the FISH equivocal test than in the IHC equivocal test suggested that genetic heterogeneity played a more significant role in the causation of equivocal FISH test. Evaluation of HER2 status, especially based on FISH equivocal test, may be beneficial for the establishment of standardized methods to improve the accuracy and consistency of interpretation of HER2 gene amplification status in breast carcinoma. Our results also showed that the genetic heterogeneity is not homogeneously present and vary among different ethnicities, indicating that genetic heterogeneity of different ethnic groups may likely have different ethnicity-specific effects. HER2 genetic heterogeneity might lead to different breast cancer subtypes that may differ in pathway activity, pro-

gression, and response to therapy. HER2 genetic heterogeneity is a peculiar feature of breast carcinomas and the correct identification of heterogeneous nature is relevant for the management of primary and metastatic breast carcinomas [15, 45, 46]. Current guidelines need to provide more clarity on the determination of the optimal cutoff percentage of HER2 genetic heterogeneity based on Trastuzumab responsiveness. HER2 genetic heterogeneity analysis based on the comparison of IHC and FISH method is an important step towards the development of better tests for HER2 status determination in breast carcinoma and maybe crucial for discovering and analyzing mechanisms involved in trastuzumab resistance.

In conclusion, HER2 genetic heterogeneity is present in a significant proportion of breast carcinomas and can complicate FISH or IHC assessment, leading to an inaccurate determination of HER2-directed therapy suitability. Our data highlighted the importance of recognizing the heterogeneous status of breast carcinoma. To our knowledge, this is the first meta-analysis to investigate the association between HER2 status and FISH or IHC testing. A total of eight independent breast carcinomas consisting of 11478 cases were involved. This meta-analysis confirms a significant correlation between FISH equivocal test and genetic heterogeneity risk. FISH equivocal test is necessary to be further standardized in order to better define HER2 status and the clinical impact on treatment outcome of genetic heterogeneity in breast carcinoma should be identified by more convincing experimental evidences in molecular level and population level.

Disclosure of conflict of interest

None.

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