

Her-2/neu amplification and breast cancer survival: Results from the Shanghai breast cancer study

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Abstract. Her-2/neu is a member of the epidermal growth factor receptor family that has been found to be overexpressed or amplified in ~20-30% of breast cancers. Negative prognosticators and a shortened survival have been shown to be associated with these changes in Her-2/neu, but previous studies have consisted of predominantly Caucasian populations. Additionally, chromogenic *in situ* hybridization (CISH) has been suggested to be a potential alternative to fluorescent *in situ* hybridization (FISH), the expensive and labor-intensive gold standard assay currently used for Her-2/neu amplification. This study evaluated breast cancer samples from 313 Chinese women participating in the Shanghai breast cancer study, of which 100 (32%) were found to have Her-2/neu amplification by either FISH or CISH methodologies. After a mean follow-up period of 6.67 years, Her-2/neu amplification was found to be significantly associated with an increased hazard of death, regardless of which assay was used to detect amplification. Patients with Her-2/neu amplification were ~60% more likely to die of the disease (HR: 1.6, 95% CI: 1.0-2.6) than patients without amplification, even after adjusting for age, stage, menopausal status, chemotherapy, radiotherapy and tamoxifen treatment. Furthermore, the negative prognostic effect of Her-2/neu varied by cancer stage, with greater risks of death evident among later stage patients. This study supports a negative prognostic role for Her-2/neu in breast cancer survival among a Chinese population, irrespective of whether FISH or CISH is used to detect amplification of the Her-2/neu gene.

Introduction

The Her-2/neu oncogene (c-erbB-2), a member of the epidermal growth factor receptor family, encodes a 185 kDa

transmembrane glycoprotein with tyrosine kinase activity (1,2). Her-2/neu has been mapped to the long arm of 17q21.1 (3) and its protein product functions as a co-receptor in the initial steps of signal transduction after growth factor binding and receptor dimerization (4-7). Functional Her-2/neu is essential for normal growth and development (8-10). However, high levels of expression result in increased cellular proliferation (11) and have been shown to be sufficient for cellular transformation (12,13). While Her-2/neu has no preferred growth factor ligand, it dimerizes with other receptors of the ErbB family and potentiates multiple proliferative and cell survival signaling cascades (4-7). The oncogenic potential of Her-2/neu has been attributed to its capacity for autophosphorylation and constitutive homodimerization (7,12,14), as well as its ability to heterodimerize with other members of the Her family, likely resulting in an increased ligand binding affinity and decreased receptor internalization and degradation (6,7,15).

Overexpression of the protein or amplification of the Her-2/neu gene has been shown to occur in ~20-30% of breast cancers (16), and to be associated with a variety of negative prognostic factors, including larger tumor size, high nuclear and histological grade, steroid hormone receptor negativity, lymph node involvement, tumor aneuploidy and high proliferation indices (17-24). Patients with Her-2/neu positive tumors have also been found to have higher risks of metastasis, disease recurrence and death (16,17,25-32). All of these studies, however, were conducted in predominantly Caucasian populations. While Her-2/neu is generally considered to be a marker of tumor aggressiveness (22), the percentage of Chinese breast cancer patients with Her-2/neu amplification and the effect of this amplification on breast cancer survival among a Chinese population, is less clear.

With the advent of trastuzumab (Herceptin®, Genentech, Inc.), a recombinant humanized monoclonal antibody against the extracellular domain of the Her-2/neu protein, patients can receive targeted cytotoxic treatment against their Her-2/neu positive breast tumors (33,34). This makes the correct assessment of Her-2/neu crucial for appropriate clinical decision making (35). Two assays for Her-2/neu evaluation are currently in clinical use: the Hercep Test™ (Dako Corp., Carpinteria, CA) uses an immunohistochemical (IHC) approach to detect the Her-2/neu protein overexpression, and the PathVysion™ test (Vysis Inc., Downers Grove, IL) uses a

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fluorescence *in situ* hybridization (FISH) method to detect the amplification of the Her-2/neu gene. IHC is simpler, faster and readily available in most pathology laboratories. However, several studies have reported that the assay is overly sensitive and has a high false positive rate (36-40). FISH is considered to be the gold standard for Her-2/neu evaluation, but it is expensive and frequently impractical for use in routine clinical settings (40-46). Chromogenic *in situ* hybridization (CISH) has been shown to be a viable alternative to FISH whereby the fluorescent signal is replaced with a chromogenic label, allowing for detection by standard microscopy (45,47-52). However, the assessment of Her-2/neu by CISH has yet to be used in a large prognostic study. To address this issue, this study was undertaken to evaluate the association between Her-2/neu amplification, as assessed by FISH or CISH methodologies, and breast cancer survival among Chinese women participating in the Shanghai breast cancer study.

Materials and methods

Study design and subjects. This cohort of primary breast cancer patients consisted of a subset of cases from The Shanghai Breast Cancer Study, Shanghai, China, 1996-2002, a large population-based study of permanent Chinese residents in urban Shanghai (53,54). Eligible women were those diagnosed with breast cancer between August 1996 and March 1998, without a previous cancer diagnosis and who were still alive at the time of the interview. Through a rapid case ascertainment system, and the population-based Shanghai Tumor Registry, 1,602 eligible breast cancer cases were identified, of which 1,459 (91.1%) completed in person interviews. Reasons for non-participation included refusal (N=109, 6.8%), death before the interview (N=17, 1.1%) and the inability to be located (N=17, 1.1%). Structured questionnaires were used to obtain detailed information on demographic factors, menstrual and reproductive histories, physical activity, tobacco and alcohol use, weight history and family history of cancer. The information on cancer diagnosis, tumor-node-metastasis (TNM) stage of disease and treatment received was abstracted by medical chart review. Cancer diagnoses were confirmed by two senior pathologists.

The 1,459 breast cancer patients were followed through July 2005 by surveys coupled with record linkage of death certificates from the Vital Statistics Unit of the Shanghai Center for Disease Control and Prevention. The majority of patients were contacted either in person or by telephone (N=1,378, 94.4%), of which 266 were found to have died. The survival status of the remaining 77 patients was determined by death registry linkage, and of these, 47 were deceased. The remaining 30 patients were assumed to be alive six months prior to the date of the registry search to allow for the possible delay of death record entry. Four subjects had insufficient information for record linkage and were considered to be lost to follow-up.

Sample collection and processing. Paraffin-embedded formalin-fixed tumor tissues were sectioned and mounted onto charged slides for Her-2/neu analysis. Due to budget constraints, only a subset of the 1,455 breast cancer patients

included in the present study were analyzed, 108 by FISH and 205 by CISH.

Fluorescence *in situ* hybridization. Dual-color FISH was performed using the PathVysion test (Vysis Inc.) according to the manufacturer's instructions. The kit included a SpectrumGreen probe for the centromeric region of chromosome 17 and a SpectrumOrange HER-2/neu locus-specific probe. Briefly, tissue sections were baked onto slides at 60°C overnight. Samples were deparaffinized by immersing them twice in Hemo-De (Fisher Scientific, Pittsburgh, PA) for 15 min at room temperature, dehydrated twice in 100% EtOH for 10 min at room temperature and air-dried. The slides were treated with microwave radiation in 10 mM citric acid (pH 6) at 700 W for 3 min followed by immersion in 2X SSC for 5 min at room temperature. Then the slides were treated with a pepsin (4 mg/ml in 0.9% NaCl, pH 1.5) digestion for 20 min at 37°C, rinsed with de-ionized water for 2 min and baked at 60°C for 20 min. Dual-color FISH was performed so that the probes and target DNA were co-denatured at 80°C for 2 min and incubated on the samples under rubber cement-sealed coverslips at 37°C overnight. After hybridization, the slides were washed twice in 1.5 M urea at 45°C for 15 min, once in 2X SSC (pH 7.0) for 5 min, and once in 2X SSC/0.1% NP-40 for 5 min. The tissue samples were then counterstained with DAPI II (Vysis Inc.). The slides were viewed with a Zeiss Axioscope ultraviolet-equipped microscope and triple bandpass filter unit (Chroma Technology, Brattleboro, VT). For each case, one H&E companion slide was used for pathological confirmation and establishment of the location of malignant tumor areas. Five non-adjacent cancer areas were randomly selected and 20 non-overlapping interphase nuclei were scored in each area. The nuclei from stromal elements were not enumerated. Green chromosome 17 centromere signals and orange HER-2/neu signals were simultaneously counted in each nucleus. The average copy number per nucleus was calculated for the two signals in all five areas. An amplification ratio was calculated by dividing the mean HER-2/neu copy number by that of the chromosome 17 centromere. A sample with an amplification ratio of <1.5 was considered to have no amplification. Samples with ratios between 1.5 and 2.0 were considered to have low; those between 2.1 and 4.0, moderate, and those with amplification ratios >4.0, high amplification. When dichotomized, the samples with no amplification were compared to those with any level of amplification.

Chromogenic *in situ* hybridization. CISH reagents were from Zymed (Zymed Laboratories, South San Francisco, CA) and were used according to the manufacturer's instructions with minor modifications. Briefly, tissue sections were baked onto slides at 60°C overnight. Samples were deparaffinized by immersing them twice in xylene for 15 min at room temperature, dehydrated twice in 100% EtOH for 10 min at room temperature and then air-dried. Slides were treated with microwave radiation in pre-warmed pretreatment buffer at 700 W for 3 min. Enzymatic digestion was carried out with pepsin at 37°C for 3 min followed by immersion of graded diluted ethanol, 70, 85, 95 and 100% at 4°C for 3 min each and then air-dried at room temperature. After the Her-2/neu



	SBCS Breast cancer cases (N=1,455)	Her-2/neu analyzed Breast cancer cases (N=313)	Her-2/neu status		p-value ^a
			Not amplified (N=213)	Amplified (N=100)	
	N (%) ^b	N (%) ^b	N (%) ^b	N (%) ^b	
Age at diagnosis					
45 or younger	583 (40.1)	145 (46.3)	98 (46.0)	47 (47.0)	0.87
Older than 45	872 (59.9)	168 (53.7)	115 (54.0)	53 (53.0)	
Menopausal status					
Premenopausal	950 (65.3)	204 (65.2)	140 (65.7)	64 (64.0)	0.76
Post-menopausal	505 (34.7)	109 (34.8)	73 (34.3)	36 (36.0)	
TNM stage of disease					
0-I	358 (24.6)	70 (22.4)	51 (23.9)	19 (19.0)	0.82
IIa	508 (34.9)	117 (37.4)	81 (38.0)	36 (36.0)	
IIb	320 (22.0)	85 (27.2)	56 (26.3)	29 (29.0)	
III-IV	165 (11.3)	24 (7.7)	16 (7.5)	8 (8.0)	
Unknown	104 (7.2)	17 (5.4)	9 (4.2)	8 (8.0)	
Chemotherapy					
Yes	1367 (94.0)	298 (95.2)	204 (95.8)	99 (94.0)	1.00+
No	70 (4.8)	12 (3.8)	8 (3.8)	4 (4.0)	
Unknown	18 (1.2)	3 (1.0)	1 (0.5)	2 (2.0)	
Radiotherapy					
Yes	566 (38.9)	127 (40.6)	85 (39.9)	42 (42.0)	0.85
No	690 (47.4)	153 (48.9)	104 (48.8)	49 (49.0)	
Unknown	199 (13.7)	33 (10.5)	24 (11.3)	9 (9.0)	
Tamoxifen treatment					
Yes	921 (63.3)	207 (66.1)	144 (67.6)	63 (63.0)	0.09
No	263 (18.1)	50 (16.0)	28 (13.2)	22 (22.0)	
Unknown	271 (18.6)	56 (18.0)	41 (19.3)	15 (15.0)	

^ap-value from χ^2 test or Fisher's exact test where indicated (+); tests of association do not include patients with unknown variable status. ^bColumn percents may not sum to 100 due to rounding error.

probe (10 ml/slide) was added, the samples were sealed under rubber cemented coverslips, denatured on a hot plate (94°C) for 3 min and incubated overnight at 37°C. The slides were washed (0.5X SSC) for 5 min and the hybridized probe was detected using the CISH detection reagents of anti-digoxigenin-FITC, anti-FITC-peroxidase and diaminobenzidine as the chromogen. The samples were counterstained with hematoxylin, mounted and evaluated by an ordinary transmitting light microscope under a 20X objective. The criteria for successful CISH analysis included the identification of at least one copy of the Her-2/neu gene per nucleus in most cancer cells and appropriate high temperature target retrieval and enzyme digestion as indicated by well-preserved cell morphology. Samples with 1-4 copies of Her-2/neu were considered to have no amplification, while those with five or more copies were considered to have Her-2/neu amplification.

Statistical analysis. The relationships between clinicopathological features of disease and Her-2/neu amplification status were evaluated with the χ^2 test of association or Fisher's

exact test when warranted. Survival time was defined as the time from diagnosis to death, or else censored at the date of the last contact. Survival functions were generated by the Kaplan-Meier method and differences between strata were evaluated by the log-rank test. Cox proportional hazards regression was used to model the risk of death associated with Her-2/neu in both unadjusted models and in models including other known or potential prognostic factors, such as age at diagnosis, stage of disease, menopausal status and types of treatment received, such as chemotherapy, radiotherapy and tamoxifen treatment. All tests were conducted with a significance level of $\alpha=0.05$ and all p-values were based on two-tailed tests of significance.

Results

The clinicopathological characteristics of all cases from the Shanghai Breast Cancer Study, as well as the subset of patients included in this analysis, are shown in Table I. The distribution of menopausal status, stage of disease, chemotherapy, radio-

Table II. Survival analysis of breast cancer patients by Her-2/neu amplification status.

Her-2/neu assay (N)	Cases	Deaths	5 year survival %	HR (95% CI) ^a	Overall survival HR (95% CI) ^b	HR (95% CI) ^c
FISH (N=108)						
Not amplified	63	10	85.5	1.0 reference	1.0 reference	1.0 reference
Amplified	45	12	78.3	1.9 (0.8-4.3)	1.9 (0.8-4.3)	1.7 (0.7-4.2)
CISH (N=205)						
Not amplified	150	33	88.1	1.0 reference	1.0 reference	1.0 reference
Amplified	55	18	76.4	1.6 (0.9-2.8)	1.7 (0.9-3.0)	1.7 (0.9-3.1)
CISH or FISH (N=313)						
Not amplified	213	43	86.6	1.0 reference	1.0 reference	1.0 reference
Amplified	100	30	78.1	1.6 (1.0-2.6)	1.6 (1.0-2.6)	1.6 (1.0-2.6)

^aUnadjusted, ^badjusted for age and stage and ^cadjusted for age, stage, menopausal status, chemotherapy, radiotherapy and tamoxifen treatment.

Table III. Stage-specific survival analysis of breast cancer patients by Her-2/neu amplification status.

	Cases	Deaths	HR (95% CI) ^a	Overall survival HR (95% CI) ^b
Stages 0 and I				
Not amplified	51	5	1.0 reference	1.0 reference
Amplified	19	2	1.1 (0.2-5.5)	1.1 (0.2-5.4)
Stage II				
Not amplified	137	28	1.0 reference	1.0 reference
Amplified	65	21	1.7 (1.0-3.1)	1.8 (1.0-3.2)
Stages III and IV				
Not amplified	16	8	1.0 reference	1.0 reference
Amplified	8	5	1.8 (0.6-5.7)	1.9 (0.6-6.0)
Stages II, III and IV				
Not amplified	153	36	1.0 reference	1.0 reference
Amplified	73	26	1.7 (1.0-2.8)	1.7 (1.0-2.9)

^aUnadjusted and ^badjusted for age.

therapy and tamoxifen treatment were comparable between the whole population and the study subset. Age at diagnosis was found to differ, with fewer patients >45 being analyzed for Her-2/neu. However, this difference was not statistically significant. The prevalence of Her-2/neu amplification was 26.8% in tumors assayed by CISH and 41.7% in those assessed by FISH. No differences in associations with clinicopathological characteristics between these subgroups were found (data not shown). Of the total 313 patients for whom Her-2/neu was evaluated, 100 (31.9%) were found to have gene amplification. In this study population, Her-2/neu was not found to be significantly associated with any patient or tumor characteristics.

Table II shows the 5-year survival rates as determined by Kaplan-Meier survival functions and the hazards of death associated with Her-2/neu amplification as determined by

Cox proportional hazards regression model for breast cancer cases analyzed by FISH (N=108), CISH (N=205) or either technique (N=313). Her-2/neu amplification was found to be associated with an elevated risk of death, and this relationship persisted after adjustment for patient age, disease stage, menopausal status, chemotherapy, radiotherapy or tamoxifen treatment. The results were similar irrespective of the method employed to detect Her-2/neu amplification. Generally, women with Her-2/neu amplification were ~60% more likely to die of the disease than patients whose tumors did not have Her-2 neu amplification (HR: 1.6, 95% CI: 1.0-2.6). This was also evident from the percentage of patients alive 5 years after their cancer diagnosis: 78.1% of those with amplification, compared to 86.6% of those without.

To further characterize the negative prognostic effect of Her-2/neu amplification, stage-specific survival analysis was

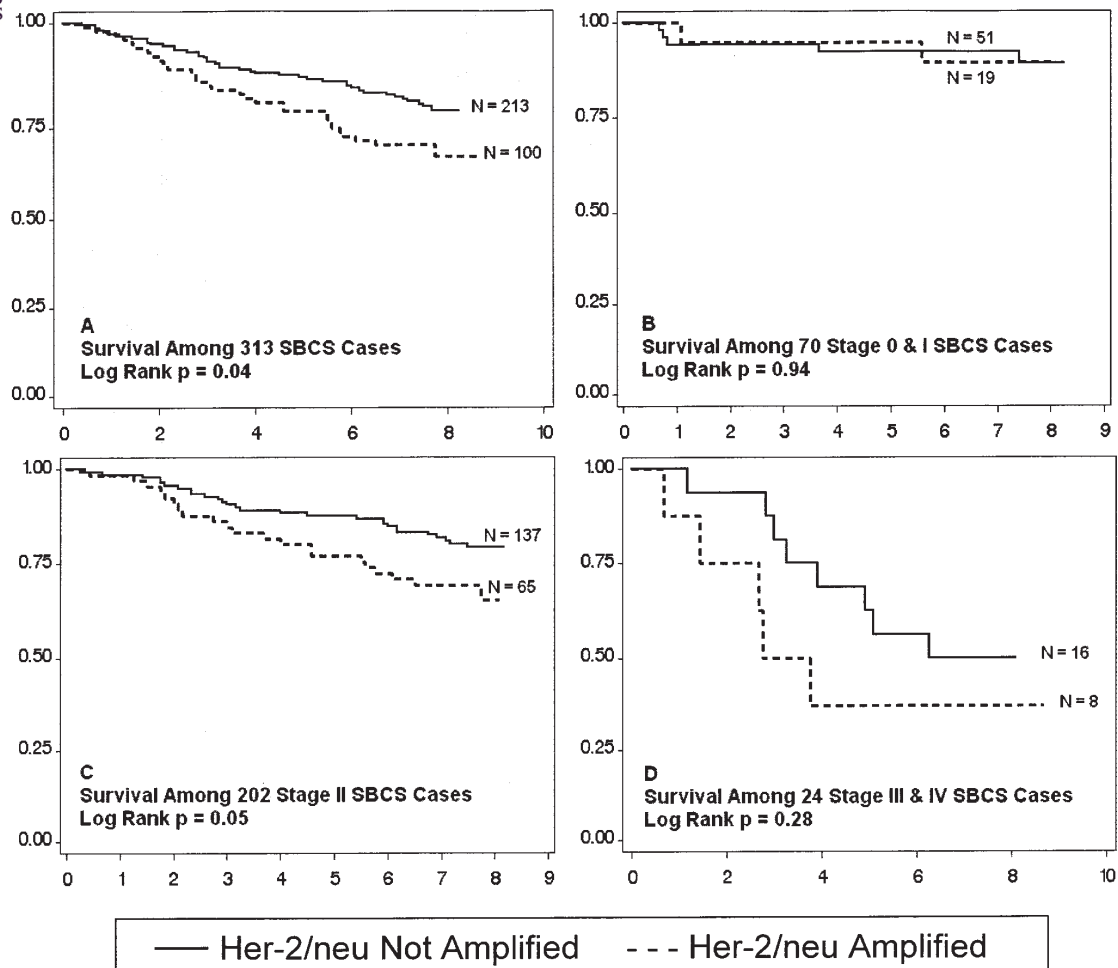


Figure 1. Kaplan-Meier survival functions of breast cancer patients by Her-2/neu amplification status and clinical stage of disease. The survival distribution function is the y-axis and the x-axis is years of survival. The solid line represents patients without while the dotted line represents those with Her-2/neu amplification.

conducted. As listed in Table III, Her-2/neu amplification among early stage breast cancer patients was not found to be associated with survival. However, Her-2/neu prognostic effects were evident among later stage patients. Among stage II patients, Her-2/neu was significantly associated with survival and women with amplification were ~80% more likely to die than those without Her-2/neu amplification (HR: 1.8, 95% CI: 1.0-3.2). Among stage III and IV patients, the hazard associated with Her-2/neu amplification was large (HR: 1.9, 95% CI: 0.6-6.0), although the sample size of this strata (N=24) precluded the estimate from reaching significance. When stage II, III and IV breast cancer cases were combined, a marginally significant hazard of death was found for Her-2/neu and amplification was associated with an ~70% increase in the risk of death (HR: 1.7, 95% CI: 1.0-2.9). Stage-stratified analysis included adjustment for patient age only, as the limited sample size within strata precluded adjustment for additional variables.

Kaplan-Meier survival functions were found to be in agreement with the results from Cox proportional hazards regression models. Overall survival was significantly worse for patients with Her-2/neu amplification ($p=0.04$) as assessed by either FISH or CISH (Fig. 1A). Among patients with *in situ*

or stage I disease, Her-2/neu amplification did not seem to adversely affect prognosis (Fig. 1B), and these results were unchanged when only stage I patients were included (data not shown). However, among stage II patients (Fig. 1C), those with Her-2/neu amplification fared worse ($p=0.05$). This difference was also seen in late stage patients (stage III and IV), but again, not to a significant extent (Fig. 1D).

Discussion

Three hundred and thirteen Chinese women with breast cancer were evaluated for their Her-2/neu status and followed up for a mean of 6.67 years (standard deviation, 1.96). Almost 32% (N=100) were found to have Her-2/neu amplification by either FISH or CISH and this was significantly associated with poorer breast cancer survival. Patients with Her-2/neu amplification were ~60% more likely to die (HR: 1.6, 95% CI: 1.0-2.6) than those without, even after adjusting for the effects of age, stage, menopausal status, chemotherapy, radiotherapy and tamoxifen treatment. The relationship between Her-2/neu and breast cancer survival did not depend upon which assay was used to detect amplification. Taken together, the results of this study support a negative prognostic

role for Her-2/neu in breast cancer survival among Chinese women. To the best of our knowledge, this is the first study to evaluate the relationship between Her-2/neu amplification and breast cancer survival in a large Asian population. Previously, Suo *et al* reported that Her-2/neu expression was weakly associated with poor prognosis among 107 Chinese breast cancer cases regardless of nodal status, although estimates of the effect were not presented (55). Similarly, Chang *et al* assessed Her-2/neu with CISH in 104 Korean patients and reported that those with amplification had a worse prognosis, although only median survival times were available (56). In the current study, not only was a significant survival disadvantage found for Chinese breast cancer patients with Her-2/neu amplification, but a stage-specific variation in the hazard of death was also suggested. Among early stage patients (94.3% stage I), Her-2/neu was not associated with survival, whereas, among stage II patients, amplification was associated with an ~80% increase in the risk of death (HR: 1.8, 95% CI: 1.0-3.2). Her-2/neu amplification conferred almost a doubling of the hazard of death among stage III and IV patients (HR: 1.9, 95% CI: 0.6-6.0), although the estimate was not significant. These stage-specific associations are perhaps not surprising as >91% of stage 0 and I patients survive for >5 years, while <84% of stage II and 52% of stage III and IV patients survive 5-years after their breast cancer diagnosis.

While differences were seen when our results were stratified with the stage of disease, the hazard of death associated with Her-2/neu amplification did not vary with the type of assay used. Current clinical guidelines to determine Her-2/neu amplification status of breast tumors call for the use of immunohistochemistry (IHC) to determine those that are definitely negative (0 or 1+) or strongly positive (3+), followed by FISH to evaluate amplification in the remaining cases (35,46). CISH has been proposed as an alternative method in assessing Her-2/neu for IHC weakly positive tumors (39,45,47,57). CISH alleviates the need for a fluorescence microscope and camera, creates results with permanent signal intensity and allows underlying histomorphology to be simultaneously assessed while being less expensive and faster to conduct (41,45-47,57). One possible limitation to CISH is that while FISH uses two signals, one for Her-2/neu, the other for the centromeric region of chromosome 17, the CISH Her-2/neu copy number does not consider the ploidy of chromosome 17. However, results between CISH and FISH have been highly consistent when directly compared (47,48,50,52,57-60). The results of two large studies of 157 breast tumors and 193 breast cancer cases found that results from CISH and FISH were 93.6% concordant (κ -coefficient, 0.81) and 93.8% concordant (κ -coefficient, 0.88), respectively (47,58). Several small studies (N<100) have reported κ statistics between 0.85 and 0.91 and concordance rates between 96 and 100% (50,57,59). Additionally, two larger studies have used tissue microarrays (TMA) to compare FISH and CISH methodologies and one found nearly perfect concordance (99%) among 110 samples (52) while the other had 94.1% concordance for 188 breast carcinomas (60). Together, these reports indicate that Her-2/neu amplification evaluated by FISH and CISH is highly comparable, and the assays generally have concordance rates of 93% or greater. As results from the two assays were in

agreement regarding their relationships with clinicopathological characteristics, as well as the association between Her-2/neu and breast cancer survival, we combined FISH and CISH data in the current study to generate a larger sample size and yield more precise estimates of association.

In addition to being a negative prognostic factor, Her-2/neu amplification has also become an indicator for selecting patients likely to respond to trastuzumab treatment. Patients included in our study were diagnosed and prospectively followed prior to the implementation of trastuzumab. Thus, the association between Her-2/neu amplification and breast cancer survival would not have been affected by this newly introduced targeted therapy. Additionally, the stage-dependent effects seen in our study suggest that among early stage patients, there may not be a survival benefit conferred by trastuzumab treatment as Her-2/neu amplification was not a significant prognosticator for these patients. The costs and risks associated with anti-Her-2/neu therapy may be avoided for these early stage patients, although further research on this hypothesis is needed before clinical recommendations can be made. In summary, Her-2/neu amplification, as assessed with either FISH or CISH, was shown to be related to an unfavorable prognosis among Chinese breast cancer patients.

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