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Original Article

Concordance of immunohistochemistry and fluorescence *in situ* hybridization for assessment of HER2 status in breast cancer patients in Xinjiang autonomous region, China

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Abstract: Objective: To investigate the concordance rate between Immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) for assessment of HER2 status in breast cancer in Uighur ethnic minority and Han population in Xinjiang province, China. Methods: Between January 2010 and June 2016, paraffin-embedded specimens form 1032 cases of breast cancer at the Tumor Hospital Affiliated to the Xinjiang Medical University were examined for HER2 status via both IHC and FISH using standard methodologies. The concordance between the two methods was then statistically evaluated. Results: The IHC 0, 1+ and 3+ cases had a high concordance rate of 100%, 90.6% and 91.7%, respectively. The IHC 2+ had a low concordance rate (64.4%). 78.6% (293/373) HER2 positive specimens (2+ and 3+) showed HER2 gene amplification and 95.1% (627/659) HER negative cases (0 and 1+) were HER2 non-amplified, yielding a kappa score of 0.7581 ($P < 0.0001$). IHC negative 0 and FISH had a high concordance rate of 100% in both populations. Han and Uighur population had similar concordance rate between IHC (0, 1+ and 3+) and FISH (100% vs. 100%, 89.0% vs. 94.7% and 91.0% vs. 94.6%). Both populations had low concordance rate (63.1% vs. 71.0%) for IHC 2+ cases. HER2 status showed significant correlation with tumor size ($P = 0.000$). The Uighur minority had a lower HER2 amplification rate compared with Han population. Conclusion: IHC can be used as an initial screening test of HER2 status for both populations in Xinjiang province. However, for cases with a HER2 IHC 2+ result, a FISH test is required for confirmation.

Keywords: Breast cancer, HER2 gene, fluorescence *in situ* hybridization, immunohistochemistry, anti-HER2 therapy

Introduction

The HER2/neu oncogene encodes a receptor tyrosine kinase that induces cell division and stimulates cell motility [1]. HER2 gene amplification and protein overexpression occurs in approximately 25% to 30% of primary breast cancer patients [2, 3]. Both HER2 amplification and overexpression is associated with an aggressive clinical phenotype and poor prognosis with reduced disease free survival and overall survival [4, 5]. This subset of breast cancers is sensitive to HER2-targeted treatment such as the monoclonal trastuzumab, resulting in significantly improved progression free survival and overall survival [6, 7], whereas these drugs offer no clinical benefit to HER2-negative patients [8]. Therefore, accurate assessment of HER2 status is important in order to identify breast cancer patients who might benefit from the anti-HER2 therapy.

Currently, immunohistochemistry (IHC, measuring protein-level) and fluorescence *in situ* hybridization (FISH, measuring the gene-level) are the most commonly used methods for determining the HER2 status in breast cancer [9, 10]. Although IHC for Her-2/neu protein is widely used in most diagnostic centers, it may produce false positive and false negative results primarily due to the antigenic changes that occurred during formalin fixation [11].

Moreover, the IHC requires standard criteria for data interpretation. On the other hand, FISH has an excellent sensitivity and specificity in detecting HER2 amplification, but is largely limited by its relatively high cost and requirement of special instrumentation [2]. Comparative studies have revealed a concordance rate of 73%-98% between IHC and FISH in detecting the HER2 state [12-14], and a concordance rate of 68.97-97.7% in China [15]. Nevertheless, no such studies have been previously performed in Xinjiang Vygur Autonomous Region in China, especially the indigenous Uighur ethnic minority living in the area. Therefore, this study was undertaken to assess the HER-2/neu gene status and to determine the concordance rate between FISH and IHC techniques in Xinjiang.

Patients and methods

Tissue specimens

This study included paraffin-embedded specimens form 1032 breast cancer patients, aged between 16-90 years, who were diagnosed and treated at the Tumor Hospital Affiliated to the Xinjiang Medical University between January 2010 and June 2016. No patient had received preoperative chemotherapy or radiotherapy. The 1032 patients included 788 from the Han population, and 244 from the Uighur population. All samples were subjected to both IHC and FISH. A positive sample with confirmed HER2 gene expression and a non-cancerous self-tissue were used as positive and negative control. This study was approved by the Ethics Committee at the Tumor Hospital Affiliated to the Xinjiang Medical University.

IHC protocol

The HER2 protein expression in paraffin sections was detected by immunostaining using Vectastain ABC kit (PK 4002, Vector Laboratories, Burlingame, CA, USA). Paraffin sections were dewaxed in xylene, rehydrated in serial concentrations of ethanol (100%, 90% and 80%), and treated with Saponin (Sigma, Shanghai, China) at room temperature for 30 min. Immunostaining was performed by incubation with rabbit anti-human monoclonal anti HER-2 (Maixin Biotech., Fuzhou, China, 1:200). The slide was observed under an inverted light microscope. The results were interpreted by two of the authors (BZ and HX) in a blinded fashion according to the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) Clinical Practice Guideline [16]. HER2 expression was identified as yellow-brown and granular staining in the cell membrane. HER2 expression was classified as level 0 to 3+ based on the proportion of positive cells and the staining intensity: 0: no staining or incomplete and faint staining within ≤ 10% of tumor cells; 1+: incomplete and/or faint membrane staining within > 10% of tumor cells; 2+: incomplete and/or weak/moderate circumferential membrane staining within > 10% of tumor cells or complete and intense circumferential membrane staining within ≤ 10% of tumor cells; and 3+: intense and complete circumferential membrane staining within > 10% of tumor cells. 0 and 1+ were regarded as negative, 3+ as positive, and 2+ as equivocal.

FISH protocol

FISH was performed using the US FDA approved PathVysion HER2 DNA probe kit (Abbott Molecular Inc., Des Plaines, IL, USA), a dual-coloured probe comprising locus specific identifier (LSI) HER2/neu SpectrumOrange and centromere enumeration probe (CEP) 17 SpectrumGreen. Briefly, tissue sections were baked at 65°C for 3 min, dewaxed in xylene twice (10 min each time), and boiled at 100°C for 30 min. Mounted slides were then treated with proteinase K for 5 min, and washed twice with 2×SSC wash buffer, baked again at 56°C for 5 min. The probe was denatured in hybridization buffer in a 78°C water bath for 5 min. Next, the tissue sections were dehydrated slightly with 70% ethanol at -20°C. The probe mixture was placed on slides and hybridized overnight in a 42°C thermostat. The slides were washed successively with 0.3% NP40/0.4×SSC, 0.1% NP40/2×SSC and 70% ethanol in the dark. The slides were dried naturally in the air, and subjected to probe labeling. Two types of DNA probes were used for overnight hybridization. Cell nucleus was stained by DAPI. The slides were observed under a fluorescent microscope (Axio 100, Carl Zeiss). Red and green signals of 30 randomly selected cell nuclei in each of the two distinct microscopic areas on each slide were collected. The ratio of the average HER2 number (red signal) to that of CSP 17 (green signal) was calculated. According to the ASCO/CAP guidelines, a ratio less than 1.8 was reported as non-amplification, a ratio higher

Table 1. Clinical and histopathologic characteristics of patients, and correlation with Her-2 status

Parameter	Number of patients n (%)	HER2 amplification by FISH		p (X^2)
		Non-amplified n (%) 707	Amplified n (%) 325	
Age (years)				
≤ 50	560 (54.3)	395 (55.9)	165 (50.8)	0.127 (2.334)
> 50	472 (45.7)	312 (44.1)	160 (49.2)	
Tumor size (cm)				
< 2	189 (18.3)	145 (20.5)	44 (13.5)	0.000 (29.893)
2-5	532 (51.6)	374 (52.9)	158 (48.6)	
> 5	245 (23.7)	132 (18.7)	113 (34.8)	
Not known	66 (6.4)	56 (7.9)	10 (3.1)	
Histological grade				
I	44 (4.3)	24 (3.4)	20 (6.2)	0.123 (4.192)
II	578 (56.0)	401 (56.7)	177 (54.5)	
III	410 (39.7)	282 (39.9)	128 (39.3)	
LN metastasis				
Positive	407 (39.4)	278 (39.3)	129 (39.7)	0.910 (0.013)
Negative	625 (60.6)	429 (60.7)	196 (60.3)	
Number of LNs				
≤ 3	837 (81.1)	571 (80.8)	266 (81.8)	0.680 (0.170)
> 3	195 (18.9)	136 (19.2)	59 (18.2)	
Population				
Han	788 (76.4)	525 (74.3)	263 (80.9)	0.019 (5.480)
Uighur	244 (23.6)	182 (25.7)	62 (19.1)	

LN, lymph node.

Table 2. Comparison of concordance between IHC and FISH in detecting HER-2 status

IHC (%)	Cases	FISH		Concordance
		Positive n (%)	Negative n (%)	
0 (30.9)	319	0 (0)	319 (100)	100% (319/319)
1+ (32.9)	340	32 (3.1)	308 (96.9)	90.6% (308/340)
2+ (17.4)	180	116 (11.2)	64 (88.8)	64.4% (116/180)
3+ (18.7)	193	177 (82.8)	16 (17.2)	91.7% (177/193)
100	1032	325 (31.5)	707 (68.5)	

than 2.2 was reported as amplification, and a ratio between 1.8 and 2.2 was considered equivocal. For the equivocal cases, a recounting of additional 40 cell nuclei (a total of 100) was done or a new FISH test was performed until a conclusive result was obtained.

Statistical evaluation

All statistical calculations were performed with the SAS (Statistical Analysis System) software version 9.3. Rate between groups was compared by chi-square test. Paired comparison tests were used to analyze the difference between IHC and FISH detection. The sensitivity was analyzed by Fisher exact probability. The Kappa constant was used to evaluate the concordance between IHC and HER2 detection. Kappa criteria were as follows: < 0 indicated no agreement; 0-0.2 slight agreement; 0.21-0.4 fair agreement; 0.41-0.6 moderate agreement; 0.61-0.8 substantial agreement; and 0.81-1 almost perfect agreement. $P < 0.05$ was considered statistically significant.

Results

Clinical and histological parameters

All patients were females with a mean age of 50 years (range: 16 to 90 years). Twenty-six patients were 635 years, while 54.3% were ≤ 50 years of age, 45.7% were > 50 years of age. The tumor size was < 2 cm in 18.3% of all cases, was between 2 and 5 cm in 51.6%, and

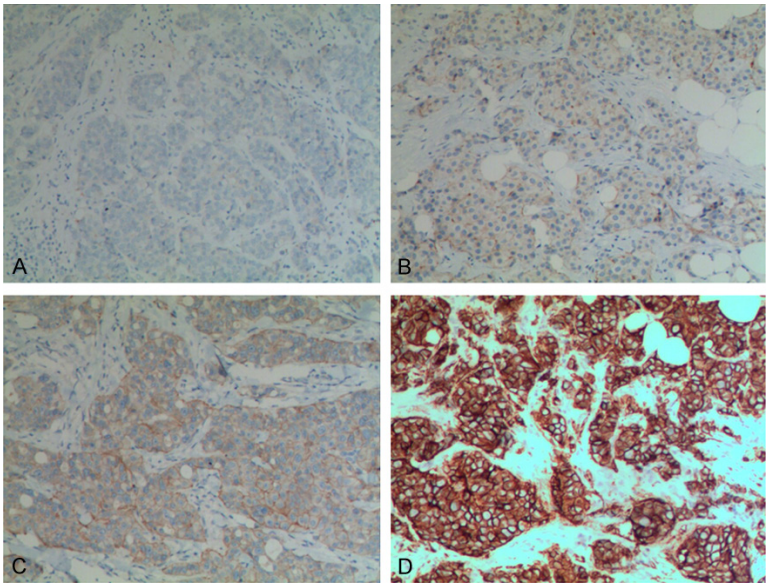

Figure 1. Immunohistochemical staining to detect HER2 protein expression in breast cancer specimens. HER2 positive expression is identified when uniform yellow-brown granules are found in the cell membranes. HER2 positive expression is classified into 4 levels according to the proportion of positive expression cells and magnitude of stain. A: 0, no staining; B: 1+, any percentage of infiltrating carcinoma cells showing partial membrane staining or weak staining; C: 2+, weak or moderate but not uniform and complete cell membrane yellow-brown staining in greater than 10% of the infiltrating carcinoma cells, or strong complete brown staining of the membranes in less than 30% of the infiltrating carcinoma cells; D: 3+, strong complete brown staining of the membrane in greater than 30% of the infiltrating carcinoma cells (SP stain, hematoxylin counter-stain, 20×).

> 5 cm in 23.7%. As regards histological grade, 4.3% of cases were grade I, 56.0% were grade II, and the remaining 39.7% were grade III. While lymph node metastasis was negative in 625 cases (60.6%), but present in 407 cases (39.4%). While cases with number of lymph nodes (LNs) ≤ 3 represented 81.1% of call cases, LN > 3 was reported in 18.9%. Among the patients, 76.4% were Han population, and 23.6% were Uighur minority.

IHC evaluation

IHC evaluation was performed on all the 1032 breast cancer specimens. As shown in **Table 2**, 319 cases (30.9%) were negative 0 (**Figure 1A**), 340 (32.9%) negative 1+ (**Figure 1B**), 180 (17.4%) weakly positive (2+, **Figure 1C**), and 193 (18.7%) strong positive (3+, **Figure 1D**).

FISH evaluation

The FISH interpretation criteria indicated HER2/CSPI7 < 1.8 as non-amplification and HER2/CSPI7 > 2.2 as amplification. As shown in **Table 2**, 707 out of the 1032 cases (68.5%) were non-amplified (**Figure 2A**), and 325 (31.5%) showed HER2 gene amplification by FISH (**Figure 2B**).

Comparison of HER2 status by IHC and FISH

The IHC and FISH results were compared using FISH as the gold standard. As shown in **Table 2**, the IHC negative 0 (30.9%), negative 1+ (32.9%) and IHC 3+ (18.7%) cases had a high concordance rate of 100%, 90.6% and 91.7%, respectively. In contrast, the IHC weakly positive cases (2+) had a low concordance rate (64.4%).

The results were further analyzed by combining the IHC 2+ and 3+ cases as positive cases. Comparison between IHC positive cases and FISH amplified cases showed that 78.6% (293/373) of HER2 positive specimens showed HER2 gene amplification and 95.1% (627/659) HER negative cases were HER2 non-amplified, indicating a high concordance between the IHC and FISH in detecting the HER2 status (**Table 3**). The kappa analysis yielded a kappa score of 0.7581 ($P < 0.0001$), indicating a substantial agreement between the two methods.

Further, the concordance between the IHC and FISH was analyzed in the Han and Uighur population, respectively. As shown in **Table 4**, the IHC negative 0 and FISH had a high concordance rate of 100% in both populations. The IHC negative 1+ and positive 3+ also had similar concordance rate (89.0% vs. 94.7% and 91.0% vs. 94.6%) in Han and Uighur population, respectively. The IHC weakly positive cases (2+) had a low concordance rate of 63.1% and 71.0% in Han and Uighur population, respectively.

Correlation between HER2 status and patient parameters

The correlation between HER2 status and various histopathologic parameters was analyzed.

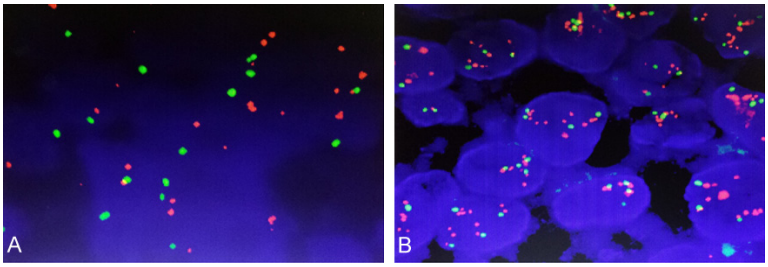

Figure 2. Detection of HER2 gene amplification by FISH. A: Chromosome 17 diploid cells, HER2/CSPI7 < 1.8 shows no HER2 overexpression in the specimen (1000×); B: Chromosome 17 diploid cells, HER2/CSPI7 > 2.2 shows HER2 overexpression in the specimen (1000×).

Table 3. Comparison between IHC and FISH in detecting HER-2 status

IHC	FISH		Kappa score, *p*
	Positive n (%)	Negative n (%)	
Positive (2+, 3+)	293 (78.6%)	80 (21.4%)	0.7581, $P < 0.0001$
Negative (0, 1+)	32 (4.9%)	627 (95.1%)	

As shown in **Table 1**, HER2 status showed significant correlation with tumor size ($P = 0.000$), but was not associated with age, histological grade, LN metastasis, or the number of LNs (all $P > 0.05$). Moreover, the proportion of Uighur patients in HER2 non-amplified cases was significantly lower compared with amplified cases ($P = 0.019$), suggesting that the Uighur minority had a lower HER2 amplification rate compared with Han population.

Discussion

The occurrence and progression of breast cancer is closely related to the expression of the HER2 gene [17, 18]. Breast cancers which are HER2 positive (protein overexpression or gene amplification) represent cancer types with special biological behavior and clinical features. Therefore, the therapy method against those cancers is different from the treatment of other breast cancer types. The US Food and Drug Administration (FDA) has approved the application of humanized anti-HER2 monoclonal antibody trastuzumab for HER2 protein overexpression patients. An accurate evaluation of the HER2 gene status is guarantying that patients will benefit from trastuzumab treatment.

The IHC and FISH methods are two main HER2 status detection techniques approved by the US FDA. The National Comprehensive Cancer Network (NCCN) guidelines recommend the IHC as the screening test, and FISH as the gold standard for determining HER2 status. In this study, we found that patients with IHC 3+ presented a 91.7% amplification rate detected by FISH. This value for IHC 2+ patients was 64.4%, for IHC 1+ it was 90.6%, and for IHC 0 it was 91.7%. These results point to big variations when comparing the HER2 status detection concordance between IHC and FISH. Some researchers have reported low overall concordances between IHC and FISH with values ranging from 27.1% to 44.4% [19, 20]. Others have found results similar to ours that the concordance between IHC and FISH for IHC 3+ samples (91.7%) is much higher than the concordance for IHC 2+ samples (64.4%) [21]. Many factors have been described that may affect the detection of HER2 status [22, 23], such as IHC tissue fixation method and type of McAb (monoclonal antibody), the stability and repeatability of IHC as a semi-quantitative measurement, and the dependence on the examiner.

Xinjiang is an Autonomous Region in China with Uighur ethnic minority and Han population as the main residents. This research aimed at establishing the concordance between IHC and FISH in detecting the HER2 status of breast cancer patients in Xinjiang province. The concordance rate between IHC 0/1+ and FISH was the highest (95.1%); and for IHC 3+ and IHC 2+, it was 91.7% and 64.4%, respectively. This assessment agreed with previous evidence showing a concordance of 93.9% for IHC 3+, 66% for IHC 2+ and 85.9% for IHC [24]. Given the high concordance between IHC (0/1+ and 3+) and FISH, IHC can be used alone as a screening test of HER2 status in these cases. However, due to the poor concordance between IHC 2+ and FISH, for cases with a HER2 IHC 2+ result, it is better to perform a FISH test afterwards in order to rule out the possibility of a wrong assessment of HER2 status. Although FISH are considered as the gold standard for determining HER2 status, its accuracy may be

Table 4. Comparison of concordance between IHC and FISH in detecting HER-2 status in Han population and Uighur population in Xinjiang

IHC (%)	FISH							
	Han population				Uighur population			
	Cases	Positive (%)	Negative (%)	Concordance (%)	Cases	Positive (%)	Negative (%)	Concordance (%)
0 (30.9)	237	0 (0)	237 (100)	100 (237/237)	82	0 (0)	82 (100)	100 (82/82)
1+ (32.9)	246	27 (11.0)	219 (89.0)	89.0 (219/246)	94	5 (5.3)	89 (94.7)	94.7 (89/94)
2+ (17.4)	149	94 (63.1)	55 (36.9)	63.1 (94/149)	31	22 (71.0)	9 (29.0)	71.0 (22/31)
3+ (18.7)	156	142 (91.0)	14 (9.0)	91.0 (142/156)	37	35 (94.6)	2 (5.4)	94.6 (35/37)
	788	263 (33.4)	525 (66.6)		244	62 (25.4)	182 (74.6)	

affected by factors such as the polysomy of chromosome 17 (where HER2 is located). It has been reported that FISH for HER2 on chromosome 17 polysomy cells yields false-positive results [25]. Hence, for these patients, IHC assessment is needed to determine the HER2 status, or alternatively, use of the HER2/CEP 17 ratio is recommended to prevent an incorrect evaluation by FISH.

We evaluated the correlation between HER2 gene amplification with clinicopathologic parameters in breast cancer patients in Xinjiang. It was shown that HER2 status was significantly correlated with tumor size, but was irrelevant to age, histological grade, LN metastasis, or the number of LNs. Our findings are consistent with previous studies [26]. More importantly, we found that the Uighur minority had a lower HER2 amplification rate compared with Han population. To our best knowledge, ours is the first report comparing the HER2 status in Uighur minority and Han population in Xinjiang province.

In conclusion, HER2 status was significantly correlated with tumor size. The Uighur minority had a lower HER2 amplification rate compared with Han population in Xinjiang province. IHC can be used as an initial screening test of HER2 status for both populations in Xinjiang province. However, for cases with a HER2 IHC 2+ result, it is better to perform a FISH test for confirmation. Our study has great clinical significance for accurate assessment of HER2 status in order to provide a more accurate anti-HER2 target therapy for HER2 positive breast cancer patients in the Xinjiang province.

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Disclosure of conflict of interest

None.

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