RESEARCH COMMUNICATION

Her2 Amplification Status in Iranian Breast Cancer Patients: Comparison of Immunohistochemistry (IHC) and Fluorescence *in situ* Hybridisation (FISH)

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Abstract

Introduction: Her2/neu is a biomarker which is amplified and/or overexpressed in a subset of breast cancer patients who are eligible to receive trastuzumab. Her-2 gene amplification analysed by fluorescence in situ hybridisation (FISH) and/or protein over-expression detected by immunohistochemistry (IHC) are the two main methods used to detect Her-2 status in clinical practice. The concordance rate between the two techniques is controversial. Methods: FISH analysis were performed on 104 tumoural samples from breast cancer patients with known IHC results to determine the Her2 gene status. The FISH/IHC analyses results were then compared and the concordance rate was determined. Results: Her2 gene amplification was detected in 0 of IHC score 1+, 24/86 (27.91%) 2+, and 8/13 (61.54%) 3+. The IHC and FISH results concordance rates were 100%, 27.9%, and 61.5% for IHC scores of 1+, 2+, and 3+ respectively. Conclusion: The results of this study suggest that IHC 1+ should be considered as negative while IHC 2+ results need further confirmative analysis by FISH. Further quality control and standardization of IHC technique are required to improve the concordance rate between the two methods.

Keywords: Breast cancer - Her2 - Trastuzumab - immunohistochemistry - FISH

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Introduction

Breast cancer is the most common cancer in women and the second most common in total worldwide. It is the leading cause of cancer-related death in women (Ferlay et al., 2010). Identification of novel molecular markers will improve the diagnosis and therapeutic interventions.

ERBB2 (commonly referred to as Her2) is a prognostic and predictive biomarker in breast cancer which is amplified and/or overexpressed in 20-25% of breast cancer patients (Owens et al. 2004). It belongs to the tyrosine kinase family of proteins and is located on the chromosome 17q21 (Slamon et al. 1987). Her2 becomes activated through homo- or heterodimerisation with other epidermal growth factor receptors (EGFR) and subsequently activates downstream signalling via the phosphatidylinositol 3-kinase (PI3K)-AKT and the mitogen-activated protein kinase (MAPK) pathways; which in turn affect cellular processes including proliferation and survival (Wieduwilt and Moasser, 2008; Hynes and MacDonald, 2009).

Several genetic alterations in the Her2 gene can lead to the constitutive activation of the receptor (Hynes and MacDonald, 2009). However, Her2 gene amplification and subsequent receptor overexpression is the main alteration in the majority of breast cancer patients (Pauletti et al., 1996). Tumours overexpressing Her2 are less differentiated, demonstrate a high proliferative capacity, and are associated with more aggressive features and poor prognosis (Slamon et al., 1987). Her2 overexpression and/or amplification determine the patient response to conventional chemotherapy regimens (Ménard et al., 2001; De Laurentiis et al., 2005; Pritchard et al., 2006; Villman et al., 2006). More importantly, only Her2 overexpressing breast cancer patients benefit from and thus are eligible to receive anti-Her2 targeted antibody (Cobleigh et al., 1999; Piccart-Gebhart et al., 2005; Smith et al., 2007). The humanised monoclonal antibody against extracellular component of Her2, trastuzumab (Herceptin®, Genentech, South San Francisco, CA) is only indicated in Her-2 positive breast cancer women. American Society of Clinical Oncology/ College of American Pathologists (ASCO/CAP) recommends that the status of Her2 be accurately determined in all newly diagnosed breast cancer patients (Harris et al., 2007; Wolff et al., 2007). Immunohistochemistry (IHC) and fluorescence in situ hybridisation (FISH) are the most frequently used methods in the clinical setting.

IHC evaluates the Her2 protein expression on the tumoural cell surface. It is a relatively low cost

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technique which is routinely available in the community pathology laboratories. However, there are several drawbacks namely: factors that can affect the test result, the subjective interpretation of the experiment and a wide range of false positive rate (Pauletti et al., 2000; Tubbs et al., 2001). FISH detects the Her2 copy number through genetic methods whereby a fluorescent-labelled oligonucleotide probe anneals to the DNA region of interest in a sequence specific manner. Thus it yields a nonambiguous result which is less variable among different observers. ASCO/CAP guideline suggests IHC to be performed as a screening test and FISH to be reserved for patients with equivocal IHC results. However, this requires a 95% concordance between IHC and FISH results (Wolff et al., 2007). High concordance rate between the two tests has already been reported when a central laboratory is responsible for performing IHC (Couturier et al.,2000; Roche et al., 2002; Press et al., 2005). However, the data on the performance of these tests in the setting of community, low volume laboratories are scarce (Phillips et al., 2009).

The aim of this study was to establish a central laboratory to examine Her2 gene status in newly diagnosed primary or recurrent invasive breast cancer patients by means of FISH. Secondly, we evaluated the concordance rate of IHC and FISH results in the community clinical practice setting.

Materials and Methods

Between September 2004 and October 2010, 104 cases of breast cancer were referred to the molecular cytogenetic laboratory for evaluation of the Her2 gene status by FISH. They were referred by external laboratories or their physicians. One case was referred with the impression of tumour of unknown origin; who was excluded from this study. The immunohistochemistry assessment of Her2 protein had been performed in various community laboratories and the results were reported as 1+ (negative), 2+ (weakly positive), and 3+ (positive).

Her2 gene status was examined in all patients by fluorescent hybridisation in situ (FISH) on formalin-fixed paraffin-embedded specimens using Repeat-free™ POSEIDON™ probes (Kreatech Diagnostics, The Netherlands) following the manufacturer's instructions with minor modifications. For each patient, 2 positively charged slides, each mounted with a 4-μm tissue section, were received. One slide had been previously stained with haematoxylin and eosin (H & E) by a pathologist. The area of tumoural cells was identified on the H & E stained slide and marked on the FISH slide. Slides were baked at 56ºC for 2 hours, deparaffinised in two 10 minute changes of xylene, and dehydrated in successively decreasing concentration of ethanol washes. Subsequently, the slides were washed in double distilled water (DDW) for 3 minutes at room temperature.

The slides were then immersed in 0.2 M hydrochloric acid at room temperature for 20 minutes, washed in DDW for 3 minutes at room temperature, immersed in 8% sodium thiocyanate at 80°C for 30 minutes and finally washed in 2x sodium saline citrate (SSC; pH 7.0) for 3 minutes. The tissue sections were then covered with pepsin solution at room temperature for 50 minutes, washed in DDW for 1 minute at room temperature, in 2x sodium saline citrate (pH 7.0) for 5 minutes and finally were dehydrated through 70%, 85% and 100% ethanol series. They were left to dry at room temperature.

After pretreatment, 10μl of probe was applied onto each slide. The cover slip was placed and sealed. The slides were heated at 80° C for 7 minutes and they were incubated at 37°C overnight. The following day, the slides were washed in 72° C preheated 0.4x SSC/Tween 20 solution for 2 minutes and then in 2x sodium saline citrate (2x SSC; pH 7.0) for 1 minutes. Slides were dehydrated through ethanol series and left to dry at room temperature. After hybridisation overnight, the slides were counterstained with 15 μl (1 μg/ml) 4',6- diamidino-2-phenylindole dihydrochloride (DAPI).

Microscopy was performed with Leica DM 6000B (Leica Microsystems, Germany). Leica CW4000 software was used for image analysis. Fifty invasive tumour cells were located and the ratio of average Her2 signals/CEP17 probe signals were determinedand a ratio of more than 5:2 was interpreted as positive.

Results

One hundred and four cases of breast cancer patients were analysed in this study (103 female, 1 male). The median age of patients was 48 years (27-79). The patients were diagnosed at varying clinical stages. Tumours were histologically classified as 82 invasive ductal carcinomas, 3 medullary carcinomas, 9 lobular/tubulolobular carcinomas, and 10 others. One hundred and one cases were primary breast cancer and 3 cases were recurrent or metastatic. Pathological tumour grading was reported in 73 cases: 9, 42, and 22 were reported as having grade 1, 2, and 3 respectively. Axillary lymph node involvement was reported in 49 cases.

All patients had an IHC assessment of the Her2 protein overexpression. Overall, 5 (4.81%), 86 (82.69%), and 13 (12.50%) had IHC score of 1+ (negative), 2+ (weakly positive), and 3+ (positive) respectively. FISH was performed in all cases. FISH was positive in 0 (0%) 1+, 24 (27.91%) 2+, and 8 (61.54%) 3+ IHC results. FISH negative cases were re-evaluated for chromosome 17 polysomy as defined by CEP17>2.25. No case of polysomy of the chromosome 17 was identified in the FISH negative group. Five (38.5%) cases of IHC 3+ were FISH negative. Concordance between IHC and FISH results is defined as 1) IHC negative (1+) and

Table 1. Concordance Rate between IHC and FISH Results for Her2/neu in Breast Cancer Patients

		FISH		Concordance
		Negative	Positive	Rate
IHC	1+	5	0	5/5 (100%)
	2+	62	24	24/86 (27.9%)
	3+	5	8	8/13 (61.5%)

IHC, immunohistochemistry; FISH, fluorescence in situ hybridisation

FISH negative or 2) IHC positive (2+ or 3+) and FISH positive. In this study, the concordance rate was 100% in 1+, 27.91% in 2+, and 61.54% in 3+ IHC scores (Table 1).

Discussion

Accurate and reproducible detection of Her2 amplification and/or overexpression is crucial in breast cancer patients as to determine their future course of treatment. Gene amplification is the cause of protein overexpression in the majority of cases. However, in approximately 3% of Her2 positive breast cancers, the protein overexpression is achieved by means other than gene amplification. It is generally argued that detection of gene amplification by using in situ hybridisation techniques such as FISH is the most accurate. Nevertheless, to detect the 3% of the patients without gene amplification, it is necessary to still perform protein analysis methods such as IHC.

This study was performed in the setting of community clinical practice and compared the concordance rate of IHC, performed by multiple local laboratories to FISH results performed by a referral centre. We identified Her2 gene amplification in 0/5 (0%), 24/86 (27.91%), and 8/13 (61.54%) breast cancer samples with IHC scores 1+, 2+, and 3+ respectively.

In a large cohort of breast cancer samples, Her2 gene amplification was identified in 11.5, 23.3, and 91.7% of IHC negative, 2+, and positive respectively (Owens et al. 2004). It is well known that IHC can have false positive and negative results due to technical inconsistencies. In this study we did not identify Her2 gene amplification by FISH in samples for which the IHC score was reported to be negative (1+). However, the discordance between IHC and FISH results are prominent in 3+ cases, with the false positive rate of 38.5%. When IHC is carried out in a central laboratory, a high concordance rate with the FISH results is attained (Paik et al. 2002; Dybdal et al. 2005). However, when IHC is performed by local laboratories, the concordance rate considerably drops. In accordance with our findings, the National Surgical Adjuvant Breast and Bowel Project (NSABP) Protocol B-31 trial showed that only 79% of locally scored as positive, scored 3+ when re-evaluated at a central laboratory. In addition, 18% were scored as 0 or 1+ and did not show any gene amplification by FISH (Paik et al. 2002). In the Breast Cancer International Research Group (BCIRG) study, Her2 gene was amplified in 6.1, 16.7, and 77.1% of 1+, 2+, and 3+ IHC scores (Press et al. 2005). Likewise, the Breast Intergroup Trial N9831 also revealed a low concordance of IHC and FISH results between local versus central laboratory (Roche et al. 2002). Dolan and Snover examined 129 breast cancer samples for which IHC had been performed at local laboratories. Concordant IHC and FISH results were observed in 100, 8, and 38% in scores 1+, 2+, and 3+ respectively (Dolan and Snover 2005). It is noteworthy that there is a certain degree of discrepancy even among the expert, high volume laboratories. In a multi-centre study where 20 breast cancer samples were evaluated by five internationally well known laboratories, only 9 were scored similarly in IHC. More importantly, for 11 samples there was at least one definite IHC score (either positive or negative) while others assessed them as weakly positive (2+). Strikingly, for 3 out of 9 samples with IHC score of 2+, there was a conflicting FISH results from different centres. All three had a borderline FISH score (Dowsett et al., 2007). This study, further demonstrates the great inter-laboratory variability of IHC and to a lesser extent FISH results especially when the score is borderline.

The cutoff point to determine the FISH positive result can influence the number of Her2 positive cases identified. Owens et al. have observed that the mean Her2/CEP17 ratio is lowest among FISH positive IHC negative cases and highest in the IHC positive cases. The most discriminatory difference is when the cutoff point is the Her2/CEP17 ratio of more than five. In this case less than 5, 20, 40, and 80% of IHC 0, 1+, 2+, and 3+ are considered positive (Owens et al., 2004). Perez et al. defined the borderline Her2/CEP17 ratio (1.30-2.00) as Her2 gene duplication. They performed FISH for samples with equivocal IHC score. They observed that 25% had gene duplication and 12% had high levels of gene amplification (Perez et al., 2002). However, the relevance of the level of Her2 gene amplification/protein overexpression to the clinical response to targeted therapies is unclear. Nevertheless, it is plausible that patients should be further stratified according to their level of gene amplification/protein overexpression.

In our study, the majority of cases who were referred for FISH analysis were IHC 2+. Due to the limited concordance rate in the group of IHC 3+ in the setting of local laboratories, it is advisable and cost effective to confirm the Her2 positivity by FISH analysis untill the establishment of strictly quality controlled and audited central laboratories.

As more personalised approaches to cancer diagnosis, prognosis, and treatment are emerging; it is of utmost importance to well integrate the advanced technologies in the routine clinical practice. Trastuzumab therapy in Her2 positive breast cancer patients as an example should lead to better understanding of how to stratify patients and how best to evaluate them. In addition, Her2 oncoprotein has been reported to be amplified and/or overexpressed in a multitude of other malignancies (Moelans et al., 2011). It is essential to have a foresight in determining the infrastructures that would presumably be required to assess Her2 amplification and/or overexpression in other settings. A genetic approach with a reproducible and more accurate result, such as FISH, can be easily optimised and employed in those circumstances.

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