RESEARCH ARTICLE

Determination of HER2 Gene Amplification in Breast Cancer using Dual-color Silver Enhanced *in situ* Hybridization (dc-SISH) and Comparison with Fluorescence ISH (FISH)

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Abstract

Background: The two basic methods that are currently accepted to identify the HER2 status are immunohistochemistry and flyorescence *in situ* hybridization (FISH) . The aim of this study was to perform the dual-color silver *in situ* hybridization (dc-SISH) technique as an alternative to FISH. Materials and Methods: A total of 40 invasive breast carcinoma cases were assessed for HER2 gene amplification by FISH and dual-color SISH. Results: Significant correlation was found in the HER2 expression results obtained with the two approaches (p=0.001, p<0.05). The concordance rate was 92.3%. Conclusions: Foutine practical use of the dc-SISH method, which is much easier to apply, score, and evaluate, has many advantages. HER2 and CEN17 status can be evaluated simultaneously with the newly developed "Dual-Color Probe". All these specifications and the reliable results obtained support the widespread use of SISH technique in clinical practice.

Keywords: Dual color SISH - breast cancer - Her2

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Introduction

Amplification of the HER2/neu gene in 18-20% of patients with breast cancer produces the primary mechanism of HER2 protein overexpression (Penault-Llorca et al., 2009; Thang et al., 2011; Dekker et al., 2012). The HER2 gene is a tumor marker associated with rapid tumor growth, increased recurrence rate after surgery, and decreased life expectancy (Penault-Llorca et al., 2009; Thang et al., 2011). HER2 positivity has a powerful predictive value related to a good response to anthracycline-based chemotherapy and a poor response to hormonal therapy (Slamon et al., 1987; Paik and Park, 2001). Trastuzumab is a human monoclonal antibody specifically developed against HER2. Sensitive, reliable and the most accurate identification of the HER2 gene is crucial in the selection of patients who will benefit from trastuzumab therapy, since the response to the treatment is directly related to HER2 overexpression. As a result, HER2 amplification in neoadjuvant therapy is directly related to the response to trastuzumab therapy. Baseline HER2/neu status identification with protein and/or genomic status is performed with immunohistochemistry and in situ hybridization (ISH), respectively (Dietel et al., 2007; Carbone et al., 2008; Francis et al., 2009; Bilous et al., 2012). The gold standart for HER2 gene copy number evaluation is considered to be FISH . Dual-color silver in situ hybridization (dc-SISH) technique as an alternative to the fluorescence in situ hybridization (FISH) method, which is a generally accepted and commonly used in situ hybridization technique, and to compare the results of each method. HER2 and CEP17 status can be evaluated simultaneously with the newly developed "Dual-Color Probe". This method enables calculation of HER2/CEP17 ratios and detection of chromosome 17 polysomy on a single slide. The primary aims of this study are the introduction of the routine use of the SISH method, which has many convenient technical methods, and to evaluate and select the best actual patients to benefit from the trastuzumab treatment, which has a very important therapeutic role in invasive breast cancer.

Materials and Methods

Case selection

Forty patients with the diagnosis of "invasive ductal carcinoma" were included in the study. The fluorescence in situ hybridization (FISH) technique and the silver in situ hybridization (SISH) technique were applied to the same cases, which were identified as Her2 (+2) through immunohistochemical evaluation, and the results were compared.

FISH

A HER2 fluorescent probe kit (Kreatech) was used to identify HER2 gene amplification. Paraffin sections

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in 4 micron thicknesses were warmed to 80°C and incubated for 30 minutes in 1 mol/L of NaSCN solution after deparaffinization and dehydration, followed by a 20 minutes standby in a pepsin solution at 37°C. The microscope slides were passed through an ethanol series (70%, 85%, and 100% alcohol) for dehydration. Slides were air dried. Ten microliters of probe were instilled and closed with a lamella, then fixed with rubber cement. The glass slides were incubated for 16-20 hours in 37°C in a moist hybridization box following 5 minutes of co-denaturation at 80°C. The glass slides were kept in a 2×SSC/0.3% NP-40 solution at room temperature for 2 minutes and then in a 2×SSC/0.3% NP-40 solution at 72°C for 2 minutes. Later, they were passed through the ethanol series for dehydration. The slides were air dried and closed with a solution formed of DAPI (4, 6-diamidino-2-phenylindole) and antifade (Phenylenediamine) compounds to count the nuclei. The counting of the FISH signals specific to each locus (HER2/CEP 17) was performed with the appropriate filters (DAPI/Orange/Green). A count of 50 or more cells that do not superpose was performed. The presence of amplification was identified if the rate of HER2/CEP 17 was equal to or greater than 2.2, and amplification was deemed negative if this rate was less than 2.2.

SISH

SISH staining was performed with an automated slide stainer (Ventana Medical Systems, Tucson, AZ, USA) by taking sections in 4 μm from the most appropriate block selected for HER2 study. SISH protocol was as follows: deparaffinization, citrate treatment, and incubation with ISH protease, addition of HER-2 DNA and/or CEP17 probe and incubation for hybridization, incubation with Silver C, incubation with Hematoxylin as contrast dye, and incubation with bluing after contrast dyeing. Incubation periods with proteases and DNA probes were adjusted for each material for the optimal time period that the signals would be visible with preserved tissue morphology. Evaluation of the tissues with SISH application was performed under the light microscope, with a 40× objective. A tumoral field with higher signal deposit was selected for evaluation and HER2 and centromere 17 signals were counted separately in 20 cell nuclei. The proportion of these values found was then calculated. Single signal, minor signal cluster, and major signal clusters were counted as 1, 6, and 12, respectively. The total HER2 score was divided in the total CEP 17 score and the HER2/CEP 17 proportion was calculated. Evaluations were performed as shown in Table 1. If the value was between 1.8 and 2.2, another group of 20 cells was also counted and the proportion is calculated again. This re-counting process was repeated until a clear result was achieved.

Statistical evaluation

Data was analyzed using SPSS program (version 18.0) (SPSS Inc., Chicago, USA). Frequency distribution and percentages were used to describe the sample. Values of the two techniques were shown in crosstabs with frequencies and percentages. The associations of the FISH and SISH groups were analyzed with chi-square tests. A confidence interval of 95% (α=0.05 error rate) was used to define the differences ($p<\alpha$). A value of $p<0.05$ was accepted as significant. The correlations of the results of FISH and SISH techniques were analyzed.

Results

Among the cases analyzed with the FISH technique, the number of negative cases (i.e. HER2/CEP17 less than 1.8) were identified as 31 (79.5%) and the number of positive cases (i.e. HER2/CEP17 greater than 2.2) were identified as 8 (20.5%). The SISH technique was applied to all cases to find the HER2/CEP17 rate and 28 (71.8%) patients with a rate less than 1.8 and 11 (28.2%) patients with a rate greater than 2.2 were identified (Table 2). No signals were received in one patient because of damage to the specimen during follow-up, and this case was excluded from the study. Twenty-eight (71.8%) of the FISH negative cases were SISH negative, while 3 (7.7%) were SISH positive. Among all of the 8 FISH positive cases (0.5%), positive test results were obtained with SISH technique as well. The correlation of the FISH and SISH techniques was analyzed with a chi-square test and a statistically significant correlation was found in the HER2 expression results obtained by the two groups ($p=0.001$, $p<0.05$). The concordance rate between two methods was 92.3%.

Table 1. Evaluation of HER2 Amplification

HER 2/CEP 17 ratio	Amplification
<1.8	absent
>2.2	avaliable
1.8-2.2	again*

*This re-counting process was repeated until a clear result was achieved

Table 2. HER2 Scoring using FISH Versus SISH

		SISH		Total
		Negative	Positive	
FISH	Negative	28 (71.8%)	3 (7.7%)	31 (79.5%)
	Positive	0 (0%)	8 (20.5%)	8 (20.5%)
Total		71.8%	28.2%	100.0%

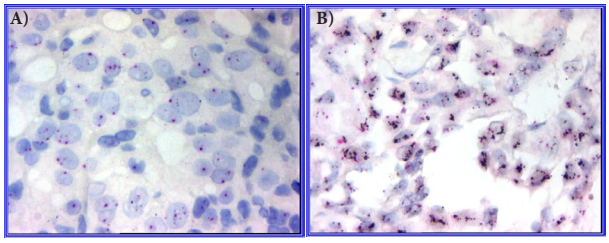

Figure 1. HER Gene Amplification A) Negative and B) Positive, (dc-SISH, x400)

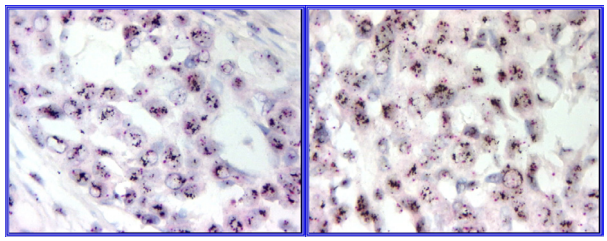

Figure 2. HER Gene Amplification Positive, Small and Large Clusters, (dc-SISH, x400)

Discussion

The human epidermal growth factor receptor 2 (HER2) is a valuable gene, with its amplification associated with aggressive disease and poor prognosis, which identifies the prognosis in cases with invasive breast cancer (Dekker et al., 2012; Lee et al., 2012). HER2/neu oncogene codes the trans-membrane protein that has tyrosine kinase activity (Thang et al., 2011). HER2/Neu (cerbB2), a member of the epidermal growth factor receptor family, which plays an important role in breast cancer pathogenesis, is localized in the long arm of the 17th chromosome (17q11.2-q12). HER2, which is a 185kDa transmembrane growth factor receptor with tyrosine kinase activity, has a signal transduction role in cell coupling. The protein 185kDa is expressed in nonneoplastic mamary epithelial cells and other normal cells. This protein creates tyrosine kinase activation when it is overexpressed in carcinoma cells and results in increased mitogenic signal transduction. The protein is overexpressed as a result of HER2/neu gene amplification, thus the normal complement of each gene for each chromosome 17 is increased (Coussens et al., 1985; Slamon et al., 1989; Gschwind et al., 2004). HER2 overexpression parallels poor prognosis, short survival and relapse, and is a sigificant marker of the response to anticancer treatment (Penault-Llorca et al., 2009; Park et al., 2011; Dekker et al., 2012).

Herceptin (trastuzumab) is a monoclonal antibody developed against HER2. The response to herceptin treatment is directly related to the HER2 status of the patient (Dietel et al., 2007; Thang et al., 2011). Therefore, it is imperative to identify the method that defines the HER2 status with the greatest safety; this issue remains controversial. The two basic methods that are currently accepted are immunohistochemistry and florescein in situ hybridization (FISH) to identify the HER2 status (Francis et al., 2009). Immunohistochemistry shows the HER2 protein expression on the cell surface, while FISH demonstrates the degree of HER2 gene amplification. Both methods have a high safety and specificty, provided that current protocols are followed.

Immunohistochemical staining results are easily affected by the variations of the method. Some of these variations are the storage conditions of the tissue samples, the specifications of the fixative used, duration of fixation and the antibody used (Penault-Llorca et al., 2009; Park et al., 2011; Dekker et al., 2012). The FISH technique is more quantitative compared to immunohistochemistry. It identifies the HER2 gene count associated with the copy of the chromosome 17 centromere. It is more stable than the DNA protein. Pre-analytic factors affect the test results less than immunohistochemistry. Nevertheless, the FISH technique is more expensive, it necessitates special equipment and educated personnel, its application process lasts longer, and a fluorescent microscope is required for the evaluation of the test (Francis et al., 2009; Papouchado et al., 2010; Ying et al., 2010; Arnould et al., 2012; Schiavon et al., 2012). The SISH method, which can be an alternative to the FISH technique, is comprised of a multimer technology, combined with enzyme metallography, which allows for the identification of the HER2 oncogene amplification with silver marking. The most important advantage of the SISH method is its ease of evaluation under a light microscope. Spot-like fluorescent signals are evaluated under highly sensitive fluorescent microscopes in the FISH method; while shining opaque silver is easily seen under the light microscope in the SISH method. Moreover, the results can be evaluated quickly and histopathological evaluation of the same tissue section can be performed at the same time (Francis et al., 2009; Papouchado et al., 2010; Ying et al., 2010; Lee et al., 2012; Schiavon et al., 2012).

Until now, some studies have been performed to compare FISH and SISH methods with a correlation ratio of 89-98% (Dietel et al., 2007; Carbone et al., 2008; Papouchado et al., 2010; Ying et al., 2010; Park et al., 2011; Lee et al., 2012; Tvrdík et al., 2012). A correlation ratio of 92.3% was found in this study between FISH and SISH.

The routine practical use of the SISH method, which is much easier to apply, score, and evaluate, has many advantages in many ways. Additionally, it presents the possibility of archiving and storing the preperates under room conditions for many years. Moreover, HER2 and CEP17 status can be evaluated simultaneously with the newly developed "Dual-Color Probe". All these specifications and the reliable results obtained support the widespread use of dual-color SISH technique in practice.

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