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Comparison of Chromogenic *In Situ* Hybridisation with Fluorescence *In Situ* Hybridisation and Immunohistochemistry for the Assessment of Her-2/neu Oncogene in Archival Material of Breast Carcinoma

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The successful treatment of breast cancer is dependent upon a number of complex factors. *Her-2/neu* gene amplification is known to be one of the most common genetic alterations associated with breast cancer and its accurate determination has become necessary for the selection of patients for trastuzumab therapy.

The aim of this study was to prove the consistency of chromogenic *in situ* hybridisation (CISH) technique after analyzing the overexpression of the *Her-2/neu* proto-oncogene in 100 invasive breast carcinomas and by comparing CISH results with immunohistochemistry (IHC) and fluorescence *in situ* hybridisation (FISH). Moreover, it was done to evaluate the possible correlation of estrogen (ERs) and progesterone receptors (PRs), the proliferation marker Ki67 and the tumour suppressor gene p53 with HER-2/neu status of these breast carcinomas.

Of the 100 breast carcinomas that were analysed, 22 cases showed HER-2/neu amplification, 66 cases showed no amplification, whereas 12 cases were non-interpretable in both assays (FISH and CISH). Consequently, the overall concordance between FISH and CISH was 100%. Additionally, it was observed that when HER-2/neu gene was overexpressed, there was an association with negative PRs and ERs status, negative p53 protein expression and high Ki67 labelling index.

It is concluded that patients with tumours scoring 2+ with the CBE356 antibody (borderline immunohistochemistry-tested cases) would also benefit from CISH as it is shown to be highly accurate, practical and can be easily integrated into routine testing in any histopathology laboratory. Finally, CISH represents an important addition to the HER2 testing algorithm.

Key words: breast cancer, CISH, FISH, *HER-2/neu*

I. Introduction

Transmembrane receptors play a major role in regulating cellular proliferation, differentiation, cell migration and cellular death. Changes in the structure and the expression of the genes that encode those receptors result in signal transduction disorders and contribute to tumorigenesis [20]. The oncogene HER-2/c-erbB-2 encodes a transmembrane tyrosine kinase growth factor receptor that belongs to the EGFR/HER family, which is comprised of 4 proteins known as HER-1/c-erbB-1, HER-2/c-erbB-2, HER-3/c-erbB-3, and HER-4/c-erbB-4. C-erbB-2 participates in a network of signalling when homodimerised or dimerised with other members of the erbB protein family. Amplification of the gene leads to amplified transcriptive activity, tumorigenesis and tumour metastasis.

Amplification or overexpression of HER-2/neu is seen

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in approximately 30% of invasive breast cancers and studies have shown that HER-2/neu overexpression is an adverse prognostic factor in patients with node positive disease [17]. HER-2/neu overexpression has been reported to be associated with positive lymph nodes, high histologic grade, high proliferation rate, lack of expression of estrogen and progesterone receptors, and shorter survival rates. There is also fairly consistent evidence that HER-2/neu overexpression is predictive of sensitivity to anthracyclines [5]. Additionally, HER-2/neu represents an ideal therapeutic target because it is accessible as a cell surface receptor and is expressed at high levels in breast tumours. A monoclonal antibody, known as trastuzumab, has been shown to be effective as a single agent in the treatment of patients with metastatic breast cancer, who failed to respond to treatment with chemotherapy [3]. Moreover, Herceptin® plus chemotherapy has been shown to be more effective than chemotherapy alone as first-line therapy in the metastatic setting [21]. The accurate testing of Her-2/neu by FISH ensures that costly and potentially toxic trastuzumab treatment will not be given to patients with no amplification of HER-2/neu proto-oncogene.

The aim of the present study was to validate whether CISH is a practical alternative technique to FISH for assessing *HER-2/neu* gene amplification in negative, positive and borderline immunohistochemistry-tested cases. The results were compared with FISH testing carried out on a series of the same 100 cases of breast carcinoma. Finally, it evaluated the possible correlation of ERs and PRs, the proliferation marker Ki67 and the tumour suppressor gene p53 with HER-2/neu status.

II. Materials and Methods

Patients

One hundred cases of invasive ductal breast carcinomas diagnosed between 2001 and 2007 were randomly selected from the pathology department of Helena Venizelou Hospital, Athens, Greece. The age of the women ranged from 34 to 80 years (mean age 59.2 years).

Immunohistochemistry

Immunohistochemical stainings were performed on 4 μm thick tumour sections after microwave antigen retrieval (0.01 M citrate buffer, pH 6.0 for 15 min) using the commercially available monoclonal antibodies to ER (1D5, 1:100 dilution; DAKO), PR (1A6, 1:20 dilution; Biogenex, San Ramon, CA), external domain of HER-2/neu (CBE356 mouse monoclonal antibody, clone 10A7, 1:200 dilution; Novocastra, Newcastle upon Tyne, UK), p53 (DO7, 1:50 dilution; DAKO), and Ki67 (MIB-1, 1:80 dilution; DAKO).

The staining for ER, PR, and p53 was classified as positive if more than 10% of the tumour cells exhibited nuclear overexpression and the proliferation index was determined by exactly measuring the percentage of Ki67 immunostained nuclei using the CAS 200 image analyzer.

Evaluation of HER-2/neu immunohistochemical expression was performed by semiquantitative scoring by BD (based on the scoring guidelines of DAKO) as follows: Score 0: no staining or membrane staining in <10% of tumour cells; Score 1+: faint membrane staining in >10% of tumour cells; Score 2+: weak—moderate complete membrane staining in >10% of tumour cells and 3+: strong, complete membrane staining in >10% of tumour cells. Scores of 0 and 1+ were considered as negative for HER-2/neu expression, 3+ as immunopositive, while 2+ were weakly or borderline positive.

Fluorescence in situ hybridisation

Paraffin-embedded tissue sections (4 μm thick) were analysed using FISH protocol (Vysis, Downers Grove, IL). The slides were deparaffinised in fresh xylene (3×, 5 min each), dehydrated in absolute ethanol and air dried. After several washes in 2×SSC, the tissue sections were incubated in 1 M NaSCN (pre-treatment reagent) at 80°C for 30 min. Cytoplasm surrounding the interphase nuclei was removed by protease digestion (protease solution) at 37°C for 10 min, increasing the accessibility of the probes to the targeted sequences and decreasing any background signals. The slides were then rinsed in dH_2O for 5 min and allowed to air dry.

The hybridisation mixture (including a centomere 17-specific, SpectrumGreen-labelled DNA probe and a HER-2/neu-specific, SpectrumOrange-labelled DNA probe) was applied to the pre-treated slides, a coverslip was added and the edges of the hybridisation area were sealed with rubber cement. To allow hybridisation, the slides were incubated for 16–24 hr in a humidified chamber at 37°C. After hybridisation, the slides were washed twice for 5 min each time in 0.05×SSC at 42°C. The slides were then rinsed in 2×SSC/0.3% NP-40 and embedded in mounting medium containing DAPI (0.5 μg/ml, Vysis) for nuclear counterstaining.

The slides were stored at –20°C until enumerated using Zeiss-Axioscope fluorescence microscope. At least 60 cells were scored in each slide and the copy numbers of HER-2/neu and CEP17 for each cell were recorded. HER-2/neu was quantified using the ratio of HER-2/neu to CEP17 signal counts. HER2/CEP17 ratio greater than 2 was interpreted as positive for gene amplification. Polysomy of chromosome 17 was defined as the presence of three or more CEP17 signals in >6% of the tumour cells evaluated.

Chromogenic in situ hybridisation

CISH was performed on 4 μm thick archived formalin-fixed paraffin-embedded tumour samples. Sections were deparaffinised 3× in fresh xylene for 5 min each, dehydrated in two changes of absolute ethanol for 5 min each and allowed to air dry. The slides were then incubated in pretreatment buffer (1 M sodium isothiocyanate; Vysis) for 13 min at 80°C, and were immediately washed with deionised water for 2 min. Enzymatic digestion was performed by incubating sections with protease (Vysis) for 2 min at 37°C. The slides were then washed with deionised water, dehydrated with a graded series of ethanol and air-dried. Fifteen microlitres of digoxigenin-labelled HER-2/neu probe (Zymed, South San

Francisco, CA, USA) were applied to the sections and the slides were denatured at 74°C for 5 min. After overnight hybridisation at 37°C, the slides were washed in 2×SSC/0.3% NP-40 (Vysis) at 73°C for 2 min, followed by three washes in distilled water. Then, the sections were blocked with 3% H_2O_2 in absolute methanol and incubated with a blocking reagent for 10 min at an ambient temperature. The hybridisation signals were detected after sequential incubations with mouse anti-DIG (60 min at room temperature), polymerised HRP (horseradish peroxidase) anti-mouse antibody (60 min at RT) and 3-3-diaminobenzedine (DAB), according to the manufacturer's instructions (Zymed). The sections were counterstained with hematoxylin.

The CISH hybridisations were evaluated using light microscope under a 40× objective. Unaltered gene copy number was defined as one to five signals per nucleus (verify chromosome 17 aneuploidy in cases with 3–5 copies of HER-2/neu gene/nucleus). Low level amplification was defined as 6 to 10 signals per nucleus or small gene clusters in more than 50% of tumour cells (verify true HER-2/neu amplification with absence of chromosome 17 aneuploidy). High level amplification of HER-2/neu was defined as numerous (>10) signals per nucleus or large gene clusters in more than 50% of tumour cells. It should be also noted that both low and high levels of amplification of the *HER-2/neu* was regarded as amplification-positive.

III. Results

HER-2/neu by IHC and correlation of IHC and FISH results

A total of 100 samples of invasive ductal breast carcinoma were analysed. HER-2/neu staining by IHC was graded as HER-2/neu negative (score 0 and 1+) in 55 tumours, 14 tumours had equivocal Her-2/neu status (2+) and 31 tumours were HER-2/neu positive (3+).

Of the 100 breast carcinomas, it was also observed that when HER-2/neu gene was overexpressed, there was an association with negative progesterone and estrogen receptor status, negative p53 protein expression and high Ki67 labelling index, although these correlations were not statistically significant (data not shown).

The correlation of IHC and FISH results of the 100 samples is presented in Table 1, where 12 cases were non-interpretable, due to high background and low signal intensity. Moreover, one negative case of 50, none of the 11 cases graded as 2+ and 21 out of the 27 3+ cases have shown gene amplification by FISH.

HER-2/neu by CISH and FISH

After CISH assay, 69 cases showed no gene amplification, whereas one of the 52 0/1+ cases, none of the 12 2+ and 21 of the 27 3+ were HER-2/neu gene amplified. The one case interpreted as 1+ (CISH positive) showed low-level gene amplification, whereas of the 21 cases interpreted as 3+ (CISH positive), 1 showed low-level gene amplification and 20 showed high-level gene amplification. Nine samples were not interpretable by CISH (Table 2). Of the 100 cases with invasive ductal breast carcinoma, which were analysed, 88 were assessable for FISH and CISH, 9 cases were not successful in either technique and 3 showed HER-2/neu non-amplification by CISH, while on FISH assay no hybridisation signals were detected. Of the 88 breast carcinoma, 22 cases showed HER-2/neu amplification in both assays, whereas 66 cases showed no amplification (Table 3). The overall concordance between FISH and CISH was 100%. Tumour specimens showing HER-2/neu amplification and non-amplification by FISH and CISH are shown in Figures 1 and 2.

Table 1. *Comparison between HER-2/neu amplification by FISH and overexpression by IHC/CBE356*

IHC/CBE356	FISH (PathVysion, Vysis)		
	Non-amplification	Amplification Positive	Non-interpretable
0/1+	49	1	5
2+	11	0	3
3+	6	21	4

* CBE356: mouse monoclonal antibody, clone 10A7 (Novocastra, Newcastle Upon Tyne, UK) to the external domain of HER2.

Table 2. *Comparison between HER-2/neu amplification by CISH and overexpression by IHC/CBE356*

IHC/CBE356	CISH		
	Non-amplification	Amplification Positive	Non-interpretable
0/1+	51	1*	3
2+	12	0	2
3+	6	21†	4

* This case (1) was low-level gene amplified.
† One case (1/21) was low-level amplified, whereas 20/21 cases were high level gene amplified.

Table 3. *Comparison between FISH and CISH results*

FISH	CISH	
	Non-amplification	Amplification
Non-amplification	66 (75.0%)	0
Amplification	0	22 (25.0%)

IV. Discussion

The accurate and rapid determination of HER-2/neu amplification has become necessary for the prognostic evaluation and selection of patients, who are candidates for treatment with Herceptin® that has been shown to significantly prolong survival in HER-2/neu positive breast carcinoma. In routine clinical practice, immunohistochemical study and fluorescence *in situ* hybridisation are used to evaluate the

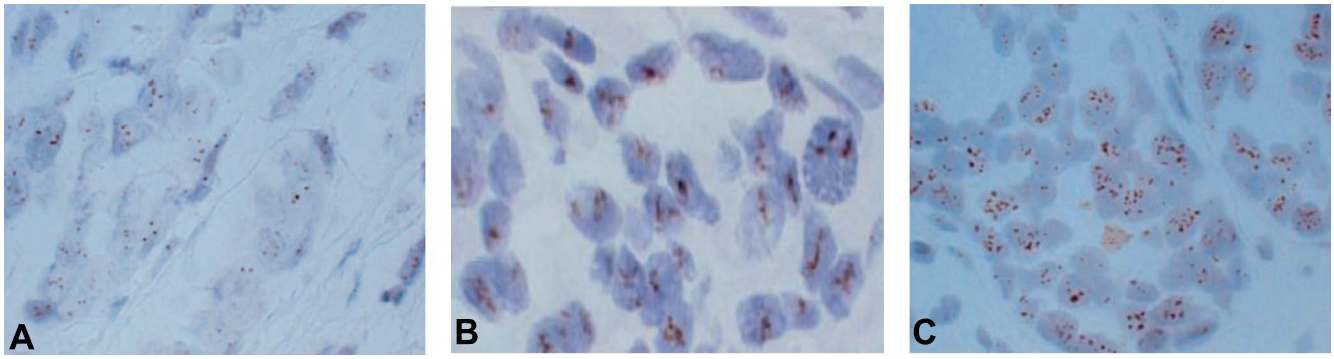

Fig. 1. CISH detection of *HER-2/neu* oncogene in invasive breast carcinomas. (**A**) A tumour with one to two clearly identifiable copies of *HER-2/neu* oncogene (no amplification). (**B**) A typical *HER-2/neu* amplification appeared either as a peroxidase positive cluster of gene copies, or as multiple individual gene copies (**C**). Counterstained with haematoxylin.

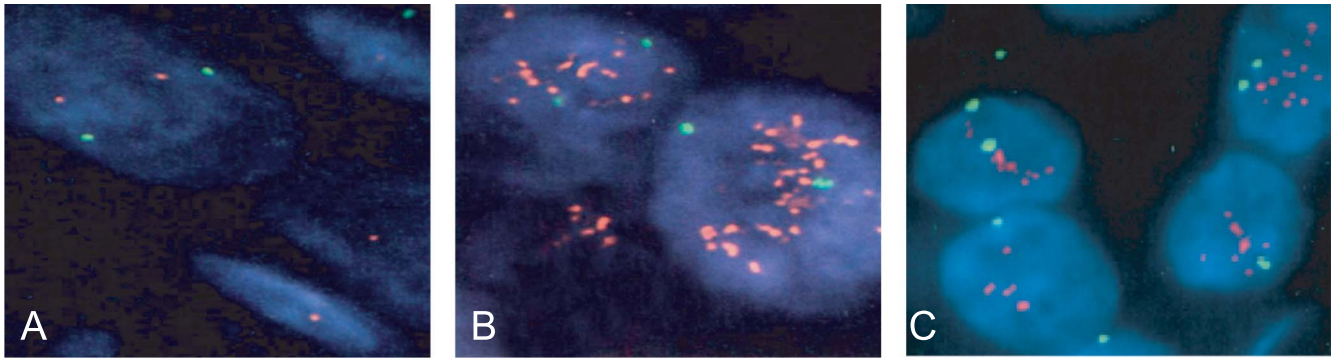

Fig. 2. FISH detection of *HER-2/neu* oncogene in invasive breast carcinomas. (**A**) Non-amplified tumour. The HER-2/neu gene signal is red and chromosome 17 centromere is green. (**B**) High level HER2 gene amplification as the HER2/CEP17 ratio is greater than 2. (**C**) Low level HER2 gene amplification.

HER-2/neu status as both of them have been FDA-approved. Although IHC and FISH should yield similar results because HER-2/neu overexpression results from gene amplification, there are discrepancies between IHC and FISH results. There are several studies comparing IHC and FISH in paraffin embedded tissues and the concordance is reported to be from 73% to 98% [9, 14, 16, 25].

Immunohistochemical staining can be significantly affected by technical issues (tissue fixation) and interpretation can be problematic, particularly in cases with low-level amplification, as there is subjectivity in grading, since there are no available quantitative computer-assisted imaging techniques. Furthermore, a number of commercially available antibodies have shown differences in specificities and sensitivities and identify different c-erbB2 protein domains [1, 10, 12, 15, 19, 23, 26]. The present study tested the CBE356 mouse monoclonal antibody, clone 10A7, that showed strong positive membrane staining compared to CB11 that is prone to heavy background. Moreover, IHC staining of *HER-2/neu* with CBE356 has proved to be significantly cheaper than HercepTest. However, Gancberg *et al.* and Tubbs *et al.* have shown discordance between gene amplification and the detection of protein amplification by immunohistochemistry [7, 24]. In these cases, in order to avoid false-positive cases, it has been suggested to perform FISH in 2+ and 3+ IHC staining tumours. Nevertheless, the significant cardiotoxicity of trastuzumab emphasises the need of accurate assessment of HER-2/neu status.

An alternative method to FISH, called CISH, has been introduced and validated that allows detection of HER-2/neu gene copies by using a simple immunohistochemistry-like peroxidase reaction, enumeration of gene copy number with simultaneous histologic examination by regular bright-field microscopy and permanent storage as the CISH signal intensity that does not diminish over time. Moreover, the most difficult category in CISH is the low-level amplification (6 to 10 gene copies/cell), in which accurate enumeration of the gene copies is necessary, as in routine diagnostic enumeration of gene copies exceeding 10 is not needed. However, even in these cases the microscopic evaluation is much faster than that of FISH.

In addition, in the present study, as the HER2 CISH (Zymed) is based on single colour detection, approximately half of the nonamplified tumours showed one to two signals per cell, and half showed three to five copies/nucleus. The latter is because of chromosomal aneuploidy and should be

regarded as no HER-2/neu amplification, which is due to DNA replication during S and G2/M phases of the cell cycle. Also, a small proportion (10% to 30%) of aneuploid cancer cells may contain five to eight signals/nucleus (polysomic cases); this should also be interpreted as a negative finding (no HER-2/neu amplification). The theoretical advantage of Vysis' two-colour FISH is its ability to distinguish chromosomal amplification from aneuploidy using a differentially labelled reference probe (chromosome 17 centromere). However, as yet there are no large comparisons between Vysis' two-colour FISH and the CISH-like single-colour FISH, which could confirm the advantage of the two-colour system. On the other hand, a recent study by Elkin *et al.* demonstrated that it is more cost-effective to use FISH alone or as a confirmation of all positive HercepTest results, than to use FISH to confirm only weakly positive (2+) results or HercepTest alone [6].

In the current study, after analysing one hundred cases of invasive ductal breast carcinomas, a high level of concordance between CISH and FISH (100%) was observed. This was also found by several other studies [4, 8, 22, 26]. The complete agreement of FISH and CISH results points out that the chromogenic ISH technique seems to be sensitive and specific for detection of HER-2/neu amplification in human archival tumour samples and could potentially fulfill the same role as FISH in the HER2 testing algorithm. As can also be seen from our results, the present study includes a false positive rate (as detected by FISH) of 19.3% (i.e. 6/31 cases, Tables 1 and 2) for the CBE356 3+ cases. The number of discrepancies was similar if CISH was used instead of FISH and the current literature suggests a 10–16% false-positive rate for these patients that may be due to the subjective score interpretation of IHC [2, 18, 24]. In view of the clinical consequences, this would subject these patients to an unnecessary Herceptin® treatment without any benefit while importantly they would be exposed to the risk of cardiac toxicity. Based on this, it is suggested to apply the HER2 testing algorithm to all positive IHC scored cases to elucidate their gene amplification status.

Furthermore, it was noted that a total of 12 cases were non-interpretable by either FISH or CISH. This was due to truncated nuclei, which were small in diameter and should not be scored. Additionally, in some cases, nuclei that were not intact were observed; these contained central unstained areas and should not be evaluated as a missing area which may have contained signals that cannot be assessed.

Additionally, in the current study, it was found that when HER-2/neu was overexpressed, there was a tendency (not statistically significant) for negative estrogen and progesterone receptor status and in those cases there was also a high proliferative activity (Ki67) and p53 protein expression. Similar data has been reported elsewhere and these findings negate the widely held belief that ER and PR positive tumours are rarely HER2 positive [11, 13].

Although a further number of cases would allow unbiased assessment of sensitivity and specificity of CISH versus FISH, the superiority of one HER-2/neu test over another can best be solved, in a future study, by using the clinical response to trastuzumab therapy as reference. Conclusively, the concordance of CISH with FISH in this study is especially encouraging and it was shown that CISH is an appealing alternative to FISH and that the combined use of IHC and CISH can be considered, even though the recommendations of the College of American Pathologists regarding HER2 testing in breast cancer and UK guidelines dictate FISH assay alone to analyse cases for which IHC results are inconclusive (borderline 2+ cases).

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VI. References

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