Concomitant Oncoprotein Detection with Fluorescence *in Situ* Hybridization (CODFISH)

A Fluorescence-Based Assay Enabling Simultaneous Visualization of Gene Amplification and Encoded Protein Expression

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We sought the validation of a three-color fluorescence-based system that simultaneously profiles Her-2/*neu* oncogene copy by fluorescence *in situ* hybridization (FISH) and Her-2/*neu* encoded protein by the use of a versatile alkaline phosphatase chromogen fast red K in either fluorescence or bright-field mode. Nuclei were counterstained with DAPI. Nineteen infiltrating ductal carcinomas of breast were comprehensively evaluated for Her-2/*neu* amplification/overexpression by direct and indirect FISH using digoxigenin (DigFISH) and direct fluorescently labeled probes, autoradiographic RNA:RNA *in situ* hybridization, and immunohistochemistry using monoclonal antibody CB11. CODFISH results correlated well with DigFISH, direct-label FISH, mRNA expression, and oncoprotein expression as assessed with CB11, and enabled simultaneous visualization of gene copy and protein. In addition, qualitative immunohistochemistry may be followed by CODFISH gene copy enumeration to clarify ambiguous cases. (J Mol Diag 2000, 2:78–83)

Currently available assays for defining gene amplification and overexpression of encoded protein focus on only one aspect of the process. That is, either the gene copy is profiled directly, or the expression of the encoded protein is identified with immunofluorescence or immunohistochemistry, or (much less frequently) the corresponding

mRNA is profiled by *in situ* hybridization. Such assays can be performed from extracted materials,^{1,2} *in situ* methods visualizing DNA amplification and RNA or protein expression provide the important advantage of simultaneous assessment of morphology.^{3–5}

For most genes, gene copy number, message, and encoded protein measurements are concordant in that increased gene copy leads to enhanced message production and overexpression of protein. For other genes, external modulating influences on transcription and translation may be reflected in enhanced expression of mRNA and overproduction of encoded protein in the presence of two apparently normal gene copies measured by fluorescence *in situ* hybridization (FISH). A technique that could allow simultaneous assessment of gene copy number and production of its encoded protein could facilitate visualization of the process on a cell-by-cell level, enhancing understanding of both physiological and pathophysiological mechanisms.

A three-color fluorescence-based assay is described herein that combines conventional DNA:DNA FISH with immunohistochemical detection of the Her-2/*neu* cell membrane-associated oncoprotein using the bifunctional chromogen of alkaline phosphatase fast red K that has both chromogenic and fluorescent properties. In sections of formalin-fixed paraffin tissue counterstained with DAPI, the three-color fluorescence assay permitted precise quantitation of gene copy by FISH and simultaneous visualization of the encoded protein by immunohistochemistry (IHC).

Concomitant oncoprotein detection with FISH (CODFISH) represents a versatile assay for simultaneous assessment of Her-2/*neu* or other genomic amplification and oncoprotein expression. For Her-2/*neu*, the method is readily adaptable to a 2-stage algorithm whereby bright field IHC with CB11 can be followed by CODFISH to detect gene copy for clarification of ambiguous cases,

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significantly decreasing possible false negative or false positive interpretations now linked to important therapeutic decisions.

Materials and Methods

Patient samples from 19 primary infiltrating mammary carcinomas of ductal type were selected for study based on preliminary immunohistochemistry results using the monoclonal antibody CB11 (Ventana Medical Systems, Tucson, AZ)^{6,7} and a semiquantitative scoring system approved by the Food and Drug Administration for interpretation of HercepTest (Dako) results.^{8,9} Paraffin blocks of formalin-fixed cell lines SiHa and BT-474 containing normal and eightfold increased Her-2/*neu* gene copy were used also in preliminary experiments and as in-run controls. Each paraffin block from the 19 cases included sufficient redundant paraffin-embedded archived tissue to do all of the components of the study. All tissues were first evaluated with a presectioning hematoxylin and eosin (H&E)-stained section, as well as a section following preparation of the last unstained section, to confirm the presence of infiltrating carcinoma in material to be studied.

CODFISH

Unstained paraffin sections on electrostatically charged slides were deparaffinized and rehydrated in 3 changes each of xylene, absolute alcohol, and 95% and 80% alcohol, and then placed in 1× phosphate-buffered saline (PBS) for 5 minutes. Cell conditioning was achieved through the use of microwaving in citrate buffer for 15 minutes, followed by cooling of the slides in the citrate solution at room temperature for 20 minutes. The monoclonal antibody CB11 at manufacturer's concentration was dispensed onto the sections and allowed to incubate for 1 hour at room temperature. Sections were then washed in 1× PBS, 2 changes of 3 minutes each, and biotinylated anti-mouse IgG (Ventana) applied at a concentration of 15 µg/ml for 30 minutes. Two changes of 1× PBS wash at room temperature were followed by the addition of avidin-alkaline phosphatase (Ventana) at a concentration of 3000 µg/ml for 30 minutes. Sections were then washed twice for 3 minutes each, in 1× PBS at room temperature, and then the chromogen reaction product developed from the fast red K proprietary solution (Ventana), 10 minutes at room temperature. Sections were then washed briefly in distilled water and counterstained with hematoxylin for approximately 30 sections. Sections were then dehydrated in graded alcohols.

Ten microliters of digoxigenin-labeled Her-2/*neu* probe in proprietary probe solution (Oncor/Ventana) were applied to the section and coverslipped, then the probe solution and target tissue were codenatured at 90° centigrade for 6 minutes followed by overnight hybridization at 37° in a humidified chamber. The coverslip was removed by soaking the slides in 2× standard saline citrate (SSC) for 5 minutes at room temperature. Washes of 0.5× SSC for 5 minutes at 72° centigrade followed. Slides were

prewashed in 1× PBS containing 0.5% Tween 20 for 3 minutes at room temperature. Fluorescein-isothiocyanate-antidigoxigenin 1:50 in 1× bovine calf solution containing 0.5% bovine serum albumin was applied to the section, per slide, and allowed to incubate for 1 hour. The sections were then washed 3 times, 5 minutes each, in 1× PBS containing 0.5% Tween 20 (Sigma, St. Louis, MO). The sections were then counterstained with 20 µl DAPI in antifade solution (Oncor, Gaithersburg, MD) and mounted for FISH analysis.

The fast red K bright red chromogenic reaction product of alkaline phosphatase also displays brilliant pink-red fluorescence on excitation at 530 nm. The sections were screened by immunofluorescence without knowledge of the immunohistochemistry results, and scored in the same manner as described above.^{8,9} The CODFISH Her-2/*neu* copy number was acquired through FISH analysis without knowledge of the DigFISH results. Signals were visualized on an Axioskop (Zeiss, Oberkochen, Germany) photomicroscope equipped with a triple bandpass filter. Images were captured through a photometric digital cooled CCD camera using a SmartCapture system (Vysis).¹⁰ Two representative fields were identified in the area preselected for probe application, and gene copy enumerated for a total of 40 nuclei, 20 in each of the two representative fields averaged.

DNA:DNA FISH (DigFISH), Site I

The same procedure sans the CB11 immunohistochemistry portion of the CODFISH protocol was used for the DigFISH assay.

Direct Two-Color Her/2-*neu*/Chromosome 17 Fluorescence Hybridization, Site II

FISH was performed using fluorescent-labeled DNA probes obtained from Vysis, Inc. (Downers Grove, IL). Five-micron paraffin sections were deparaffinized, dehydrated, incubated in 2× SSC at 75°C for 15 minutes, digested in pepsin solution (4 mg/ml in 0.9% NaCl, pH 1.5) for 15 minutes at 37°C, rinsed in 2× SSC at room temperature for 5 minutes, and air-dried. Dual-probe hybridization was performed using a Spectrum Green-labeled probe for the centromere region of chromosome 17 (CEP17) and a Spectrum Orange-labeled Her-2/*neu* probe. The probe mixture was hybridized to an area containing invasive carcinoma as determined on adjacent H&E-stained section. Probes and target DNA were denatured simultaneously in an 80°C oven for 5 minutes, and hybridization performed by incubation at 37°C overnight. Posthybridization washes were performed using 1.5 mol/L urea/0.1× SSC at 45°C for 30 minutes and 2× SSC at room temperature for 2 minutes. Nuclei were counterstained with 4,6-diamidino-2-phenylindole and antifade compound *p*-phenyldiamine. The number of HER2 signals and CEP17 signals were counted in 60 nonoverlapping nuclei, and an overall mean HER2/

CEP17 ratio was calculated. Lesions with ratios ≥ 2.0 were defined as having HER2 amplification. Site I used the CEP17 probe in combination with the DigFISH method described. All other procedural steps were identical for both Sites I and II.

RNA:RNA in Situ Hybridization

The formalin-fixed, paraffin-embedded breast biopsy blocks were serially sectioned at 4 to 5 μm , and the sections were mounted on 3-aminopropyltriethoxysilane-coated slides. These sections served as the substrate for *in situ* hybridization. A final serial section was stained with H&E to verify preservation of the morphology.

Her2/neu in Situ Hybridization, Site III

In situ hybridization for Her-2/neu mRNA was carried out using well-characterized methods.¹¹⁻¹³ The Her-2/neu DNA construct, pHER2-436-1 (originally in pUC12), was obtained from the American Type Culture Collection (Manassas, VA; stock number 59297). The 1.45-kb construct was recloned into pCDNA1, which was grown up in bulk, and template plasmids were prepared by cesium banding. Templates for riboprobe transcription were prepared by restriction digestion of the DNA with *Hind*III for antisense transcription using SP6 polymerase and *Xba*I for T7 directed sense orientation probes. *In vitro* transcription to generate ^3H -labeled riboprobes was carried out in the presence of ^3H -UTP and ^3H -CTP (approximately 30 or 60 Ci/mmol/L, respectively), yielding probes with a specific activity of 1.1×10^8 dpm/ μg . The *in situ* hybridization mixture contained 50% formamide, 0.3 mol/L NaCl, 20 mmol/L Tris-Cl, 1 mmol/L EDTA, pH 8.0, $1 \times$ Denhardt's solution, 500 ng/ml yeast tRNA, and 10% dextran sulfate. Hybridization was carried out at $\sim T_m - 25^\circ\text{C} = 45^\circ\text{C}$. The

final probe concentrations were adjusted to just fully saturate the target messages (0.2 $\mu\text{g}/\text{ml}/\text{kb}$ complexity). Following hybridization, the specimens were washed at high stringency, estimated to be $\sim T_m - 5^\circ\text{C} = (0.1 \times \text{SSC at } 65^\circ\text{C})(1 \times \text{SSC} = 0.15 \text{ mol/L NaCl, } 0.0015 \text{ mol/L Na-citrate})$. Parallel hybridizations were simultaneously performed using a 1.8-kb actin antisense probe. This was used to assess RNA preservation and for normalization as a means to judge overexpression (see below). The slides were then overlaid with Kodak NTB-2 autoradiography emulsion, exposed for 2 weeks at 4°C , developed photographically, and lightly stained with H&E.

All *in situ* hybridization results were evaluated independently (M. H. S.) without any knowledge of the parallel results of the multiple FISH or IHC analyses. Under the conditions of these experiments, a semiquantitative scale was developed and normalized to the level of actin expression in the adjacent section as follows: Neg/0 = < 5 grains/cell, trace+ = 5–10 grains/cell, 1+ = 10–15 grains/cell, 2+ = 15–20 grains/cell, and 3+ = 20–25 grains/cell. The typical breast biopsy had an actin signal of at least 1+. Cases with trace or negative actin were considered RNA degraded. Cases were considered to have strong overexpression of Her-2/neu if the level was two or more steps higher than the actin level on the same day/experiment. A one-step difference was graded as weak overexpression. Normal tissues within the sections typically had no overexpression and served as internal controls. Positive controls included an identically processed breast cancer cell line, BT-474, that has known eightfold amplification of Her-2/neu and a known high-grade ductal carcinoma that immunohistochemically was consistently 3+. These controls reproducibly were weak and strong Her-2/neu mRNA overexpressors, respectively. These controls were performed in each run, as were

Table 1. CODFISH Assay Results

Case	COD	FISH	DigFISH	Direct FISH	FISH 17 Site I	FISH 17 Site II	mRNA	CB11 IHC
1	0	2.0	2.2	2.0	1.7	2.0	Degraded	0
2	0	2.2	2.6	2.6	1.8	2.5	Negative	0
3	0	1.8	2.0	2.3	1.7	2.2	Negative	0
4	0	4.0	6.0	2.8	1.9	2.5	Negative	0
5	0	2.1	3.7	2.6	2.0	2.4	Negative	0
6	0	1.9	2.7	2.0	1.9	2.0	Negative	0
7	0	3.2	3.6	3.0	2.3	2.8	Negative	0
8	0	1.8	2.0	2.0	1.9	2.0	Negative	0
9	0	2.8	4.6	2.8	1.9	2.4	Negative	0
10	0	1.9	5.1	3.1	2.6	2.8	Negative	0
11	0	3.5	4.6	2.2	1.6	2.1	Negative	0
14	0	2.0	1.9	2.1	1.6	1.9	Negative	0
12	0	2.0	4.9	2.9	2.1	2.6	Weak	0
13	0	3.1	4.3	2.1	1.8	2.0	Weak	0
15	3	8.2	5.3	19.9	2.3	3.3	Strong	
16	3	15.5	11.6	21.0	1.4	1.7	Strong	3
17	3	15.0	14.0	20.6	2.7	2.3	Strong	3
18	3	13.4	15.8	21.0	2.1	2.6	Strong	3
19	3	16.9	18.0	21.0	2.2	2.7	Strong	3

DigFISH and CODFISH were performed at Site I, Direct FISH at Site II.

COD, concomitant oncoprotein detection; immunohistochemistry using CB11 and fast red K as alkaline phosphatase chromogen; FISH, Her-2/neu gene copy as determined using digoxigenin-labeled probes and CODFISH; DigFISH, Her-2/neu gene copy as determined using digoxigenin-labeled probes and FISH; FISH 17, Chromosome 17 copy as determined using direct label FISH.

Figure 1. Fluorescence confocal microscopy photomicrograph, cell line known to have normal Her-2/*neu* gene copy and absence of oncoprotein expression. One, three, or four Her-2/*neu* gene copies are identified in each nucleus (green). No surface oncoprotein is present.

sense orientation controls to assess for background binding of probe.

Immunohistochemistry Using Monoclonal Antibody CB11

Unstained paraffin sections on electrostatically charged slides were deparaffinized and rehydrated in three 1-minute changes each of xylene, absolute alcohol, and 95% and 80% alcohol, and then placed in 1× PBS for 5

Figure 2. Fluorescence confocal microscopy photomicrograph, BT-474 cell line. Numerous Her-2/*neu* gene copies (green) are simultaneously visualized along with abundant surface membrane and some cytoplasmic oncoprotein expression (red).

Figure 3. Fluorescence confocal microscopy photomicrograph. Infiltrating ductal carcinoma of breast demonstrating normal Her-2/*neu* gene copy and absence of oncoprotein expression.

minutes. The modified labeled avidin biotin method was used for detection. Cell conditioning was achieved through the use of microwaving in citrate buffer for 15 minutes, followed by cooling of the slides in the citrate solution at room temperature for 20 minutes. The monoclonal antibody c-erb-B2 at manufacturer's proprietary concentration (Ventana) was dispensed onto the sections and allowed to incubate for 1 hour at room temperature. Sections were then washed in 1× PBS, two changes of 3 minutes each, and biotinylated goat anti-mouse IgG (Ventana) applied at a concentration of 15 μ g/ml in PBS containing 0.05 proclin 300 for 30 minutes. Two changes of 1× PBS wash at room temperature were followed by the addition of avidin-alkaline phosphatase (Ventana) at a concentration of 3000 μ g/L PBS with 0.05 proclin 300 for

Figure 4. Fluorescence confocal microscopy photomicrograph. Abundant Her-2/*neu* gene copy (green) and oncoprotein (red) overexpression are simultaneously visualized. Infiltrating ductal carcinoma of breast.

Table 2. CODFISH Statistical Comparison (Her-2/*neu* Gene Copy Enumeration Assay Component)

Correlation test (Fisher's <i>r</i> to <i>z</i>)			
	<u>Correlation</u>	<u>P value</u>	
CODFISH and DigFISH	0.945	<0.0001	
CODFISH and Direct FISH	0.956	<0.0001	
Paired means comparison			
	<u>CODFISH</u>	<u>DigFISH</u>	<u>Direct FISH</u>
Negative mRNA CB11 weak/negative group versus	0.0019	0.0068	<0.001
CB11/mRNA positive group			

30 minutes at room temperature. Sections were then washed twice for 3 minutes each, in 1× PBS at room temperature, and then the chromogen reaction product developed from fast red K proprietary solution (Ventana), 10 minutes at room temperature. Sections were then washed briefly in distilled water and counterstained with hematoxylin for approximately 30 sections. After dehydration in graded alcohols and xylene, coverslips were mounted with Permount and examined by conventional optical microscopy.

Statistics

Statistical comparisons were made using Statview (SAS Institute, Cary, NC).

Results

The data comparing the various *in situ* hybridization and immunohistochemical methods are summarized in Table 1 and illustrated in Figures 1-4. There was overall excellent agreement among the different modalities evaluated. All cases were reviewed by standard histopathological evaluation and graded by the Scarff-Bloom-Richardson system before the submission for special ancillary studies. Material evaluated by the various techniques were consecutive sections.

CODFISH, DigFISH, and CB11 immunohistology were performed at site I. Direct FISH and CB11 immunohistology were performed at site II. mRNA ISH was performed at site III.

Comparison of Standard Immunohistochemistry Results and Oncoprotein Detection by CODFISH

Five breast carcinomas and one positive cell line showed a strong circumferential thick layer of cytoplasmic membrane staining with both a monoclonal and polyclonal antibodies, and with the CB11 component of CODFISH. Identical patterns and intensity of staining were noted. However, HercepTest did also stain (grade 2 intensity) two cases negative by all FISH methods, and by CB11 and the Zymed polyclonal antibody sans epitope retrieval.

Gene Copy Enumeration by *in Situ* Hybridization

Three methods were used to determine copy number. A direct two-color system, an indirect digoxigenin-labeled FISH system (DigFISH) and the DigFISH component of CODFISH were used for this purpose. There was excellent concordance among the three methods used (Table 2). The presence of the intense fluorescent emission from fast red K did not interfere with either capture or enumeration of the digoxigenin-based gene enumeration by CODFISH.

Interrelationships between Gene Amplification, mRNA Expression, and Protein Detection

One case had degraded mRNA. Results were normalized for actin message as described in Materials and Methods. All five clinical specimens and the known positive cell line displayed abundant Her-2/*neu* mRNA. Ten of 12 specimens that were negative for the oncoprotein and for gene copy amplification were also negative for Her-2/*neu* mRNA expression. Two cases showed weak mRNA expression in the absence of an increase in gene copy or oncoprotein cell membrane expression.

Discussion

Approaches to the identification of gene expression are legion, ranging from techniques that focus on amplification at the genomic level, identification of increase in Her-2/*neu* RNA, and profiles of protein production encoded by the gene. Such approaches may use extracted DNA, RNA, or protein, or may focus on detection *in situ* by either DNA:DNA *in situ* hybridization, RNA:RNA *in situ* hybridization, or IHC. But even for real time measurements of such events in living cells, such approaches focus on only one of the determinants along the gene-message-protein pathway. Here, we present a method whereby gene amplification and encoded protein production are simultaneously visualized.

We chose Her-2/*neu* as a model system for first developing the assay in previously characterized cell lines, then extending the technique to a series of human invasive breast carcinomas for which gene copy number,

mRNA expression, and Her-2/*neu*-encoded protein production were well established. The current controversy surrounding the accuracy of an immunohistochemistry assay for profiling this oncogene's overproduction as a means for screening women for serotherapy with a humanized monoclonal antibody highlights the potential benefits of an assay that simultaneously profiles both characteristics.^{3,5,8,13}

We were concerned that differences in cell conditioning and the use of immunohistochemistry reagents might adversely effect the DNA *in situ* hybridization portion of the assay. In fact correlations between DigFISH and the digoxigenin-based: DNA *in situ* hybridization component of CODFISH were excellent. The leveraging of the fluorescent properties of fast red K may have numerous advantages over other applications whereby FISH and labeling of specific cellular populations may be of value. Figures 1–4 used confocal microscopy; in clinical practice, the expense and time required for confocal microscopy is not necessary, as gene copy counting is easily performed by examining multiple nuclear focus planes.

We have also concluded that the CODFISH assay could be part of a cost-effective algorithm in the clinical approach to profiling gene amplification and oncoprotein overexpression. Concern has been expressed in the relevant literature regarding the clinical import of a 2+ *versus* a 3+ IHC result in the management of patients with breast carcinoma; a significant portion of such cases appear to be false-positive IHC results. The cardiotoxicity of Herceptin, alone or in combination with Adriamycin, itself a cardiotoxic drug, requires the greatest possible accuracy in selection of patients for Herceptin serotherapy. The CODFISH assay could be used in an algorithm whereby qualitative IHC assessment is done with bright-field mode, and any cases demonstrating borderline or equivocal results by immunohistochemistry could be very easily subjected to the balance of the CODFISH assay (DigFISH), correlating the ambiguous IHC results with definitive gene copy enumeration.

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