Microwave Enhancement of CISH for HER2 Oncogene

Anthony S.-Y. Leong, MD, FRCPA, FRCPath, Zenobia Haffajee, HT, and Megan Clarke, HT

Abstract: We describe a modification to the prescribed procedure for the Zymed Spot-Light HER2 chromogenic in situ hybridization kit (84-0146, Zymed Laboratories, San Francisco, CA) by substituting the heat pretreatment step with MW irradiation in citrate buffer 10 mmol/L at pH6.0 at 120°C for 10 minutes and repeating the procedure afterenzyme digestion with time and temperature controlled in the Mega T/T oven (Milestone s.r.l., Sorisole, Italy). The subsequent procedure leading up to hybridized was as per manufacturer's instructions. Invasive breast carcinoma previously scored by immunohistochemistry for HER2, comprising 18 cases of 3+, 18 cases of 2+, and 12 cases of 1+, were examined by chromogenic in situ hybridization using this modified procedure, with a parallel set of cases examined by the prescribed Zymed method. The introduction of the "MW retrieval" steps resulted in consistently a greater number of hybridization signals in amplified tumor cells with benign epithelial cells and lymphocytes displaying 2 clear dots compared with the weaker and less consistent signals obtained with the standard procedure. MW exposed sections showed larger numbers of large and small clusters that often allowed identification of amplified tumors without having to count single dots with crisp staining and absence of background precipitation.

Key Words: MWs, CISH, HER2, immunohistochemistry, breast cancer

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The HER2 oncogene is a member of the epidermal growth factor receptor family and detection of its amplification is a major criterion for the selection of breast cancer patients for treatment with trastuzumab (Herceptin). It may also be of importance for treatment with anthracycline-based and hormonal regimens in patients with metastatic disease.[1] HER2 amplification may be of prognostic relevance,[2] and more recent reports showing that trastuzumab therapy in combination with or after chemotherapy significantly improves disease-free survival in women with HER2-positive breast cancer[3,4] places even greater therapeutic and prognostic importance on the accurate identification of HER2 oncogene amplification.

Fluorescence in situ hybridization (FISH) for the detection of HER2 amplification has a sensitivity of about 96% and specificity of 100%[5] and is regarded as the "gold standard". The technique has several important advantages including its application in fixed paraffin-embedded tissue sections, and direct visualization of amplified genes and chromosomes within individual cell nuclei. The impermanence of fluorescence, requirement for specialized fluorescence microscope and filters, and high costs (about US$100 per test for reagents alone) need consideration. There are also difficulties associated with the accurate separation of invasive from in situ cancer cells in fluorescence microscopy that make chromogenic in situ hybridization (CISH) a viable alternative to FISH. Several publications attest to the validity of this latter technique.[6–10] As CISH employs a peroxidase reaction to visualize the chromogen, it allows the convenience of bright field microscopy and the direct visualization of gene amplification and corresponding tissue morphology. It is relatively less expensive and provides a permanent record.

This study describes a modification to the prescribed procedure for CISH, using MW (MW) irradiation to enhance the staining of HER2. We compare the results with parallel sections in which the manufacturer's standard protocol was followed and report, for the first time, enhanced CISH signals after theexposure to MWs.

MATERIALS AND METHODS

Forty-eight cases of breast carcinoma of all histologic types and grades in which immunostaining for HER2 had been performed, were randomly picked from material accessioned at the Immunohistology Unit, Hunter Area Pathology Service, Newcastle, Australia during January 2003 to August 2005. These cases had HER2 scores of 1+ (12 cases), 2+ (18 cases), and 3+ (18 cases). Two parallel sets of 5-μm-thick, formalin-fixed, paraffin-embedded sections were cut from the tumor blocks used for HER2 staining and examined by CISH using the Zymed protocol and our modified protocol with the Zymed Spot-Light HER2 CISH kit (84-0146, Zymed Laboratories, San Francisco, CA). The Zymed protocol was carried out as per manufacturer's instructions. Briefly, the steps involved deparaffinization of the sections with xylene and hydration, followed with heat pretreatment by boiling in a proprietary reagent, and enzyme digestion at room temperature, the latter 2 steps identified as "the most critical steps for successful CISH perfor-

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From the Immunohistology Unit, Division of Anatomical Pathology, Hunter Area Pathology Service, Newcastle, Australia.
Reprint: Professor Anthony S.-Y. Leong, MD, Hunter Area Pathology Service, Locked Bag 1, HRMC, Newcastle 2310, Australia (e-mail: aleong@mail.newcastle.edu.au).
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mance."[11] Denaturation was conducted at 95°C followed by hybridization at 37°C for 16 h with a digoxigenin-labeled HER2 probe. Signals were detected with a mouse antidigoxigenin-peroxidase system with diaminobenzidine as chromogen.

In our modified procedure, the heat pretreatment step was replaced by immersing the deparaffinized sections in citrate buffer 10 mmol/L at pH6.0 and exposed to MWs for 10 minutes at 120°C. Allow cooling to room temperature on the bench. Wash with phosphate buffered saline (PBS) (0.01 M, pH7.4) ×3. After enzyme digestion as prescribed in the Zymed protocol,[11] and washing in PBS ×3, the MW irradiation step was repeated by irradiating in citrate buffer 10 mmol/L at pH6.0 and exposed to MWs for 10 min at 120°C. MW irradiation was done in the Milestone Mega T/T (Milestone, s.r.l, Sorisole, Italy), which allowed accurate time and temperature control. After cooling to room temperature the subsequent steps of denaturation and hybridization leading to signal demonstration were performed according to the manufacturer's protocol with Meyer hematoxylin as the counterstain.

A multitissue block comprising sections of breast cancer with unaltered gene copy, low-level amplification and amplified HER2 as validated by FISH was employed as control.

CISH signals were enumerated by examining at least 50 invasive tumor cells with a ×20 or ×40 objective. When >50% of the cells in the chosen area exhibited >10 single dots, or large clusters (considered equivalent to 5 to 10 single dots relative to the size of a single dot), or small clusters (considered equivalent to 3 to 5 single dots relative to the size of a single dot), HER2 was recorded as amplified. When there were 6 to 10 dots per nucleus in >50% of cells, low amplification was recorded, and 1 to 5 dots per nucleus was regarded as non amplified.[6,11]

In Australia all cases of breast cancer with a HER2 score of 2+ are routinely sent to the FISH Reference Laboratory, St Vincent's Hospital, Sydney, where FISH testing is performed gratis. In addition, random cases with 0 to 1+ and 3+ HER2 scores were also submitted for validation, all cases tested by FISH were also tested by CISH.

RESULTS

The results of CISH testing are shown in Table 1. Using established criteria[6,11] that defines amplification of HER2 as those tumors in which >50% of the invasive tumor cells contain >10 dots per nucleus, we did not find any difference between the 2 CISH protocols. There was high concordance with FISH. All tumors with HER2 scores of 1+ showed unaltered gene copy by both FISH and CISH. Among the cases with 2+ scores, CISH failed to identify 2 tumors that were found to be amplified by FISH. One was a micropapillary carcinoma whose section lifter of the slide and was technically unsuitable for CISH and the other was a tumor assessed as showing low amplification by FISH. Among the cases with HER2 scores of 3+, one case was found to show amplification by CISH that was nonamplified by FISH and vice versa in another tumor.

TABLE 1. Breast Cancer Examined by HER2 Score, CISH, and FISH

HER2 score	CISH		FISH	
	Tested	Amplified	Tested	Amplified
1+	12	0	3	0
2+	18	4*	18	6†
3+	18	17‡	11	9§

*micropapillary carcinoma section lifted and technically unsuitable for CISH.
†One tumor with low-level amplification by FISH.
‡One case found to be non-amplified by CISH was also non-amplified by FISH.
§One case found to be non-amplified by FISH was found to be amplified by CISH.

The difference in the results obtained with the 2 CISH methods was obvious on casual examination and we performed comparative counts in 10 random tumors with amplified HER2 to demonstrate the difference (Table 2). The number of signals per tumor cell obtained after the MW protocol was consistently greater and easier to identify in both amplified and nonamplified tumors, and there was more consistent demonstration of signals in benign epithelium, lymphocytes and stromal cells (Figs. 1, 2). The MW protocol produced more frequent large and small clusters so that counting of signals in amplified tumors was often not necessary (Figs. 2 to 4). In nonamplified tumors, the MW protocol resulted in crisp and distinctive signals that were consistently present compared with that obtained with the conventional protocol. The rigors of exposure to MWs resulted in slightly more swelling of the tumor cells which somewhat aided enumeration of signals, and the occasional lifting in areas of the tissue section was no more than with the conventional method and did not impede assessment as there were generally preserved areas of tumor present. There was no background precipitation with either protocol.

DISCUSSION

In addition to the many applications of MWs in the pathology[12] that include fixation and tissue processing for light and electron microscopy, MW irradiation is used to enhance immunostaining of proteins in paraffin-embedded tissue sections. This development was hailed as "revolutionary"[13] and has resulted in a level of consistency that has enabled immunohistology to become an indispensable adjunct to morphologic diagnosis.[14]

The use of MWs in molecular analyses is a more recent development. MWs were initially employed to achieve the high temperatures necessary to denature probe and tissue DNA, providing a method with ease of control and rapidity of heating.[15,16] This application has also been adopted for the accelerated detection of mRNA.[17]

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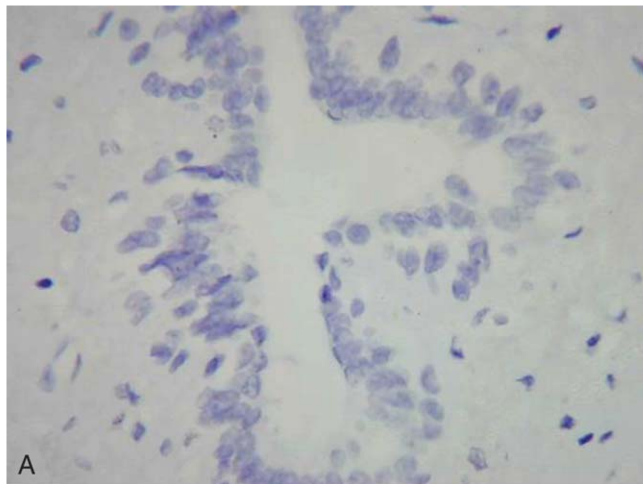
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59

TABLE 2. Means Signal Counts by CISH/50 Tumor Cells in 10 Random Cases of Infiltrating Breast Carcinoma With Amplified HER2 Gene Copies*

Case	Conventional Method	MW Method
1	282	474
2	301	450
3	460	610
4	340	476
5	365	550
6	290	396
7	181	370
8	176	398
9	210	494
10	430	668
Mean per 50 cells	3035	4886

*Signal enumeration was performed on 50 invasive tumor cells, avoiding areas of necrosis and overlapping nuclei. More than 50% of the cells enumerated in all cases by both methods contained > 10 dots/nucleus.



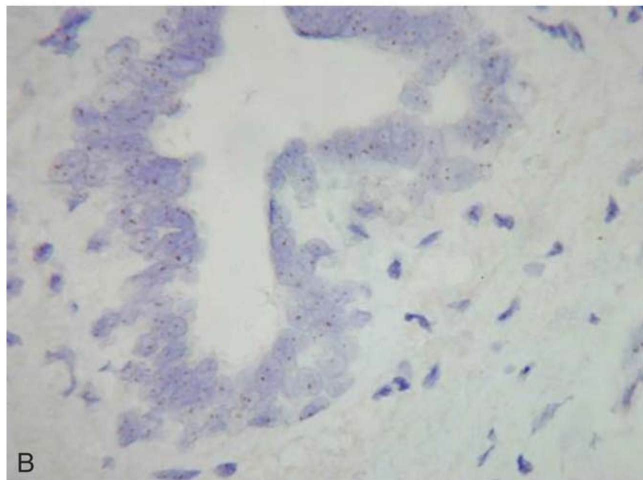


FIGURE 1. Benign breast duct showing no discernable signals with the Zymed method (A) compared with 2 distinct dots in the mirror image section afterthe microwave method (B).

Formaldehyde-fixed tissues remain the most common source of material for molecular studies and protease digestion is an essential procedure for unmasking the cross-linking effects of this fixative before in situ hybridization (ISH) can be performed. Recent studies demonstrated that the exposure of formalin-fixed, paraffin-embedded sections to MWs in citrate buffer in a manner similar to that applied for antigen retrieval produced enhanced signal detection for both mRNA[18] and DNA.[17,19]

The combination of MW irradiation followed by short proteolytic digestion produced a cumulative effect on tissue and target sequences that resulted in a significantly improved ISH signal detection compared with enzyme digestion alone or MW retrieval alone.[20–22] Unlike proteolytic digestion which needs to be applied for sufficient durations to produce the desired staining intensity, the combined method of MWs and shortened

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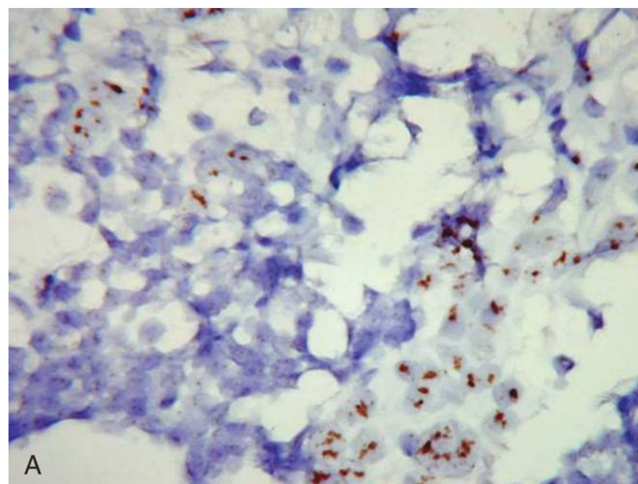

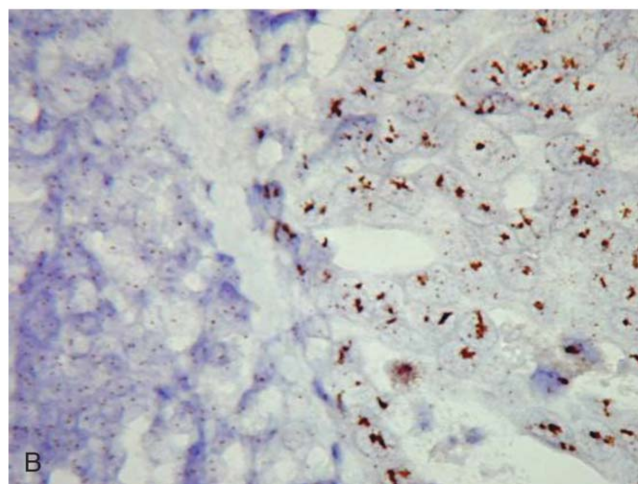

FIGURE 2. Although amplification is found in the invasive tumor cells in both methods, the signals are barely discernable in the adjacent lymphocytes afterthe Zymed method (A) compared with the distinct signals in the lymphocytes in the microwave preparation (B).

periods of enzyme digestion resulted in morphology that was significantly better than the proteolytic method alone as the latter tended to result in over digestion and destruction of morphology.[20] MW treatment generally decreased the amount of background staining simply by reducing the time required for enzymatic digestion. Prolonged enzyme digestion disrupts cellular integrity, allowing target molecules to migrate into the background thereby increasing nonspecific background staining and decreasing signal specificity.

Sperry et al[21] examined the effects of MWs, enzyme digestion and simple heating in sodium chloride-sodium citrate on the detection of RNA and DNA in formalin-fixed, paraffin-embedded tissue. They found that a combination of MW treatment for 15 to 20 minutes in 10-mM citrate buffer at pH 6.0 with a shortened digestion with proteinase K produced the best results. Not only were the positive signals enhanced but the number of positive cases detected was also increased and nucleotide sequences were detected with probe concentrations that were ineffective with other methods of retrieval. They found a 10-fold difference in the minimum concentration of albumin probe using MWs compared with the other 2 methods studied. The order of the combined method was not important and enhanced signals were obtained irrespective of the order in which digestion and MW irradiation was carried out.[20] MW pretreatment in conjunction with enzyme digestion gave positive results in all cases for which ISH without the MW pretreatment was not successful.[23] Experimentation with various buffer solutions for retrieval, enzyme digestion, and durations of MW exposure revealed that the optimal sequence and combination of buffer/duration/power depended on the target RNA and tissue. Their results suggested that MWs may also facilitate the combination of ISH and immunohistochemical labeling on the same slide. Others have obtained similar results for mRNA in human infant brain tissue after 12 minutes of MW pretreatment in citrate and Tris/EDTA buffers.[24] The same retrieval method was equally effective for the demonstration of Epstein-Barr virus EBER RNA with quantitative confirmation of the increased sensitivity render by MW pretreatment.[25] Importantly, MW irradiation renders RNA-ISH a more consistent and reliable procedure.[26]

MW irradiation has also been employed for the ISH demonstration of chick *Sox* 11 and *Sox* 12 gene mRNA in semithin plastic sections.[27] Compared with MW irradiation in 10 mM citrate buffer at pH 6.0, heated for 20 minutes at 450 W and digestion with proteinase K at 10 mg/mL at 37°C for 15 minutes, superheating at 121°C in a pressure cooker in 10 mM citrate buffer at pH 6.0 for 3 minutes proved to be the most effective method of enhancing the target signals even in tissue blocks prepared some months previously where reactivity seemed to be lost. Although the number of papers describing in ISH procedures in plastic sections are few, it seems that it is possible to attain good results if the tissue is embedded in methyl methacrylate and when pretreatment with superheating in a MW oven is employed for the enhancement of target signals.[22]

MWs have also aided the identification of DNA by other molecular techniques. The exposure of serum to MWs facilitated the detection of hepatitis B virus DNA with the polymerase chain reaction,[28] and direct irradiation of whole blood and hair shafts allowed sensitive genomic amplification by polymerase chain reaction.[29] MW irradiation allowed DNA extraction from paraffin-embedded tissues,[30] including genomic DNA from *Aspergillus fumigatus*.[31] MW denaturation of metaphase chromosome preparations resulted in reproducible comparative genomic hybridization analysis with a potential application in paint and DNA probe hybridization to chromosome spreads, and to RNA in tissue sections.[32] Recently, it was shown that MW irradiation of the sample before incubation with the DNA probe allowed the detection of estrogen receptor and cyclic adenosine monophosphate-responsive element binding protein by Southwestern histochemistry, whereas, no signal was detected in the absence of the MW treatment.[33]

In this study, we show, for the first time, that MWs can be employed to achieve signal enhancement in CISH for HER2. By substitution of the pretreatment heating step with irradiation by MWs in citrate buffer for 10 minutes and the repetition of this step after ashort enzyme digestion, we obtained enhanced signals in both neoplastic and benign tissues with no background precipitation. The mean signal count was higher in the MW protocol compared with that prescribed by the manufacturer, and a greater number of large and small clusters were revealed, allowing most amplified tumors to be identified without resorting to counting of signals. There was no significant deterioration in tissue morphology.

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