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High concordance between HercepTest immunohistochemistry and *ERBB2* fluorescence *in situ* hybridization before and after implementation of American Society of Clinical Oncology/College of American Pathology 2007 guidelines

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Human epidermal growth factor receptor 2 (*HER2, ERBB2*) is an important critical predictive marker in patients with invasive breast cancer. It is thus imperative to ensure accuracy and precision in HER2 and *ERBB2* testing. In 2007, the American Society of Clinical Oncology and College of American Pathologists (ASCO/CAP) proposed new guidelines for immunohistochemistry and fluorescence *in-situ* hybridization scoring in an effort to improve accuracy and utility of these companion diagnostic tests. The goal of the 2007 guidelines was to improve concordance rates between the diagnostic tests and decrease the number of inconclusive cases. This study examines the impact in concordance rates and number of inconclusive cases based on the recent change in guidelines in a large study cohort. HER2 immunohistochemistry and *ERBB2* fluorescence *in-situ* hybridization were performed on all specimens from our facility from years 2003 through 2010 ($n=1437$). Cases from 2003–2007 ($n=1016$) were scored using Food and Drug Administration guidelines, with immunohistochemical 3+ cases staining >10% of tumor cells and fluorescence *in-situ* hybridization amplification cutoff value of 2.0. The 2007 guidelines were implemented and scored accordingly for cases from 2008–2010 ($n=421$), with immunohistochemical 3+ cases staining >30% of tumor cells and fluorescence *in-situ* hybridization amplification cutoff value of 2.2. We compared concordance rates before and after 2007 guidelines. For the 2003–2007 study population, the concordance rate between the assays was 97.6% with a corresponding kappa coefficient (*k*) of 0.90. For the 2008–2010 study population, concordance rate was 97.6% with a corresponding *k* of 0.89. There was no significant difference in number of inconclusive rates before and after 2007 guidelines. In our study, implementation of the new ASCO/CAP 2007 HER2 guidelines did not show a significant difference in concordance rates and did not decrease the number of inconclusive cases.
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Human epidermal growth factor receptor 2 gene, *ERBB2* (frequently referred to as *HER2*), is a proto-oncogene located on chromosome 17, with a resultant 185-kDa glycoprotein. HER2 protein overexpression was initially recognized as a prognostic marker of poor clinical outcome.[1–3] This is characterized by amplification of the *ERBB2* gene and accompanied by abnormally high levels of the glycoprotein.[4,5]

The discovery of trastuzumab (Herceptin), a monoclonal antibody to HER2 for the treatment of HER2-positive breast cancer, heralded the additional significance of HER2 as a critical predictive marker in patients with invasive breast cancer who can

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benefit from this therapy.[3] Studies have validated the efficacy of trastuzumab in HER2-positive breast cancer in both adjuvant and metastatic settings.[6–8] Furthermore, HER2 overexpression has been found to confer a relative resistance to endocrine therapies.[9] Thus, the accurate assessment of HER2 status is essential in the clinical treatment algorithm for patients with breast cancer.

In 2007, the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) proposed new recommendations for HER2 immunohistochemistry and *ERBB2* fluorescence *in-situ* hybridization scoring in an effort to improve accuracy of these companion diagnostic tests as a predictive marker for patients with invasive breast cancer.[10] The goal of the new guidelines was to improve the concordance rate between the diagnostic tests for HER2 and to decrease the number of inconclusive cases.

Several publications have focused on the concordance rates between HER2 immunohistochemistry and *ERBB2* fluorescence *in-situ* hybridization analysis, concentrating mainly on the analysis of different methods and the level of agreement between *ERBB2*-testing platforms.[11–14] At our institution, all cases of patients with invasive breast carcinoma undergo both HER2 immunohistochemical staining and *ERBB2* gene fluorescence *in-situ* hybridization testing. The objective of this study is to assess the impact of ASCO/CAP 2007 guidelines on HER2 concordance rates and number of inconclusive cases in a single large institution.

Materials and methods

Samples

Formalin-fixed, paraffin-embedded human breast cancer tissue specimens from 1437 patients with invasive breast carcinoma were used in the study. Both immunohistochemical analysis for HER2 protein and fluorescence *in-situ* hybridization for *ERBB2* gene were performed on all specimens from our facility at David Geffen University of California at Los Angeles (UCLA) Medical Center from years 2003 through 2010 ($n=1437$). All samples were collected from 2003–2010, with testing results available for each sample. Optimal tissue handling requirements (eg, time to fixation) were followed and recorded, especially after publication of the ASCO/CAP guidelines, on 1 January 2008. To decrease pre-analytic variables in testing, optimal internal validation procedures, internal quality assurance procedures, external proficiency assessment, laboratory accreditation, and immunohistochemistry and fluorescence *in-situ* hybridization testing requirements were met in accordance with the ASCO/CAP guideline recommendations. A retrospective review of data was performed. No further testing has been done in relation to the study. An application to the Internal Scientific Peer Review Committee was submitted and approved. Subsequently, the study was conducted according to Office of Human Research Protection Program, and was approved by the University of California at Los Angeles Institutional Review Board.

Immunohistochemical Analysis

The US FDA-approved HercepTest was performed using DAKO A0485 polyclonal antibody kit (DAKO Corp, Carpenteria, CA, USA). Cases from 2003–2007 ($n=1016$) were scored by US Food and Drug Administration (US FDA) guidelines before publication of ASCO/CAP guidelines, with immunohistochemistry of 3+ cases staining >10% of tumor cells. The new ASCO/CAP guidelines were implemented and scored accordingly for cases from 2008–2010 ($n=421$), into three categories: negative for HER2 protein overexpression (scores 0 and 1+), indeterminate (2+), and positive for HER2 protein overexpression (3+).

Score 0: No staining is observed in invasive tumor cells.

Score 1: Weak, incomplete membrane staining in any proportion of invasive tumor cells, or weak, complete membrane staining in less than 10% of cells.

Score 2: Complete membrane staining that is nonuniform or weak but with obvious circumferential distribution in at least 10% of cells, or intense complete membrane staining in 30% or less of tumor cells.

Score 3: Uniform intense membrane staining of more than 30% of invasive tumor cells.

Fluorescence *In-Situ* Hybridization Analysis

Fluorescence *in-situ* hybridization was performed using the US FDA-approved PathVysion *HER-2* DNA Probe Kit (PathVysion Kit), which is designed to detect amplification of the *ERBB2* gene via fluorescence *in-situ* hybridization in formalin-fixed, paraffin-embedded human breast cancer tissue specimens. Fluorescence *in-situ* hybridization analysis with VYSIS dual-color probes specific for chromosome 17 centromere and the *ERBB2* gene (17q11.2) was performed and examined by two independent technologists, and signed out by clinical pathologists (non-surgical anatomic pathologists) at the UCLA Cytogenetics Laboratory. Slides containing 4 μm sections were submitted for fluorescence *in-situ* hybridization analysis. For each slide, based on the corresponding hematoxylin and eosin slide, the invasive tumor area(s) was circled with a secureline marker. Areas containing ductal carcinoma *in-situ* or normal tissue were excluded from fluorescence *in-situ* hybridization testing, as HER2 protein overexpression and *ERBB2* gene amplification are seen more frequently in ductal carcinoma *in-situ* (50–60%) than in invasive carcinoma of the breast

Table 1 Correlation of HercepTest and HER2 immunohistochemical status *vs ERBB2* gene status by PathVysion fluorescence *in-situ* hybridization based on US Food and Drug Administration Guidelines (2003–2007)

HER2 immunohistochemical status	*ERBB2 fluorescence in-situ hybridization*		*Total,* n *(%)*
	Non-amplified, n *(%)*	*Amplified,* n *(%)*	
Negative (0, 1+)	756 (74)	21 (2)	777 (76)
Equivocal (2+)	69 (7)	52 (5)	121 (12)
Positive (3+)	0 (0)	118 (12)	118 (12)
Total	825 (81)	191 (19)	1016 (100)

Table 2 Correlation of HercepTest and HER2 immunohistochemical status *vs ERBB2* gene status by PathVysion fluorescence *in-situ* hybridization based on American Society of Clinical Oncology/College of American Pathologists Guidelines (2008–2010)

HER2 immunohistochemical status	*ERBB2 fluorescence in-situ hybridization*		*Total,* n *(%)*
	Non-amplified, n *(%)*	*Amplified,* n *(%)*	
Negative (0, 1+)	324 (77)	6 (2)	330 (79)
Equivocal (2+)	32 (7)	15 (4)	47 (11)
Positive (3+)	3 (1)	41 (9)	44 (10)
Total	359 (85)	62 (15)	421 (100)

(25–30%).[15] Slides were baked overnight at 60 °C and pretreated using the VP2000 tissue processor as per manufacturer's protocol (Abbott Molecular, Abbott Park, IL, USA). Amplification of the *ERBB2* gene was detected by using the PathVysion Kit; the instructions in the package insert were followed for the hybridization, post-hybridization washing, and analysis steps (Abbott Molecular).

Cases from 2003–2007 ($n=1016$) were scored according to US FDA guidelines for HER2 immunohistochemistry and using *ERBB2* gene fluorescence *in-situ* hybridization amplification cutoff value of 2.0 (Table 1). The new ASCO/CAP guidelines were implemented and scored accordingly for cases from 2008–2010 ($n=421$) (Table 2). *ERBB2* gene amplification was defined as *ERBB2*/CEP17 ratio of ≥ 2.2. For the purposes of this study, no amplification was defined as an *ERBB2*/CEP17 ratio of <2.2.

A comparison of the data between 2003–2007 using US FDA Guidelines and 2008–2010 using ASCO/CAP Guidelines is shown (Table 3).

Statistical Analysis

The calculation of concordance rates and k between the HER2 immunohistochemical analysis and *ERBB2* fluorescence *in-situ* hybridization assays, and χ^2-tests, were performed on SPS software. The results of discordant cases were included in the data analysis before trouble shooting.

Table 3 Summary table with comparison of data between 2003–2007 scored by US Food and Drug Administration Guidelines and 2008–2010 Scored by American Society of Clinical Oncology/College of American Pathology Guidelines

Immunohistochemistry	*2003–2007 US FDA Guidelines (*n = *1016) (% = number of cases/total* n*)*		*2008–2010 ASCO/CAP Guidelines (*n = *421) (% = number of cases/total* n*)*	
	FISH unamplified, n *(%)*	*FISH amplified,* n *(%)*	*FISH unamplified,* n *(%)*	*FISH amplified,* n *(%)*
0	346 (34.1)	3 (0.3)	121 (28.7)	2 (0.5)
1+	410 (40.3)	18 (1.8)	203 (48.3)	4 (0.9)
2+	69 (6.8)	52 (5.1)	32 (7.6)	15 (3.6)
3+	0 (0)	118 (11.6)	3 (0.7)	41 (9.7)
Total	825 (81.2)	191 (18.8)	359 (85.3)	62 (14.7)

Results

Concordance Rates

Overall, our concordance rate between non-equivocal HER2 immunohistochemical analysis and *ERBB2* fluorescence *in-situ* hybridization results was 97.6%. Thirty of 1269 (2.4%) non-equivocal total cases displayed immunohistochemical scores discordant with fluorescence *in-situ* hybridization results, ie, 27 cases had negative immunohistochemical scores but positive (amplified) fluorescence *in-situ* hybridization results (false negative), while 3 cases had positive immunohistochemical scores with negative (non-amplified) fluorescence *in-situ* hybridization results (false positive).

For the 2003–2007 study population, the concordance between the HER2 immunohistochemical analysis and *ERBB2* fluorescence *in-situ* hybridization assays was 97.6% with a k of 0.90, corresponding to near perfect agreement (Table 4). The equivocal immunohistochemical 2+ cases were excluded from analysis ($n=121$; 12% of cases). For the 2008–2010 study population, the concordance between the two assays was 97.6% with a k of 0.89 (Table 4). The equivocal immunohistochemical 2+ cases were excluded from analysis ($n=47$; 11% of cases). Thus, the k between cases scored in 2003–2007 *vs* 2008–2011, $k=0.90$ *vs* $k=0.89$, were essentially similar. This was reflected in the identical concordance rates of 97.6% between cases scored in 2003–2007 *vs* 2008–2010.

Changes in Percentages of Inconclusive/Equivocal and Positive Cases

Overall, cases with inconclusive immunohistochemical score of 2+ comprised 168 of the total 1437

Table 4 Comparison of false-positive rates, false-negative rates, HER2 immunohistochemistry and *ERBB2* fluorescence *in-situ* hybridization testing concordance rates (excluding 2+ equivocal/inconclusive cases), and Kappa coefficients based on US Food and Drug Administration and American Society of Clinical Oncology/College of American Pathologists Guidelines (2003–2007) and (2008–2010)

	2003–2007	*2008–2010*
False-positive rate (IHC+/FISH non-amplified)	0%	0.7% (3/421)
False-negative rate (IHC-/FISH amplified)	2.1% (21/1016)	1.4% (6/421)
HER2 and *ERBB2* testing concordance rate	97.6% (995/1.016)	97.6% (412/421)
Kappa coefficient	0.90	0.89

(11.7%). The percentage of inconclusive/equivocal cases was 12% (121/1016 cases) using US FDA guidelines and was 11% (47/421 cases) using ASCO/CAP guidelines. A χ^2-test demonstrated no significant decline in inconclusive/equivocal cases ($P=0.64$).

Overall, cases with positive HER2 immunohistochemical score of 3+ comprised 162 of the total 1437 (11.3%). The percentage of positive cases by immunohistochemistry decreased from 12% (118/1016) to 10% (44/421) after implementation of ASCO/CAP guidelines, albeit not reaching statistical significance ($P=0.46$). However, a change in the scoring of *ERBB2* fluorescence *in-situ* hybridization cases resulted in a decrease of *ERBB2*-amplified cases from 19% (191/1016) to 15% (62/421), which is significant ($P=0.03$) (Table 5).

Table 5 Comparison of Data of HER2 HercepTest equivocal/inconclusive cases, HER2 HercepTest-positive cases, and *ERBB2* fluorescence *in-situ* hybridization-amplified cases between 2003–2007 (Food and Drug Administration Guidelines) and 2008–2010 (American Society of Clinical Oncology/College of American Pathologists Guidelines)

	2003–2007	*2008–2010*	P *value*
HER2 immunohistochemistry 2+ equivocal (cases/total)	12% (121/1016)	11% (47/421)	0.64
HER2 immunohistochemistry 3+ positive (cases/total)	12% (118/1016)	10% (44/421)	0.46
ERBB2 fluorescence *in-situ* hybridization-amplified cases (cases/total)	19% (191/1016)	15% (62/421)	0.03

Discordant Results and Comparison between US FDA Guidelines and ASCO/CAP Guidelines

Overall, when fluorescence *in-situ* hybridization result was considered as the gold standard, the false-negative rate was 27 cases of 1437 (1.9%), ie, cases which were negative on immunohistochemistry and positive (amplified) on fluorescence *in-situ* hybridization analysis (5 cases scored immunohistochemically as 0; 22 cases scored immunohistochemically as 1+) in our entire study population from 2003–2010. The false-positive rate was 3 cases of 1437 (0.2%); three cases were positive on immunohistochemistry (3+) yet proved to be negative (non-amplified) by fluorescence *in-situ* hybridization analysis.

US FDA Guidelines (2003–2007)
Of 1016 total cases, the false-negative rate was 2.1% ($n=21$) and the false-positive rate was 0% (Table 4).

ASCO/CAP Guidelines (2008–2010)
Of 421 total cases, the false-negative rate was 1.4% ($n=6$) and false-positive rate was 0.7% ($n=3$) (Table 4).

Discussion

Interlaboratory discordance rates for HER2 immunohistochemistry and *ERBB2* fluorescence *in-situ* hybridization testing have been reported to be as high as 20%.[16,17] In order to address the issue of HER2 and *ERBB2* test accuracy, the American Society of Clinical Oncology and the College of American Pathologists reviewed the existing literature on testing, and devised and published a guidance document in 2007 (ASCO/CAP 2007 guidelines), which included the modification of analysis of immunohistochemistry and fluorescence *in-situ* hybridization tests. It also contained the recommendation of 95% concordance rate for immunohistochemical testing for HER2 with another validated test such as fluorescence *in-situ* hybridization.[10] Since 2003, both immunohistochemistry and fluorescence *in-situ* hybridization tests have been performed at our institution regardless of HercepTest immunohistochemical scores. Thus, this allowed us to investigate testing concordance rates of HER2 immunohistochemical and *ERBB2* fluorescence *in-situ* hybridization analysis utilizing the US FDA guidelines from years 2003–2007, and subsequently the ASCO/CAP guidelines for testing from years 2008–2010. The purpose of this study was to examine the merit of changing the HER2 and *ERBB2* testing guidelines in order to improve concordance rates and to decrease the number of inconclusive cases (scored as 2+ on immunohistochemical analysis).

The present study indicates that our institution has excellent and identical concordance rates of 97.6% for HercepTest HER2 immunohistochemistry and PathyVysion *ERBB2* gene fluorescence *in-situ* hybridization testing utilizing both US FDA and ASCO/CAP guidelines. These rates are in accordance with the recommendation that any two diagnostic companion tests establish a concordance rate of >95%.[10]

The accuracy of HER2 immunohistochemistry is reported to be highly dependent upon both preanalytical and analytic factors. Although fixation

times and optimal tissue handling procedures were only strictly imposed after publication of the ASCO/CAP guidelines in 2007, our identical concordance rates suggest that there is minimal to no interference by pre-analytic and analytic variables. We observed a similar lack of discordance in immunohistochemical accuracy when we changed fixative types, fixation times, and ischemia times for HER2 and other hormonal receptor studies for breast cancer.[18,19]

On the basis of the change in guidelines for scoring 3+ immunohistochemical positivity in 10–30% of tumor cells, one would expect an increase in equivocal (2+) cases as well as a decrease in positive (3+) cases given the more stringent cutoff. Middleton *et al*[20] reported a 64% reduction of inconclusive cases, from 10.8–3.4%, and an increase in their concordance rate from 98–98.5% after implementation of ASCO/CAP guidelines, supporting the change in *ERBB2* testing guidelines. In our patient population, however, the percentage of inconclusive/equivocal cases by immunohistochemistry was 12% with US FDA guidelines and 11% with ASCO/CAP guidelines. Our study did not disclose a significant decline in inconclusive immunohistochemistry results.

Moreover, changing the fluorescence *in-situ* hybridization cutoff value for *ERBB2* gene amplification from 2.0–2.2 based on ASCO/CAP guidelines, one would expect fewer *ERBB2*-amplified cases. Indeed, we did see a significant decline in cases considered to be *ERBB2* amplified from 19–15% ($P=0.03$). Similarly, Atkinson *et al*[21] found 3.3% fewer *ERBB2*-positive cases when the new guidelines were implemented. Although the decrease in *ERBB2*-amplified cases was expected, the considerable decline in amplified cases may not be because of a change in fluorescence *in-situ* hybridization cutoff values alone. It may also be secondary to a change in our patient population; indeed, we have noticed a steady decline in *ERBB2*-positive breast cancers in our patient population, with a cumulative 7% reduction over the past 8 years (Table 6). This may represent an overall shift in patient populations, wherein earlier studies suggested that as many as 30% of breast cancers displayed HER2 overexpression.[3] Subsequent studies, however, have displayed a lower percentage of HER2-positive cases, from 15–25% of breast cancers to as low as 7%.[22,23]

A small number of our cases were rated as negative by HercepTest immunohistochemistry and yet demonstrated amplification by PathVysion fluorescence *in-situ* hybridization, thus considered as a false-negative result. Using a stepwise algorithm of performing immunohistochemistry first, then subsequent fluorescence *in-situ* hybridization testing based on immunohistochemistry results, a small percentage of samples would inevitably be scored as falsely negative, thereby inappropriately excluding these patients from trastuzumab (Herceptin) therapy. An estimated 3–4% of primary *ERBB2* testing are falsely negative.[24] Excluding patients because of a falsely negative result would be quite disadvantageous, as the addition of Herceptin therapy can reduce the recurrence risk by half and mortality by a third in early stage breast cancer patients.[17] In our study, the overall false-negative rate is 1.9%, well below the aforementioned 3–4% false-negative rate. Following the ASCO/CAP guidelines did yield a slight decrease in the false-negative rate from 2.1–1.4%.

Table 6 Percentage of *ERBB2* FISH-amplified cases per year from 2003 to 2010

2003	*2004*	*2005*	*2006*	*2007*	*2008*	*2009*	*2010*
21%	26%	16%	15%	16%	11%	12%	14%

On the other hand, an exceedingly small percentage of patients (0.2%) were scored as falsely positive, ie, rated as positive on immunohistochemistry, and yet proved to be unamplified by fluorescence *in-situ* hybridization. These cases of overexpression by immunohistochemistry were secondary to polysomy of chromosome 17, and not *ERBB2* gene amplification. Polysomy of chromosome 17 is a well-documented cause for false-positive immunohistochemical results, accounting for up to 2.8% of HER2 immunohistochemically positive cases in one study.[25] The utility of targeted Herceptin therapy on polysomy 17 cases is unknown, and the current data regarding response to therapy is scarce at the present time. Elucidation of effectiveness of therapy in this setting is particularly important. Trastuzumab therapy is not without adverse effects and can even lead to significant cardiotoxicity, especially when combined with other chemotherapy agents, such as anthracyclines.[26] We attribute the increase in false-positive rates from 0–0.7% from 2003–2007 to 2008–2010, respectively, solely to three cases of polysomy 17, and not because of a change in test scoring as based on ASCO/CAP guidelines.

We ascribe our high concordance rates to several factors:

(1) High volume of HER2 testing.
(2) HER2 immunohistochemistry interpretation and scoring are performed by breast subspecialty anatomic pathologists only (three pathologists at our institution).
(3) One hematoxylin and eosin slide is submitted to the Cytogenetics Laboratory on all cases, and the area of invasive cancer is marked with permanent ink. This ensures that the cytogenetic technologists and clinical pathologists focus on scoring *ERBB2* fluorescence *in-situ* hybridization in the designated areas of invasive carcinoma only, and excludes areas of normal breast tissue or ductal carcinoma *in-situ* component.

We recognized in our study that the most common type of discrepancy arose from immunohistochemistry-negative/fluorescence *in-situ* hybridization-positive cases (false negative). This is in contrast to a study by Grimm *et al*,[27] which showed that the most common type of discrepancy was immunohistochemistry positive/fluorescence *in-situ* hybridization-negative (false positive) cases because of over-interpretation of immunohistochemistry positivity. The causes of false-positive interpretational error identified in their study included cautery artifact, chatter artifact, granular staining, and over-interpretation of complete circumferential membrane staining—all of which were not problematic in our current study. Most of our false-negative cases (immunohistochemistry negative/fluorescence *in-situ* hybridization positive) were due to underestimating the score to 1+ rather than 2+, which is also an interpretational error rather than a technical error. In rare cases, where immunohistochemistry score was interpreted as '0' and subsequently proven to be fluorescence *in-situ* hybridization amplified, we speculated that a technical problem was at fault. In many of these false-negative cases, as a matter of troubleshooting, we would repeat immunohistochemical testing on the same tissue block, and additionally perform immunohistochemical analysis on a different block from the tissue containing invasive carcinoma. In the end, these cases would continue to be scored as 0 or 1+ on the repeat testing of additional samples. One of these cases was owing to monosomy 17 where fluorescence *in-situ* hybridization was reportedly amplified.

On the basis of our study, we would advocate performing fluorescence *in-situ* hybridization analysis in addition to immunohistochemistry testing to capture the small percentage of patients who may benefit from trastuzumab therapy and to exclude those who do not show true *ERBB2* amplification. We did not retrospectively review immunohistochemistry slides for changes in scoring cutoff values as the concordance rates utilizing US FDA and ASCO/CAP guidelines were essentially identical. Of interest, pre-analytic variables such as fixation time and ischemic time did not alter the concordance rates between US FDA and ASCO/CAP guidelines. Additionally, the number of inconclusive 2+ immunohistochemical cases did not show a substantial decline, contrary to the intent of the change in ASCO/CAP guidelines. To our knowledge, our study is the largest study that examines the impact of the implementation of ASCO/CAP guidelines on HER2 immunohistochemistry and *ERBB2* gene fluorescence *in-situ* hybridization testing concordance rates. At our institution, implementation of the new ASCO/CAP scoring guidelines did not significantly influence HER2 testing concordance rates nor was there a decrease in immunohistochemically equivocal/inconclusive cases.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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