Original Study

The Impact of Partial Weak Staining in Normal Breast Epithelium on the Reliability of Immunohistochemistry Results in HercepTest-positive Breast Cancer

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Abstract

We studied the significance of human epidermal growth factor receptor-2 (HER2) staining in normal epithelium and its relationship to HER2 amplification by fluorescence in situ hybridization (FISH) in breast cancer. A total of 154 cases with a normal gland component were stained for HER2 by immunohistochemistry and FISH. Thirty-four cases showed staining in normal glands, 9 of which were FISH non-amplified. This shows a strong association between normal epithelial staining and lack of HER2 amplification by FISH.

Introduction: Although normal epithelial cells do not show human epidermal growth factor receptor-2 (*HER2*) gene amplification and should lack membrane staining by *HER2* immunohistochemistry (IHC), *HER2* staining in benign breast epithelium is occasionally encountered. The significance of this occurrence has not yet been adequately studied, and its associated American Society of Clinical Oncology/College of American Pathologists recommendations are vague. Our objective is to assess the correlation between *HER2* IHC 3+ breast cancer cases with normal epithelium staining (NES) and their corresponding fluorescence in situ hybridization (FISH) results, and to suggest recommendations for interpretation. **Materials and Methods:** A total of 154 breast cancer cases with *HER2* IHC 3+ were reviewed. NES, along with other clinicopathologic characteristics, were recorded. NES was scored as present or absent. All study cases were sent for FISH testing. All cases, and particularly those that showed false positivity for IHC (positive IHC, negative FISH) were examined for NES. **Results:** Of the 154 cases, 146 cases were FISH-positive (94.8%) and 2 failed FISH testing (1.3%). Conversely, 22% (34/154) of the cases showed NES for *HER2*. Of these 34 cases, 23 (67%) were FISH-amplified, 9 (26%) were FISH not amplified, and 2 failed FISH testing. Notably, all of the false-positive (FISH-negative) breast cancer cases showed some degree of positivity in normal breast epithelium. **Conclusions:** Our findings, though descriptive, show a very strong association between NES and false-positive HER2 IHC. This confirms the need to carefully evaluate IHC-positive breast cancers for NES, and to have a low threshold for confirmatory testing by FISH.

Clinical Breast Cancer, Vol. 19, No. 5, 340-4

Keywords: ASCO/CAP recommendations, False positive HER2, Fluorescent in-situ hybridization, *HER2* gene amplification, Normal breast epithelium

Introduction

Human epidermal growth factor receptor-2 (*HER2*) is a proto-oncogene located on chromosome 17q and expressed at low levels in a variety of normal epithelia including mammary epithelium.[1-3] The role of the *HER2* gene product in the pathogenesis of a subgroup of breast cancers was uncovered by Slamon et al in 1987, and has since spawned an entire era in the modern diagnosis and targeted treatment of breast cancer.[4] *HER2*'s biologic significance lies in its amplification and protein overexpression, which significantly

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Submitted: Dec 10, 2018; Revised: Apr 24, 2019; Accepted: Apr 28, 2019; Epub: May 18, 2019

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1526-8209/$ - see frontmatter
https://doi.org/10.1016/j.clbc.2019.04.017

enhances tumor cell growth and proliferative potential.[4,5] Affecting a substantial proportion of breast cancers (20%-25%), *HER2* overexpression has been associated with high histologic tumor grade, tumor recurrence, and shortened patient survival. Fortunately, positive *HER2* status has been more recently shown to predict response to both conventional and targeted (eg, Trastuzumab) therapy, improving the prognosis of these intrinsically aggressive neoplasms.[6,7] It hence follows that validated and standardized testing modalities to accurately assess *HER2* status are vital for correct patient stratification and management.[6-8] The most common methods used to currently evaluate *HER2* expression in tumors are immunohistochemistry (IHC) and fluorescent in situ hybridization (FISH). IHC detects surface protein overexpression, whereas FISH detects gene amplification.[9-12] IHC and FISH are generally considered equivalent for unequivocally positive and negative results. Absent/weak (IHC 0/1+) and strong (IHC 3+) *HER2* immunostains are hence highly correlated with gene amplification status by FISH, the latter being the most accurate determinant of response to targeted Trastuzumab therapy.[9,13]

Despite established clinical utility and continuously improved methodology over the past couple of decades, testing by IHC and FISH still suffers from a number of inaccuracies.[3] One example is the occasional immunohistochemical reactivity of non-neoplastic breast epithelium to *HER2* antibodies. This phenomenon, generally regarded as artifactual, is thought to imply that the tumor may be overstained, casting significant doubt on the reliability of *HER2* status interpretation.[3,14] Although the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) recommended in its 2007, 2013, and 2018 guidelines, rejecting any sample with strong membrane staining of normal breast epithelium by IHC, they made no mention of focal weak (+1) staining in normal epithelium.[6,15,16] Other authors have suggested subtracting the degree of staining observed in normal breast epithelium from the staining score observed in breast cancer cells in the same section, and applying the testing algorithm accordingly.[17-20] Consensus and clear guidelines remain elusive, however.

The purpose of this study is to carefully assess the significance of weak normal epithelium staining (NES) in *HER2* IHC 3+ cases, and how this finding affects their corresponding FISH amplification results compared to cases without NES, under the most recent ASCO/CAP scoring and interpretation guidelines. By verifying the significance of the NES phenomenon on *HER2* IHC studies, we aim first to test whether a tumor's final *HER2* status can be predicted by its histologic and staining characteristics, and second to suggest a more detailed list of recommendations than the ones currently provided by the ASCO/CAP consortium.

Materials and Methods

Samples

A total of 154 breast cancer resection specimens from our institution were reported as *HER2* IHC 3+ between January 2005 and March 2011. These cases were reviewed and ensured to have a normal duct component on the *HER2* stained slide. *HER2* status was re-evaluated according to the revised ASCO/CAP guidelines of 2018.[16] FISH was performed on all cases to determine their *HER2* amplification status. Cases were specified as samples with IHC 3+ *HER2* status having NES, whereas controls were *HER2* IHC 3+ and lacked NES. Lack of NES required complete absence of membranous staining in normal breast epithelium. Weak NES was considered present when any percentage of normal breast epithelium showed weak membranous staining (only visible on $> 10\times$ magnification objective). Strong NES was considered present when any percentage of normal breast epithelium showed strong membranous staining (visible on $< 10\times$ magnification objective). The quantitative assessment of NES was not taken into consideration as it was deemed to decrease reproducibility, and because the amount of benign epithelium was small in many cases. Particular attention was given to unusual patterns of staining such as membranous granularity, heterogeneity, or concomitant strong cytoplasmic staining.

The study was approved by the Institutional Review Board at the American University of Beirut. All clinical investigations were conducted in accordance with the Declaration of Helsinki.

Immunohistochemical Analysis

Immunohistochemical analysis was performed as mentioned by the manufacturer using the HercepTest kit (Dako). Paraffin slides (3-μm) were placed overnight in an oven at 37°C. These were then deparaffinized in xylene, rehydrated in graded alcohol, incubated in citrate buffer at 95°C (in a water bath) for 20 minutes for antigen retrieval, and then washed in water for 5 minutes. Then they were placed on an immunostainer using the primary polyclonal antibody and polymer detection system. Finally, the slides were placed in hematoxylin for 1 minute, dehydrated in graded alcohol, cleared in xylene, and cover slipped. Tumors were scored as 0, 1+, 2+, or 3+ according to ASCO/CAP guidelines. Staining in normal glands was documented as present or absent. All cases were sent for FISH testing.

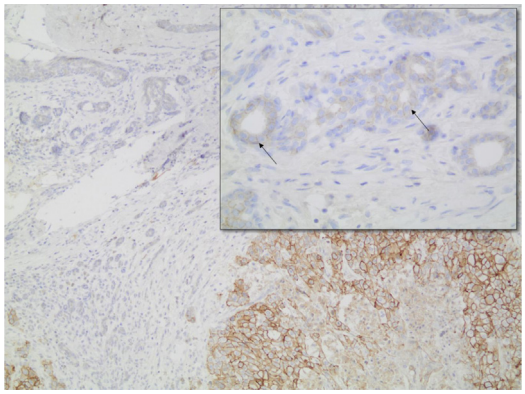
FISH

FISH was performed as specified by the manufacturer using the PathVysion kit (Vysis, Downers Grove, IL). Thick paraffin sections (4-μm) were cut on organosilane-coated slides, deparaffinized, and pretreated in 0.2N hydrochloric acid and sodium thiocyanate. Then protease solution was added at 37°C for 10 minutes. The slides were washed and fixed in neutral buffered formalin for 10 minutes. Then they were washed, dried, and denatured in 70% formamide standard saline citrate, and hybridized with the *HER2*/*CEP17* dual-probe mixture. The slides were dried, and stained with 4,6-diaminido-2-phenylindole dihydrochloride (DAPI), and cover slipped. Finished slides were stored at −20°C and evaluated within 24 hours. FISH slides were scored according to the ASCO/CAP guidelines. Total orange (*HER2*) and green (*CEP17*) signals were recorded, as was the *HER2*/*CEP17* ratio.

Results

All 154 patients were female with a median age at diagnosis of 55.5 years. Of the 154 cases, 146 (94.8%) cases were FISH-positive, and 2 (1.3%) failed FISH testing. Regarding NES, 22% (34/154) of the cases showed weak NES for *HER2*. Of these 34 cases, 23 (67%) were FISH-amplified, 9 (26%) were FISH not amplified, and 2 failed FISH testing. No cases showed strong NES. Of the 9 negative cases, 4 were grade 3, 4 were grade 2, and one was grade 1. None of the FISH-negative cases exhibited unusual patterns of staining such

Figure 1 A Photomicrograph Illustrating Normal Breast Gland Staining (Inset) in Association With a *HER2* Amplified Carcinoma



as membranous granularity or concomitant strong cytoplasmic staining. All cases showed diffuse positivity in essentially all tumor cells without significant staining heterogeneity. A photomicrograph illustrating normal gland staining in association with a *HER2* amplified carcinoma is shown in Figure 1.

Discussion

A review of the literature regarding *HER2* IHC and FISH testing reveals that, despite their widespread popularity, both modalities remain undeniably controversial and still suffer from a number of technical shortcomings.[7,21-26] Not only can each test have an error rate of up to 20%,[7,27] disagreement between different laboratories may reach 18% for IHC and 12% for FISH.[28] This is primarily owing to the tests' high dependence on both pre-analytical and analytical factors, from pre-fixation ischemic time, to duration and type of fixation, to the specific antibody used, to the experience of the interpreting pathologist/technologist.[29]

When examined for concordance, the vast majority of published papers report highly concordant results between FISH results and IHC scores of 0, 1+, and 3+.[1,7,30,31] Few reports, however, still detected a significant rate of IHC false positivity (IHC 3+, FISH-negative) ranging from 5% to a striking 22%.[32,33] This, in turn, was attributed to variability in tissue fixation and processing, intra-tumoral heterogeneity, and polysomy of chromosome 17.[34,35] All testing conditions being equal, one may still encounter over-expression of the *HER2* protein without gene amplification in at least 3% of breast cancers, as described by Pauletti et al, giving rise to "pseudo-false positive" results, the significance of which remains unclear.[9,36]

Although the ASCO/CAP guidelines do not require FISH confirmation for IHC 3+ tumors, and some studies have shown response to anti-*HER2* therapy in IHC-positive/FISH-negative tumors,[37] several studies have shown that response to Herceptin was better predicted by FISH amplification.[13,38] Short of equating *HER2* positivity with gene amplification, cases showing discrepant *HER2* IHC and FISH should be handled on a case-by-case basis, and expectations of response to treatment should be guarded.

Staining in normal epithelium, the additional potential confounder under evaluation in this study, has been addressed in very few manuscripts, using the United States Food and Drug Administration scoring system. The authors of these few studies adopted subtraction of the staining score in non-neoplastic glands from the score in neoplastic cells to reach a final *HER2* score, resulting in a significant decrease in false-positive results.[18-20] The effect was, however, markedly inflated by the fact that both 2+ and 3+ scores were considered positive by United States Food and Drug Administration criteria. No papers have yet explored the effect of NES on the interpretation of test results using both IHC and FISH in IHC 3+ tumors under the most recent ASCO/CAP scoring and specimen handling recommendations.[6] Although ASCO/CAP guidelines have improved the concordance level between IHC and FISH results,[7] some practically relevant points remain unclear. Because strong staining is associated with gene amplification, which should essentially be absent in all normal epithelium, the ASCO/CAP recommended rejecting any sample with strong normal gland staining by *HER2* IHC. However, the guidelines did not specifically mention weak staining in normal epithelium. This leaves the general pathologist in a precarious position regarding IHC 3+ tumors with any measure of staining within non-neoplastic epithelium.

Our findings are notable in more than one way. First, the relative frequency of weak NES, which is quite likely to go unnoticed if faint and focal, as is frequently the case, especially in a *HER2* 3+ tumor, was not an expected finding. The absence of strong NES was, on the other hand, expected, given that it represents an ASCO/CAP criterion for rejection. Second, and more importantly, NES was present in all cases that showed a discrepant (false positive) result. This is not to infer any causality between NES and false-positive *HER2* IHC results, as two-thirds of cases with NES were actually FISH amplified. However, it serves as a flag for cases that might benefit from confirmatory FISH testing. It is worth noting that the vast majority (8/9) of false-positive cases were of grades 2 and 3. The fact that grade 1 tumors are much less likely to be *HER2* FISH-amplified is therefore not a significant confounding variable in our series. Other confounding factors linked to the fact that *HER2* IHC interpretation often lends itself to inter-observer variability were addressed by careful review and scoring of all the cases with special attention to misleading staining patterns such as membranous granularity, prominent cytoplasmic staining, and staining heterogeneity. None of these factors appeared to contribute to an erroneously high *HER2* IHC score in any of the cases under study.

It should also be mentioned that our concordance rate of 94.8% between IHC 3+ and *HER2* FISH-amplified accurately recapitulates the percentages available in the literature, lending credence to the quality and reliability of our CAP-accredited laboratory and its adherence to the most recent ASCO/CAP recommendations. The above observations further underscore the importance of identifying NES in *HER2* tested specimens. It also reinstates the need for more specific guidelines regarding the interpretation and significance of NES. In our opinion and based on our results, weak NES should be actively looked for in any *HER2* IHC case, be it a biopsy or an excision specimen, as normal glands

are often inconspicuous on a counterstained IHC slide. Comparison with the corresponding hematoxylin and eosin stain may be helpful in finding these glands.

In case *HER2* testing is to be performed on the surgical specimen, care should be given to selecting a block that includes normal breast epithelium, not just a representative tumor sample. Although arguably difficult to implement, consideration should be given to performing confirmatory FISH testing in cases that show a *HER2* IHC score of 3+ while no normal glands are identified. Finally, any staining in a normal gland should be considered positive for NES. Although a present versus absent approach to evaluating NES may appear a little crude, we could not, given the relative small size of our sample, quantify NES in any practical or useful scheme that would be both reproducible and meaningful with respect to our results.

Even though the limitations of *HER2* IHC may sometimes seem prohibitive, and some authors have suggested the use of FISH alone to bypass the inaccuracies and technical hurdles of IHC,[36] FISH remains an expensive alternative that requires more expertise, and results in significantly longer turnaround times. Its likelihood of supplanting IHC is therefore questionable at this time.

From a clinical perspective, we should remember that a false-positive *HER2* test has more negative impact than just ascribing a worse prognosis to a breast neoplasm. Targeted therapy is financially burdensome and not devoid of side effects, as it carries potential cardiotoxicity, which is compounded by combination with anthracyclins, whose therapeutic effects it amplifies.[9,35]

Conclusion

This study sheds more light on an insufficiently known or studied occurrence of *HER2* staining in normal breast epithelium. Our findings show a non-negligible frequency of this often-subtle phenomenon, and an association with discordance between *HER2* IHC and FISH. We therefore suggest careful assessment of benign epithelium for aberrant IHC staining, and a low threshold for FISH confirmation in such cases. Also, we hope these findings will be tested in future studies and will lead to further recommendations and clarifications by the frequently revisited ASCO/CAP recommendations.

Clinical Practice Points

- HER2 staining by IHC in normal breast epithelium is a poorly described phenomenon as far as frequency and implications.
- The ASCO/CAP guidelines recommend rejecting HER2 IHC with strong staining in normal epithelium, but they do not give recommendations regarding weak staining in normal epithelium, and how to interpret it when the tumor is HER2 overexpressed (3+).
- Our findings show that weak staining in normal breast epithelium is highly correlated with discordant IHC and FISH, casting doubt on the reliability of HER2 status by IHC in the presence of staining in normal breast epithelium.
- Normal breast epithelium should be carefully examined as an internal negative control, and the presence of any degree of staining should prompt further investigation, and, ideally, FISH testing of the tumor for confirmation of HER2 amplification.

Disclosure

The authors have stated that they have no conflicts of interest.

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