The Diagnostic Utility of the Minimal Carcinoma Triple Stain in Breast Carcinomas

Dara S. Ross, MD, Yi-Fang Liu, Jennifer Pipa, and Sandra J. Shin, MD

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Abstract

Pathologists are expected to accurately diagnose increasingly smaller breast carcinomas. Correct classification (ie, lobular vs ductal or in situ vs invasive) directly affects subsequent management, especially when the focus is near a surgical margin or present in a needle core biopsy and is further challenging if the lesion is morphologically ambiguous. We assessed the diagnostic utility of a multiplex, trichromogen immunostain of 3 commonly employed antibodies (CK7, p63, and E-cadherin) developed in our laboratory to evaluate these small lesions. Of the 147 specimens containing minimal (defined as ≤3 mm in size) invasive carcinoma, 81 also contained in situ carcinoma. In each case, the Minimal Carcinoma Triple Stain was prepared with a parallel H&E-stained slide. Observations of staining characteristics in the focus of interest were recorded. The Minimal Carcinoma Triple Stain was diagnostically useful in all but 1 case. In a case of invasive lobular carcinoma in an excisional biopsy, the Minimal Carcinoma Triple Stain stained only the surrounding breast tissue (appropriately) and not the focus of interest. Also, a subset of 29 of 81 excisional biopsies had minimal invasive carcinoma located 2 mm or less from the inked surgical margin, in which all cases the Minimal Carcinoma Triple Stain was fully interpretable despite morphologic distortion due to concomitant cautery artifact and tissue disruption in some cases. The Minimal Carcinoma Triple Stain offers an accurate and tissue-conserving method to diagnose small, morphologically problematic foci of breast carcinoma while ideally leaving more tissue for additional adjunctive studies.

Breast specimens containing carcinoma of minimal size or as rare foci are increasingly encountered and diagnostically challenging to characterize. Accurate classification (ie, lobular vs ductal or in situ vs invasive) of these increasingly small foci directly affects subsequent clinical management, especially when near a surgical margin or present in a needle core biopsy specimen. This challenge is all too familiar to those who practice diagnostic pathology in other anatomic sites, such as the lung and prostate, where full histologic characterization is expected despite small-sized biopsy material.1,2

Immunohistochemistry has afforded pathologists the ability to fully characterize such minute lesions, but because of the extremely limited amount of lesional tissue in some cases, some are relegated to choosing between additional H&E-stained levels and potentially diagnostic immunostains in further evaluating a morphologically challenging focus.3 This unfortunate scenario in which the number of studies (diagnostic or prognostic/predictive) necessary exceeds the amount of lesional tissue available would invariably lead to an incomplete workup as well as complete depletion of lesional tissue in the paraffin block, leaving none for additional tests for prognostic and predictive purposes.

As a possible solution to this unsavory situation, we developed and evaluated the usefulness of a cocktail immunostain composed of 3 commonly employed immunostains (CK7, p63, and E-cadherin) in a large cohort of patients. This immunostain, called the Minimal Carcinoma Triple Stain, separately stains epithelium, myoepithelium, and E-cadherin cell membrane protein using 3 chromogens (red, blue, and brown). This combination of antibodies has not been studied previously and offers advantages over commercially available multiplex immunostains currently used in breast pathology.
Materials and Methods

Cases

A total of 175 nonconsecutive specimens containing invasive breast carcinoma 3 mm or smaller in size were identified in our surgical pathology files from 2006 to mid-2010, of which 28 were subsequently excluded for various reasons, the most common of which was insufficient lesional tissue. The remaining 147 cases that were confirmed to contain minimal (defined as \( \leq 3 \text{ mm in size} \)) invasive breast carcinoma were used in this study. Eighty-one of the 147 cases also contained coexistent in situ carcinoma of either ductal or lobular type.

Immunohistochemistry

An unstained slide was prepared from the appropriate paraffin block of each case and stained with the Minimal Carcinoma Triple Stain using the BOND-MAX autostainer (Leica Microsystems, Bannockburn, IL) after antigen retrieval was employed. Triple staining of p63/E-cadherin/CK7 was accomplished by staining the 3 antibodies sequentially using the BOND-MAX autostainer. Sections were first baked and deparaffinized. They were then subjected to appropriate antigen retrieval and incubation procedures. Table 1 shows the clone, dilution, pretreatment, and vendor for each antibody. Vector Blue, DAB, and Refine Red chromogens were used for p63, E-cadherin, and CK7, respectively. Appropriate positive and negative controls were included.

Morphologic Review

Parallel H&E-stained slides of all cases were also prepared. All H&E slides and corresponding Minimal Carcinoma Triple Stain were concurrently reviewed by 2 contributing pathologists (D.S.R. and S.J.S.), and staining characteristics in carcinoma and surrounding normal ducts (when present) were recorded. One of the 2 pathologists (S.J.S.) was blinded to the rendered pathologic diagnosis at the time of immunostain review to simulate daily practice conditions and evaluate whether the Minimal Carcinoma Triple Stain result was diagnostically useful in “real time.”

Results

Morphologic Features and Specimen Types

The features are summarized in Table 2. All 147 cases of invasive breast carcinoma were confirmed to measure 3 mm or smaller in size. Of these 147 cases, 80 were of ductal type and 67 were of lobular type. Of the 80 invasive ductal carcinomas, 44 cases were identified in excisional biopsy specimens, whereas the remaining 36 were present in needle core biopsy specimens. Of the 67 invasive lobular carcinomas, 37 and 30 were identified in excisional and needle core biopsy specimens, respectively. Twenty-seven of the 81 excisional biopsy specimens contained invasive carcinoma that measured 1 mm or less in greatest dimension (18 ductal, 9 lobular) and staged as T1mi by the 2010 American Joint Committee on Cancer guidelines. Concurrently, in 81 of 147 cases, there was in situ carcinoma (49 ductal, 32 lobular). The histologic characteristics of in situ carcinoma were not further evaluated for the purposes of this study.

Of the 81 excisional biopsies, 29 contained invasive carcinoma located 2 mm or less from the inked surgical margin. Thirteen of these 29 invasive carcinomas were

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Clone, Dilution, Pretreatment, and Vendor of Antibodies Used</th>
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<tbody>
<tr>
<td>Antibody</td>
<td>Clone</td>
</tr>
<tr>
<td>p63</td>
<td>4A4</td>
</tr>
<tr>
<td>E-cadherin</td>
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<td>CK7</td>
<td>OV-TL12/30</td>
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Table 2 | Characteristics of Breast Carcinoma Highlighted With the Minimal Carcinoma Triple Stain |
<table>
<thead>
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<tbody>
<tr>
<td>Tumor Characteristics</td>
<td>Number of Cases</td>
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<tr>
<td>Invasive ductal carcinoma</td>
<td>80</td>
</tr>
<tr>
<td>Excisional biopsy specimens</td>
<td>44</td>
</tr>
<tr>
<td>Measurement ≤ 1 mm</td>
<td>18</td>
</tr>
<tr>
<td>Needle core biopsy specimens</td>
<td>36</td>
</tr>
<tr>
<td>Invasive lobular carcinoma</td>
<td>66</td>
</tr>
<tr>
<td>Excisional biopsy specimens</td>
<td>36^a</td>
</tr>
<tr>
<td>Measurement ≤ 1 mm</td>
<td>9</td>
</tr>
<tr>
<td>Needle core specimens</td>
<td>30</td>
</tr>
<tr>
<td>Distance to margins in excisional biopsy specimens (≤ 2 mm)</td>
<td>29</td>
</tr>
<tr>
<td>Postneoadjuvant chemotherapy</td>
<td>7</td>
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</tbody>
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^a Excludes a single case of invasive lobular carcinoma in which the Minimal Carcinoma Triple Stain was technically unsuccessful.
ductal in type, and the remaining 16 were lobular. Of the 13 invasive ductal carcinomas, the average pathologic tumor size was 1.7 mm (range, single tumor cells to 3 mm). Of the 16 invasive lobular carcinomas, the average pathologic tumor size was 1.8 mm (range, single tumor cells to 3 mm).

Seven of 81 excisional biopsies were from patients who had undergone neoadjuvant chemotherapy. Of the 7 cases, 5 showed residual invasive ductal carcinoma, whereas the remaining 2 had residual invasive lobular carcinoma. The average pathologic tumor size in these cases was 1.8 mm (range, single cells to 3.5 cm).

**Immunohistochemistry**

In the vast majority of cases, normal mammary ducts and/or lobules were present elsewhere in the stained slide and served as a reliable internal positive control for all 3 immunostains (CK7 and E-cadherin for epithelium; p63 for myoepithelium) [Image 1].

The Minimal Carcinoma Triple Stain demonstrated staining patterns that were diagnostic in all but 1 case of invasive lobular carcinoma. In this excisional biopsy specimen, the Minimal Carcinoma Triple Stain was reactive only in the surrounding breast glands (appropriately) and not the focus of question, despite repeating the stain twice. Except for this single case, the Minimal Carcinoma Triple Stain was not repeated for any case due to a suboptimal staining result.

The Minimal Carcinoma Triple Stain was particularly useful in several diagnostically problematic scenarios of minimal carcinoma, in which it is imperative to make an accurate diagnosis without depleting lesional tissue. First, it is well known that immunohistochemistry is more difficult to perform on small tissue samples, such as needle core biopsy specimens, because of the increased frequency of losing, disrupting, or dislodging the tissue during the staining process. The risk is even higher for multiplex immunostains, in which multiple rounds of pretreatment and staining are required. We found that all 66 cases of needle core biopsy material withstood the staining process, and the resulting Minimal Carcinoma Triple Stain immunostains were fully diagnostic. Second, diagnostically challenging cases that require classifying invasive and/or in situ carcinoma with ambiguous morphology [Image 2] and [Image 3], especially when near a surgical margin [Image 4] and [Image 5] or in a chemotherapy-treated specimen [Image 6] that may contain secondary morphologic distortion, were clarified using the Minimal Carcinoma Triple Stain in all cases. Third, the challenge in some cases was to verify and quantitate the relative amounts of invasive and in situ carcinoma in morphologically uncertain instances [Image 7] and [Image 8], which was achieved using the Minimal Carcinoma Triple Stain in conjunction with the morphologic evaluation in all such instances.

**Discussion**

The widespread practice of breast cancer screening programs, increased sensitivity of newer breast imaging modalities (ie, magnetic resonance imaging), and sophisticated biopsy techniques have invariably led to an increase in not only the number of screen-detected breast cancers but also their decreasing tumor sizes. These trends, in turn, have made diagnostic breast pathology a busier and more challenging practice.

Although immunohistochemistry has transformed from an adjunctive tool to a diagnostic necessity in some subspecialties, such as hematopathology, the general principle that morphology on a routine H&E stain takes precedence over findings by other stains is practiced in most other areas of surgical pathology, including breast pathology. However, many would agree that in certain problematic cases, the diagnosis heavily rests on the results of immunohistochemical stains, particularly when the morphologic features are equivocal and the entities in the differential diagnosis carry widely different clinical consequences. For instance, in lung biopsy specimens it has been estimated that only about 75% of non–small cell carcinomas can be accurately subtyped as adenocarcinoma or squamous cell carcinoma by examination of routine H&E-stained material. In this scenario, the problem lies in accurately subtyping lung biopsy specimens that contain poorly differentiated areas. Equally problematic issues
exist in breast pathology, and among the most prevalent are the distinctions between invasive and in situ carcinoma and between ductal and lobular phenotypes, especially in certain settings, such as in a needle core biopsy specimen or if the lesion in question is near or at a surgical margin. Depending on the diagnosis rendered, the patient’s clinical management may include (or forgo) further surgery, lymph node mapping, and/or adjuvant therapy (tamoxifen, radiation, chemotherapy). For instance, invasive carcinoma at or very close to a surgical margin will warrant reexcision of that area, but a diagnosis of ductal carcinoma in situ (DCIS) may be managed differently in some cases. Moreover, if the rendered diagnosis is lobular carcinoma in situ (LCIS) instead of DCIS near this margin, the management will likely include observation with or without hormonal therapy (i.e., tamoxifen).

In most cases, questions of invasion and histology type are easily answered by morphologic features alone. However, more diagnostically difficult cases typically involve
Image 3: The Minimal Carcinoma Triple Stain in examples of invasive and in situ lobular carcinoma. Lobular carcinoma in situ (LCIS) (CK7 positive/E-cadherin negative) directly involving a duct (CK7 positive/E-cadherin positive/p63 positive) can be mistaken for ductal carcinoma in situ (DCIS). In addition, growth patterns of invasive lobular carcinoma (CK7 positive/E-cadherin negative/p63 negative) other than single filing can be mistaken for invasive carcinoma of ductal type (A). LCIS involving a terminal duct–lobular unit (CK7 positive/E-cadherin negative/p63 positive) and adjacent invasive lobular carcinoma (CK7 positive/E-cadherin negative/p63 negative) (B). Florid LCIS (CK7 positive/E-cadherin negative/p63 positive) directly involving and distending a duct, thus mimicking solid DCIS. Adjacent invasive lobular carcinoma (CK7 positive/E-cadherin negative/p63 negative) is also confirmed by the Minimal Carcinoma Triple Stain (C).

Image 4: Small glandular proliferation suspicious for invasive ductal carcinoma near an inked surgical margin. The Minimal Carcinoma Triple Stain demonstrates that the glands nearest to the inked surgical margin (lower) are benign (CK7 positive/E-cadherin positive/p63 positive), whereas the more distant epithelial nests represent invasive ductal carcinoma (upper).

Image 5: Evaluating morphologically distorted cells at the inked, cauterized surgical margin. Owing to the presence of invasive ductal carcinoma in this specimen, the crushed cells at a surgical margin are highly suspicious for invasive carcinoma (A). The Minimal Carcinoma Triple Stain highlights the epithelial, ductal, and invasive nature of these cells (CK7 positive/E-cadherin positive/p63 negative). Although this result is not diagnostic per se for a positive margin, this staining pattern can dramatically heighten one’s suspicion of this possibility (B).
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carcinoma with ambiguous morphology. For instance, histologic typing of in situ carcinoma with a solid architectural pattern and an intermediate to high nuclear grade can be difficult because DCIS and pleomorphic LCIS are both considerations. Because, unlike DCIS, there is currently no consensus regarding the appropriate clinical management of pleomorphic LCIS near or at a surgical margin, this distinction is clinically important.

In prior years, the number of immunohistochemical stains at our disposal was limited, but today the increasingly more common limitation is the amount of lesional tissue available for additional studies. In cases of breast carcinoma, there are further considerations even subsequent to rendering the diagnosis because assessment of biomarkers (estrogen/progesterone receptors, Ki-67, and HER-2/neu) is necessary to guide further clinical management and treatment. This is certainly true in the needle core biopsy setting as some patients are poised to receive neoadjuvant chemotherapy, and biomarker studies should be performed prior to commencing treatment. More recently, clinicians have incorporated prognostic and predictive information provided by multigene assays to guide therapy, such as the commercially available Oncotype DX (Genomic Health, Redwood City, CA). This test, as well as others to follow, requires a significant amount of lesional tissue (50 µm), much more than what would be needed for even an extensive immunohistochemical workup (4 µm for each stain). As assessment by these array-based assays becomes part of the mainstream of treatment, the problem

![Image 6](image6.png) Neoadjuvant chemotherapy-treated breast specimens containing minimal residual carcinoma. Residual carcinoma in this type of specimen can be scattered in distribution and minute in size. These are difficult to identify amid treatment-related changes (A). The Minimal Carcinoma Triple Stain highlights the presence of residual invasive ductal carcinoma (CK7 positive/E-cadherin positive/p63 negative) as scattered foci (arrows) as well as ductal carcinoma in situ (CK7 positive/E-cadherin positive/p63 positive) (lower left corner) (B).

![Image 7](image7.png) The Minimal Carcinoma Triple Stain helps to determine the proportion of invasive to in situ carcinoma. In situ carcinoma with a high nuclear grade and an associated brisk lymphocytic infiltrate is shown. A focus suspicious for microinvasive carcinoma is seen (A). The Minimal Carcinoma Triple Stain not only clearly defines the ductal nature of the in situ carcinoma (CK7-positive/E-cadherin positive/p63 positive) but also confirms the presence of microinvasive ductal carcinoma (CK7 positive/E-cadherin positive/p63 negative) (B).
of premature tissue depletion will become painfully evident. Surgical pathologists who handle lung biopsy specimens are no stranger to these issues, as whole studies have been carried out to determine the optimal number and types of immunohistochemical stains needed to accurately distinguish lung adenocarcinomas from squamous cell carcinomas in small samples while preserving enough tissue for molecular testing such as epidermal growth factor receptor mutation analyses.\(^1\),\(^5\),\(^7\)-\(^9\) In fact, the 2011 guidelines of the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society for classification of lung adenocarcinoma recommend that tissue used for stains of small biopsies be minimized to preserve as much of the specimens as possible for molecular testing.\(^10\)

One way to address this problem is to incorporate 2 or more antibodies in a single stain, known as a “double/dual” (2 antibodies) or “cocktail/multiplex” (≥3 antibodies) immunostain. These combination stains not only conserve tissue but also allow for the added advantage of seeing how multiple antibodies stain relative to each other in the same section of tissue. The latter undoubtedly allows for better scrutiny of the staining pattern in the focus of concern and, theoretically, a more precise diagnosis. Such combination immunostaining has been mostly studied in the area of uropathology, particularly in the prostate\(^2\),\(^11\)-\(^18\) and much less so in other sites.\(^19\),\(^20\) In the breast, attempts to use double immunostaining have been described by investigators for various diagnostic dilemmas, but none to date has been widely adopted by practicing pathologists.\(^21\)-\(^26\) More recently, commercially available cocktail immunostains such as the Breast Triple Stain (Clarient, Aliso Viejo, CA), ADH-5 (Biocare, Concord, CA), and LC/DC Breast Cocktail (Biocare) have become available, which further underscores the increasing need to use multiple immunostains to render a single diagnosis. We believe the Minimal Carcinoma Triple Stain is superior to not only what has been previously described in the literature but also commercially available multiplex immunostains. First, we chose 3 antibodies that have not yet been combined into a single immunostain but are routinely used to address 2 of the most common diagnostic quandaries in breast pathology: the distinctions between invasive and in situ carcinoma and the ductal vs lobular phenotype. Second, p63, CK7, and E-cadherin localize to different compartments of a carcinoma cell (myoepithelial nuclei, epithelial cytoplasm, and epithelial cell membrane, respectively), which allows for easier and more accurate interpretation. Last, the Minimal Carcinoma Triple Stain is composed of 3 chromogens, unlike other cocktail immunostains that are dual colored but stain for multiple (>2) antibodies. The crisp staining pattern of each chromogen gives way to an overall clear and unequivocal immunohistochemical result.

The purpose of our study was 2-fold. First, we endeavored to test how robust the quality of the Minimal Carcinoma Triple Stain was in a large number of cases of varying tissue sizes (needle core biopsy specimens vs excisional specimens). Second, we evaluated the interpretable consistency of premature tissue depletion will become painfully evident. Surgical pathologists who handle lung biopsy specimens are no stranger to these issues, as whole studies have been carried out to determine the optimal number and types of immunohistochemical stains needed to accurately distinguish lung adenocarcinomas from squamous cell carcinomas in small samples while preserving enough tissue for molecular testing such as epidermal growth factor receptor mutation analyses.\(^1\),\(^5\),\(^7\)-\(^9\) In fact, the 2011 guidelines of the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society for classification of lung adenocarcinoma recommend that tissue used for stains of small biopsies be minimized to preserve as much of the specimens as possible for molecular testing.\(^10\)

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of this stain in addressing important diagnostic problems in a variety of situations in which this type of multiplex stain would be particularly useful. In our study, we evaluated 66 needle core biopsy cases containing invasive carcinoma of minimal size. In all cases, the stain was consistently interpretable and demonstrated the expected staining pattern for invasion (CK7 positive/p63 negative), whereas the underlying morphology remained preserved. In addition, the histologic type was confirmed as CK7 positive/E-cadherin positive for ductal carcinoma and CK7 positive/E-cadherin negative for lobular carcinoma in all cases. None of the stains needed to be repeated for technical reasons, and none of the corresponding paraffin blocks were depleted. Furthermore, the Minimal Carcinoma Triple Stain allowed full characterization of carcinoma in certain instances when there may have been compromised morphology, such as near or at a surgical margin (concomitant cautery artifact + tissue disruption), or in postneoadjuvant chemotherapy–treated specimens in all cases studied. In 1 specimen of postneoadjuvant chemotherapy, the residual tumor was present as single cells in sclerotic stroma that was not well visualized on the H&E-stained slide but identifiable on the Minimal Carcinoma Triple Stain. Despite tissue alterations related to prior exposure to chemotherapy, there was no appreciable compromise in staining intensity or specificity in benign or malignant cells. We also found the Minimal Carcinoma Triple Stain to be helpful in precisely quantifying the amount of invasive and in situ carcinoma when morphologic separation was difficult. Quantification of each component in addition to histologic typing was achieved using the Minimal Carcinoma Triple Stain in 81 (49 ductal, 32 lobular) cases studied. Specific examples include identifying and histologically typing single invasive tumor cells in a heavy lymphocytic infiltrate, usually arising in a background of morphologically ambiguous in situ carcinoma with intermediate or high nuclear grade; identifying and histologically classifying the extent of invasive carcinoma in a background of sclerosing adenosis with or without secondary involvement of morphologically ambiguous in situ carcinoma; and identifying invasive mucinous carcinoma when arising in a background of mucin-producing DCIS. Lesional tissue was not depleted from the paraffin block in any of the cases. Although the above findings are very encouraging, we caution that a wider experience in the hands of others may reveal some limitations not realized in this study. For instance, it is well known that p63 can be attenuated and even absent in some in situ carcinomas, which could lead to a misinterpretation of invasion using the Minimal Carcinoma Triple Stain. How frequently this potential pitfall occurs with routine use of this cocktail immunostain has yet to be determined.

In sum, the results of this study firmly validate the utility of the Minimal Carcinoma Triple Stain in certain diagnostically problematic scenarios, all of which have significant clinical consequences if misdiagnoses are made. This stain consists of 3 antibodies that are heavily used in breast pathology and also advantageously localize to 3 separate compartments of the carcinoma cell. Its trichromogen constitution further allows for a clear staining result. It is a robust stain that is highly consistent in staining quality without noticeable tissue compromise that would otherwise hinder interpretation. The Minimal Carcinoma Triple Stain is a diagnostically very useful and easily interpretable cocktail immunostain in breast pathology.

From the Department of Pathology and Laboratory Medicine, The New York Presbyterian Hospital–Weill Cornell Medical Center, New York, New York.

Address reprint requests to Dr Shin: Dept of Pathology and Laboratory Medicine—Starr 1031D, The New York Presbyterian Hospital–Weill Cornell Medical College, 525 E 68th St, New York, NY 10065.

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