Immunohistochemical Distinction of Invasive From Noninvasive Breast Lesions
A Comparative Study of p63 Versus Calponin and Smooth Muscle Myosin Heavy Chain

Robert W. Werling, M.D., Harry Hwang, M.D., Hadi Yaziji, M.D., and Allen M. Gown, M.D.

Identification of myoepithelial cells using antibodies to cytoskeletal proteins, such as smooth muscle myosin heavy chain (SMM-HC) and calponin, can play an important role in distinguishing invasive carcinoma from its histologic mimics. However, antibodies to these proteins may also cross-react with stromal myofibroblasts and vascular smooth muscle cells. It has recently been demonstrated that myoepithelial cells express the nuclear protein, p63, a member of the p53 gene family. We compared the patterns of reactivity of antibodies with p63, calponin, and SMM-HC on 85 breast lesions, including 11 cases of sclerosing adenosis, 33 cases of ductal carcinoma in situ, including 10 that showed microinvasion, 6 cases of lobular carcinoma in situ, and 35 cases of infiltrating ductal carcinoma. All three antibodies were positive on the vast majority of myoepithelial cells in all cases. A small minority of cases showed focal gaps in the revealed myoepithelial cell layer, reflected in discontinuous positive immunostaining around noninvasive epithelial nests (including ductal carcinoma in situ). No case showed p63 expression by myofibroblasts or vascular smooth muscle cells, whereas myofibroblasts expressed, in 8% and 76% of cases, SMM-HC and calponin, respectively. Although no tumor cell reactivity was noted with antibodies to calponin or SMM-HC, tumor cells in 11% of cases showed at least focal p63 expression. And although antibodies to p63 offer excellent sensitivity and increased specificity for myoepithelial detection relative to antibodies to calponin and SMM-HC, they have the following diagnostic limitations: 1) they occasionally demonstrate an apparently discontinuous myoepithelial layer, particularly around ductal carcinoma in situ, and 2) they react with a small but significant subset of breast carcinoma tumor cells. p63 may represent a myoepithelial marker that can complement or replace SMM-HC and/or calponin in the analysis of difficult breast lesions.

Key Words: Breast cancer—Immunohistochemistry—p63—Myoepithelium.

The morphologic distinction between benign and malignant, or in situ and invasive malignant, disease of the breast can be problematic, particularly in the setting of core needle biopsies. Although morphology alone can yield accurate diagnosis in the vast majority of breast biopsies and excisions, we recently documented the presence of considerable interobserver disagreement in the interpretation of difficult lesions based on histology alone.41,43 Breast ductal and lobular structures are both composed of a double cell layer (an inner, luminal cell and an outer myoepithelial cell),11 and it has been conclusively demonstrated in a series of investigations over the past 20 years that the presence of an intact peripheral myoepithelial cell layer characterizes all normal and benign breast lesions (e.g., adenosis, papilloma) as well as ductal carcinoma in situ (DCIS). Loss of this outer myoepithelial layer is the hallmark of invasive carcinoma, and demonstration of this loss has been documented by immunohistochemical techniques.1,5,6,14,15,31 However, rare carcinomas, such as low-grade adenosquamous carcinomas and adenomyoepithelioma,38 may show retention of an outer myoepithelial layer; conversely, apparent loss of myoepithelium may occasionally be seen in “distended” DCIS nests.7

Various strategies for the immunohistochemical demonstration of myoepithelial cells have been used, exploiting the phenotypic differences between myoepithelial cells and other cell types present in breast tissue, e.g., luminal epithelial cells, stromal myofibroblasts, and vascular smooth muscle cells. Some of these
markers include expression of unique cytokeratin subsets, S-100 protein, CD10, and smooth muscle-specific proteins, such as smooth muscle actin, heavy caldesmon, smooth muscle myosin heavy chain (SMM-HC), and calponin.

Each of these strategies for specific detection of myoepithelial cells has its merits and limitations. In general, cytokeratin subsets manifest relatively low specificity, as it has been demonstrated that the "high molecular weight cytokeratins," as originally described in the myoepithelium, are also, to a variable degree, expressed by a subset of luminal epithelial cells. In addition, expression of these cytokeratin subsets is nonuniform by myoepithelial cells, i.e., the markers also demonstrate low sensitivity. Another putative myoepithelial-restricted marker, S-100 protein, also shows poor specificity, as it is expressed frequently in the cells of both in situ and infiltrating carcinoma, even in the absence of myoepithelium. Earlier studies had suggested that smooth muscle-restricted proteins, such as muscle-specific isoforms of actin and, later, SMM-HC and calponin, might serve as highly specific myoepithelial markers. However, the use of antibodies to smooth muscle proteins as markers of myoepithelium can be problematic because of simultaneous expression of these latter markers by smooth muscle cells and stromal myofibroblasts, occasionally causing significant diagnostic difficulties, particularly in the setting of desmoplastic and/or highly vascular stromal responses to both in situ and infiltrating malignancy. We have previously shown that among these markers SMM-HC and calponin offer the highest sensitivity and specificity for myoepithelial cells and should be considered the current "gold standard" for myoepithelial cell identification in breast lesions.

Very recently, it has been demonstrated that myoepithelial cells also express the nuclear protein, p63, which is expressed neither in luminal mammary epithelium nor in smooth muscle cells or myofibroblasts and might prove superior to all previous myoepithelial markers with respect to specificity and sensitivity. p63 is a member of the p53 gene family and is expressed constitutively in the basal epithelia of multiple organs, including prostate, skin, and uterine cervix, as well as in some carcinomas. Although its biologic functions are postulated to include maintenance of epithelium-specific stem cells, diagnostic applications of its expression include the identification of poorly differentiated carcinomas as

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**TABLE 1. Summary of antibodies used in study**

<table>
<thead>
<tr>
<th>Antibodies to</th>
<th>Clone designation</th>
<th>Source</th>
<th>Pretreatment</th>
<th>Working dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calponin</td>
<td>CALP</td>
<td>Dr. Maria Frid, Univ. of Colorado*</td>
<td>Steam 20 min in 6 mM citrate, pH 6.0, followed by 10 min room temp 0.01% Pronase</td>
<td>1:1000</td>
</tr>
<tr>
<td>SMM-HC</td>
<td>SMMS-1</td>
<td>Dr. Maria Frid, Univ. of Colorado*</td>
<td>Steam 20 min in 6 mM citrate, pH 6.0, followed by 10 min room temp 0.01% Pronase</td>
<td>1:40</td>
</tr>
<tr>
<td>p63</td>
<td>4A4</td>
<td>Neomarkers, Freemont, CA</td>
<td>Microwave pressure cooker 8 min. in 6 mM citrate, pH 6.0</td>
<td>1:2000</td>
</tr>
</tbody>
</table>

* Also available from DAKO Corp., Carpinteria, CA, USA.

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**FIG. 1.** (A and B) Results of a double-label immunohistochemical technique applied to benign breast epithelium, using antibodies to SMM-HC with a blue chromogen (Vector Blue) and antibodies to the nuclear p63 with a brown signal (3,3′-diaminobenzidine). These images illustrate that: 1) the antibodies colocalize to the same (myoepithelial) cells, and 2) the brown p63 signal is confined to the nucleus, and the blue SMM-HC signal is confined to the cytoplasm, of the myoepithelial cells.
squamous or transitional cells as well as the identification of the basal cells of prostatic glands, which are lost in invasive prostatic adenocarcinoma.

The purpose of this study was to compare the specificity and sensitivity of p63 with the most sensitive and specific markers currently used for that purpose in our laboratory, SMM-HC and calponin. Sensitivity in this context is defined as the ability of the marker to demonstrate myoepithelial cells in deparaffinized, formalin-fixed breast specimens. Specificity is defined as the degree to which the marker distinguishes between myoepithelial cells and other cells likely to be found in the vicinity of the myoepithelial cells, i.e., luminal epithelial cells, vascular smooth muscle cells, and stromal myofibroblasts.

MATERIALS AND METHODS

Case Selection/Tissues

We evaluated histologic sections from 85 breast lesions seen in consultation at PhenoPath Laboratories between March and December 2001, including 11 cases of sclerosing adenosis, 23 cases of DCIS without invasion (16 of which were high grade), 10 cases of DCIS with microscopic or focal invasion (defined as <1 mm of invasion), 6 cases of lobular carcinoma in situ, and 35 cases of infiltrating carcinoma, of which 27 were ductal, 3 were papillary, and 5 were not further specified. Specimens included 64 excisional biopsies and 21 needle core biopsies.

Single Label Immunohistochemistry

Deparaffinized 4–5-μm-thick sections of at least one block from each case were rehydrated and subjected to heat-induced epitope retrieval procedures, optimized for each antibody and summarized in Table 1. Clone designations, sources, and working dilutions for the antibodies to calponin, SMM-HC, and p63 are also summarized in Table 1. The three antibodies were applied to sequential sections taken from each block. A standard avidin biotin immunoperoxidase technique as previously described was used for antibody localization (Vector Elite reagents, Vector Laboratories, Burlingame, CA, USA). Sections were counterstained with hematoxylin. Internal positive controls were present in every case, in the form of non-neoplastic breast tissue. Negative controls were performed by replacing the primary antibody with normal mouse serum.

Double Label Immunohistochemistry

In several cases antibodies to p63 and SMM-HC were colocalized using a double immunostaining technique that used separate chromophores (DAB for the nuclear p63 and Vector Blue for the cytoplasmic SMM-HC; Fig. 1). For these cases pretreatment for epitope retrieval consisted of 8 minutes of microwave pressure cooking in 6 mmol/L citrate buffer at pH 6.0 (same as p63 alone). After cooling for 20 minutes at room temperature and rinsing in tap water, the anti-SMM-HC antibody was added at 1:20 dilution and allowed to incubate for 40 minutes. Slides were then rinsed in phosphate-buffered saline (PBS) and incubated for 30 minutes in streptavidin/alkaline phosphatase (1:1000; Vector Laboratories, Burlingame, CA, USA) and 10% mouse serum. Following a second PBS rinse, the anti-p63 antibody was then added at 1:1000 dilution, for a 40-minute incubation, followed by another PBS rinse. Sections were then incubated for 30 minutes with goat anti-mouse Envision+/horseradish peroxidase (DAKO, Carpinteria, CA, USA), followed by a PBS rinse and a 15-minute Tris-buffered saline rinse. Finally, sections were incubated for 20–40 minutes in the dark with Vector Blue (Vector Laboratories, Burlingame, CA, USA), followed by PBS rinse and a 5–10-minute incubation with DAB+ (DAKO).

Scoring of Immunoreactivity Patterns

All hematoxylin and eosin-stained and immunostained slides were reviewed by at least two observers, with scoring of results by consensus; there were no cases in which

<table>
<thead>
<tr>
<th>Antibodies to</th>
<th>Myoepithelial cells</th>
<th>Myofibroblasts (percent positive)</th>
<th>Vascular smooth muscle (percent positive)</th>
<th>Luminal epithelial cells (percent positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p63</td>
<td>100</td>
<td>10</td>
<td>0</td>
<td>10†</td>
</tr>
<tr>
<td>SMM-HC</td>
<td>100</td>
<td>7</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Calponin</td>
<td>100</td>
<td>1</td>
<td>74</td>
<td>0</td>
</tr>
</tbody>
</table>

The numbers in the table represent percentages of cases (n = 85) in which the cells in question showed positive reactivity with the relevant antibody at a score of greater than or equal to 2 (see Materials and Methods section).

† Note that only one of the 9 cases (10% of 85 cases) with p63-expressing epithelial cells showed a level of intensity approximating that seen in myoepithelial cells (see Figure 5).
significant disagreements between observers were noted. The reactivity of each antibody was scored separately on the myoepithelium, myofibroblasts, vascular smooth muscle cells, and tumor (or benign epithelial) cells in each case and assigned a score of negative (−), 1 (<25% of target cells positive), 2 (26–90% of target cells were labeled), or 3 (91–100%).

RESULTS

Double Label Immunohistochemistry

Double label immunohistochemistry, as shown in Figure 1, clearly demonstrated colocalization of the p63 and SMM-HC signal in the identical cell population.
with the p63 signal restricted to the nucleus and the SMM-HC signal restricted to the cytoplasm.

Sensitivity

Results are summarized in Table 2. In benign breast tissue and in all categories of noninvasive breast lesions, antibodies to SMM-HC, calponin, and p63 were positive on the overwhelming majority of myoepithelial cells (Fig. 2). None of the cases showed myoepithelial cells manifesting either negative or only focal positivity (score of 1). No differences were noted in the immunostaining of low-grade versus high-grade DCIS. In a small minority of cases, however, the myoepithelial cells showed incomplete positivity (score of 2) with the antibodies, resulting in apparent gaps in the myoepithelial cell layer, seen as a “discontinuous” positive signal around the nests of DCIS (Fig. 3), defined as spaces equivalent to two or more nuclei between two p63-positive nuclei. Whereas this phenomenon was seen in only one case immunostained with antibodies to calponin, it was noted in six (7%) and nine (10%) of cases immunostained with antibodies to SMM-HC and p63, respectively. Quantitative analysis indicated that antibodies to calponin yielded a slightly higher sensitivity than those to either SMM-HC or p63 (100% vs 93% and 90%, respectively). Most of the cases showing incomplete myoepithelial immunostaining with antibodies to SMM-HC and p63 were DCIS.

Specificity

Results are summarized in Table 2. Antibodies to p63 showed the highest specificity for myoepithelium of all antibodies tested, as in none of the cases was there any p63 expression by either myofibroblasts or vascular smooth muscle cells. In contrast, stromal myofibroblasts reacted in 7 of 85 cases (8%) and 65 of 85 cases (76%) with antibodies to SMM-HC and calponin, respectively (Fig. 2H). In 100% of cases, the vascular smooth muscle cells were both SMM-HC and calponin positive. We also identified a subset of cases in which vascular smooth muscle cells, positive for expression of SMM-HC and calponin and negative for expression of p63, closely approximated foci of infiltrating ductal carcinoma (Fig. 4), simulating the appearance of an interrupted myoepithelium. Although no tumor cell (or luminal epithelial cell) reactivity was noted with antibodies to calponin or SMM-HC, tumor cells in 9 of 85 cases (11%) showed at least focal p63 positivity; in only one of these cases was the tumor cell reactivity similar in intensity to that seen in the myoepithelium (Fig. 5).

DISCUSSION

Over the past decade, there has been significant progress in the discovery and application of antibody markers that can be used in the immunohistochemical identification of myoepithelial cells. Identification of the presence or absence of these cells has been demonstrated to be of great utility in the distinction of benign from malignant lesions of the breast and to distinguish between invasive and intraductal carcinoma. For optimal clinical utility, however, an ideal marker of myoepithelium would manifest absolute sensitivity and specificity for these cells. In particular, such a marker would not cross-react with other cells in the breast, such as stromal myofibroblasts, vascular smooth muscle cells, or luminal epithelial/carcinoma cells.

In selecting a marker that putatively distinguishes benign from malignant disease, the highest levels of sensitivity and specificity are required. The “traditional”
myoepithelial markers still currently in wide use, including S-100 protein, type IV collagen and laminin, high molecular weight cytokeratins and smooth muscle actins, all have incomplete sensitivities and specificities that make them imperfect candidate myoepithelial markers. For example, antibodies to muscle actin isoforms, particularly HHF-35 and 1A4, have been widely used in the various clinicopathologic settings for the identification of myoepithelial cells. In a previous publication, however, we demonstrated the superiority of antibodies to SMM-HC and calponin over actin-isoform specific antibodies in terms of improved specificity in the breast.

Indeed, all the current markers remain imperfect, largely because of cross-reactivities with stromal myofibroblasts and occasionally vascular smooth muscle cells or luminal epithelial cells. The purpose of the current study was to compare the performance of those myoepithelial markers currently considered the most sensitive and specific against p63, a nuclear transcription factor recently reported to have specificity for myoepithelial cells in the context of breast tissue. The novel use of p63 as a myoepithelial cell marker represents the first exploitation of a nuclear antigen for this purpose. As has been previously noted by Barbaresci et al., the nuclear pattern of reactivity with anti-p63 antibodies in myoepithelial cells is qualitatively different from that seen with antibodies to cytoplasmic and cytoskeletal proteins. This nuclear pattern gives the advantage of “cleaner” staining but is occasionally difficult to interpret because the nucleus of a given myoepithelial cell is not directly juxtaposed to the nuclei of its nearest neighbors; therefore, even in clear-cut cases with continuous myoepithelial cell layers, the pattern of reactivity of p63 is discontinuous. Unlike Barbaresci et al., we did not observe a subpopulation (<1%) of basal cells in the breast positive for p63 but negative for SMM-HC.

This adjustment to using a nuclear rather than cytoplasmic myoepithelial marker does not pose interpretive difficulties in most cases. It can, however, occasionally be challenging to discern the difference between the “dotted line” of nuclei of a normal p63-defined myoepithelial cell layer and the disrupted line of myoepithelial cell nuclei in DCIS with microinvasion. One scenario in which this difference may be most challenging is in the assessment of the myoepithelial layer in a dense proliferation of tubules, as in sclerosing adenosis versus tubular carcinoma. In these cases the lesional architecture may be more difficult to discern than with cytoplasmic myoepithelial markers (Fig. 6). The detection of microinvasion (Fig. 7) seems to pose less of a problem, as both markers typically show complete loss of immunostaining when compared with the adjacent DCIS.

The major advantage to p63 as a marker of myoepithelial cells is its unique and near-absolute specificity in the context of breast tissue. Although in rare cases p63 expression is detected in the nuclei of breast carcinoma cells, that expression is almost limited to a small subset of the tumor cell population and is always less intense compared with the levels of expression in myoepithelial cells. In our study of 85 cases, only one showed uniform strong expression of p63 by tumor or luminal epithelial cells (Fig. 5). In rare cases such as that one, the clinical utility of p63 as a marker of myoepithelial cells is obviously limited. By contrast, however, the presence of calponin signal on most myofibroblasts surrounding both infiltrating and in situ carcinoma nests creates a significant problem in interpretation of some breast biopsies to rule out invasion. This cross-reactivity, i.e., suboptimal specificity, is simply absent with p63 and rarely prob-

**FIG. 5.** The breast epithelium in one of 85 cases reacted strongly and uniformly with antibodies to p63 (left), while a more common finding, illustrated at right and seen in nine cases, was a qualitatively weak signal in scattered epithelial cells (<25% of cells, score of 1), unlikely to cause diagnostic difficulty in distinction from myoepithelial cells.
lematic with SMM-HC, as demonstrated in the studies reported here.

Recently, the use of a double labeling technique, using antibodies to cytokeratins and smooth muscle actins, was recommended for routine use in the distinction of invasive versus noninvasive breast cancer.\textsuperscript{32} Whereas the latter is a valid technique that can overcome some of the specificity limitations of antibodies to smooth muscle actins alone, i.e., it can help distinguish between the smooth muscle actin-positive myofibroblasts and smooth muscle actin-positive (and cytokeratin positive) myoepithelium, double labeling techniques are laborious and difficult to incorporate into the routine diagnostic laboratory.

**FIG. 6.** Two distinctive examples of adenosis, with immunohistochemical demonstration of p63 (B and F), SMM-HC (C and G), and calponin (D and H). (A–D) The case illustrates that the nuclear marker does not optimally highlight the architecture in a dense proliferation of tubules, relative to the cytoplasmic markers. By contrast, the higher power image of the case (E–H) shows consistent myoepithelial cell reactivity with all three antibodies.
In cases with discrepant results from different antibodies, no biologic gold standard is available. Fortunately, all such cases in this study could be resolved by carefully examining the hematoxylin and eosin-stained sections, using information from all three antibodies, and occasionally acquiring deeper levels in the paraffin block. Although in rare cases these strategies still may fail to illuminate a definitive diagnosis, it is clearly helpful to have multiple different antibodies available for comparison. Based on the results of this study, we recommend the use of one cytoplasmic marker (SMM-HC) and one nuclear marker (p63) for the identification of myoepithelial cells in breast pathology, using the significantly less specific but slightly more sensitive marker, calponin, only in the rare cases in which the first two antibodies fail to produce a clear-cut diagnosis.

In the majority of cases of infiltrating ductal carcinoma, myofibroblasts and vascular smooth muscle cells are not immediately juxtaposed to malignant glands; therefore, the imperfect specificity of the cytoplasmic smooth muscle markers is acceptable. However, we did note in this study, and as reported elsewhere in abstract form, a subset of cases of infiltrating ductal carcinoma with small caliber blood vessels that array themselves at the periphery of the malignant ducts in a pattern that can simulate the appearance of myoepithelium and might lead to the misinterpretation of them as representing nests of DCIS. This distinction is made without equivocation using antibodies to p63 (Fig. 4).

In summary, although antibodies to p63 offer excellent sensitivity and increased specificity for myoepithelial cell detection relative to antibodies to calponin and SMM-HC, they have the following diagnostic limitations: 1) they occasionally demonstrate an apparently discontinuous myoepithelial layer around nests of DCIS, and 2) they react with a small but significant subset of breast carcinoma tumor cells; however, this aberrant reactivity rarely causes diagnostic difficulty. Furthermore, relative to vascular smooth muscle cells and myofibroblasts, the specificity of p63 for myoepithelium in this study is almost perfect. Therefore, we conclude that p63, because of its near-perfect sensitivity and near-absolute specificity in distinguishing myoepithelial cells from myofibroblasts and vascular smooth muscle cells, represents a myoepithelial marker that can complement or replace SMM-HC and/or calponin in the analysis of difficult breast lesions. Certainly, all three of these markers have significant advantages over S-100, smooth muscle actin, and other putative myoepithelial markers still widely in use today.

REFERENCES

2. Barbareschi M, Pecciarini L, Cangi MG, et al. p63, a p53 homo-


