Usefulness of Basal Cell Cocktail (34βE12 + p63) in the Diagnosis of Atypical Prostate Glandular Proliferations

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Abstract

We evaluated the diagnostic usefulness of the 34βE12-p63 cocktail, compared with 34βE12 and p63 used alone, in 34 prostate needle biopsy (NBXs) and 3 transurethral resection specimens containing atypical glandular proliferations and in 18 NBXs containing unequivocal prostate carcinoma (PCa). Staining intensity; percentage of basal cells staining in benign, atypical, and malignant glands; number of benign glands lacking basal cell staining; and staining variance were analyzed. All NBXs with unequivocal PCa were negative with all 3 markers. Diagnoses were as follows for the atypical cases after staining for the 3 markers: PCa, 9; postatrophic hyperplasia, 12; high-grade prostatic intraepithelial neoplasia (HGPIN), 5; atypical adenomatous hyperplasia, 6; benign atypical proliferations, 4; and HGPIN with adjacent small atypical acinar proliferation suggestive of PCa, 1. The cocktail demonstrated consistently strong staining intensity and improved basal cell staining in morphologically benign and benign atypical glands compared with p63 and 34βE12 alone; it had the smallest staining variance compared with 34βE12 (F < 0.0001) and p63 (F = 0.31), although its advantage for resolving individual atypical cases was limited compared with 34βE12 and p63 alone. Of 37 atypical cases, 1 (3%) additionally was resolved as benign using the cocktail and p63. Because the diagnosis of PCa is supported by lack of basal cell staining, the immunohistochemical analysis with highest possible sensitivity and lowest variability is critical to ensure that a negative reaction is true. The cocktail provides a simple, cost-effective improvement in basal cell immunohistochemical analysis of difficult prostate lesions.

Widespread use of prostate-specific antigen screening has led to an increase in prostate needle biopsies and, subsequently, an increase in earlier detection of prostate carcinoma (PCa).1,2 This trend also has led to an increase in the number of equivocal diagnoses on prostate biopsy specimens.3 Surgical pathologists must make critical decisions on an increasing number of prostate needle biopsy specimens with only small foci of atypical glands. In this setting, the mimics of PCa must be distinguished from a small focus of adenocarcinoma.

The basal cell marker, high-molecular-weight cytokeratin antibody (34βE12) is used widely in this setting.4-7 Recent studies have shown p63, a p53 homologue, to be a useful basal cell marker.8-10 Diagnosis of PCa using both basal cell markers is supported by the lack of basal cell staining in atypical glands. However, the diagnosis using basal cell immunohistochemical analysis might not always be conclusive because the absence of staining or only patchy staining can occur, particularly in some benign and premalignant prostate lesions such as atypical adenomatous hyperplasia (AAH), high-grade prostatic intraepithelial neoplasia (HGPIN), and postatrophic hyperplasia (PAH).6,11

Recent work comparing both basal cell markers demonstrated that 34βE12 and p63 are subject to some staining variability, although p63 staining has less variability.10 Because the diagnosis of PCa is supported by lack of basal cell staining, it is critical to use the basal cell marker with highest possible sensitivity and lowest variability to ensure that negative staining is truly due to lack of basal cells and not to technical problems. To improve confidence in basal cell immunohistochemical analysis, p63 frequently is used in addition to 34βE12 in difficult prostate lesions. Zhou et al12 recently demonstrated that the combined use of 34βE12 and p63 as a
cocktail improves basal cell detection in benign prostate glands from the transition zone compared with each used separately. The goal of the present study was to evaluate the usefulness of the basal cell cocktail in the workup of clinically atypical cases.

Materials and Methods

Study Population

To study the diagnostic usefulness of the basal cell cocktail antibodies (34βE12 and p63), 34 consecutive prostate needle biopsy specimens and 3 specimens from transurethral resection of the prostate (TURP) containing foci of atypical glandular proliferations and 18 prostate needle biopsy specimens with unequivocal PCa were selected from our routine surgical pathology caseload. Three study pathologists (R.B.S., L.P.K., and M.A.R.) reviewed the original H&E-stained sections before staining cases for immunohistochemical analysis. Based on morphologic features, all cases were classified into 3 categories: (1) cancer; (2) atypical, favor benign; or (3) atypical, favor PCa. Of 37 atypical cases (34 prostate needle biopsy specimens and 3 TURP specimens), 16 were morphologically highly suggestive of PCa and 21 were morphologically atypical with a low level of suspicion for PCa. All 18 prostate needle biopsy specimens containing unequivocal PCa had small foci of PCa ranging from 5% to 10% of total submitted cores. In 16 prostate needle biopsy specimens, the primary and secondary Gleason patterns were 3, and 2 prostate needle biopsy specimens had a primary Gleason pattern of 3 and a secondary pattern of 4.

Immunohistochemical Analysis and Evaluation

As part of the routine protocol, 4 slides per needle biopsy specimen with 2 levels on each slide were cut. The first and fourth slides were used for H&E staining, and the intervening 2 slides were saved for potential immunohistochemical analysis. However, for this study, when an atypical lesion was encountered on initial H&E examination, a fifth slide was cut shortly after the initial sectioning of the block to use for immunohistochemical analysis.

Care was taken to minimize delay between initial sectioning and cutting the extra section. If the atypical focus was lost in any of the 3 immunohistochemically stained slides, the particular case was excluded from further analysis. Attempts were made to ensure that uniform working conditions were applied while staining for all antibodies. Immunohistochemical analysis was performed using the same lot of antibodies, and experiments were run with nearly similar working conditions. For the majority of cases, tests with all 3 antibodies were performed in the same run on the autostainer for a given case. However, for a few cases that were obtained retrospectively, the basal cell cocktail was performed on a separate run following recut of the block, while 34β12 and p63 were performed in the same run for these cases.

Standard avidin-biotin complex immunohistochemical analysis was performed. Antibody concentration was optimized to obtain the strongest target staining with minimal background staining. Antigen retrieval was performed by steaming the slides for 15 minutes in a 10-mmol/L concentration of sodium citrate buffer, pH 6.0, in a microwave oven. The slides then were incubated sequentially with primary antibody, biotinylated secondary antibody, avidin-biotin complex, and chromogenic substrate 3,3′-diaminobenzidine. The primary antibodies and their dilutions were as follows: 34βE12, 1:100 (DAKO, Carpinteria, CA); and 4A4 p63, 1:100 (Labvision, Fremont, CA). For the basal cell cocktail, the calculated amount needed of 34βE12 and p63 was added to a predetermined total volume in such a way that final dilution for each antibody was 1:100. An example of this calculation to make 2 mL of cocktail is as follows: 2,000 μL total volume needed × 1/100 desired dilution of 34βE12 and p63 = 20 μL of 34βE12 + 20 μL of p63 + 1,960 μL of diluents = final volume of 2,000 μL of cocktail.

Immunostaining was performed using a DAKO autostainer. The primary antibodies and the cocktail were incubated for 45 minutes at room temperature and the secondary biotin-labeled antibody for 30 minutes for 34βE12 and 45 minutes for p63. The streptavidin-labeled streptavidin-biotin amplification method (DAKO K0679) was carried out for 30 minutes, followed by peroxidase/diaminobenzidine substrate/chromogen for 34βE12 and p63. The DAKO EnVision Plus detection system was used for basal cell cocktail antibody localization according to the vendor’s protocol, followed by peroxidase/diaminobenzidine substrate/chromogen. Slides were counterstained with hematoxylin.

Two study pathologists (R.B.S., L.P.K) evaluated immunohistochemically stained slides, and the average score of the evaluation was used for the analysis. Expression of basal cell marker staining intensity was scored as follows: 1, negative; 2, weak; 3, moderate; or 4, strong. The percentage of basal cell staining in benign, atypical, and malignant glands was scored on a scale of 0% to 100%. The numbers of basal cell marker staining intensity was scored as follows: 1, negative; 2, weak; 3, moderate; or 4, strong. The percentage of basal cell staining was scored on a scale of 0% to 100%. The numbers of basal cell marker staining intensity was scored as follows: 1, negative; 2, weak; 3, moderate; or 4, strong. The percentage of basal cell staining was scored on a scale of 0% to 100%.
Statistical Methods

The Wilcoxon signed rank test for paired samples was performed using a statistical software package (SAS Institute, Cary, NC) to evaluate whether the overall basal cell staining was significantly different among 34βE12, p63, and the cocktail. The F test was used to assess the variances for 34βE12, p63, and the cocktail.

Results

34βE12, p63, and Cocktail Staining in 18 Unequivocal PCa Prostate Needle Biopsy Specimens

All 18 prostate needle biopsy specimens containing histologically unequivocal PCas were negative with all 3 markers for PCa. No aberrant basal cell staining was detected in any of the 18 PCa cases when using the antibody cocktail.

34βE12, p63, and Cocktail Staining in 37 Atypical Lesions

Of 37 atypical cases (34 prostate needle biopsy specimens and 3 TURP specimens), 16 were suggestive of PCas and 21 were favored as benign based on H&E morphologic examination.

In 9 of 16 cases suggestive of PCas, all 3 markers were entirely negative in the atypical focus and positive in adjacent surrounding benign glands, supporting the diagnosis of PCa. Of the remaining 7 cases with an atypical focus, 6 showed variable staining in the atypical focus with all 3 markers, supporting a benign diagnosis. One atypical prostate needle biopsy specimen could not be resolved with all 3 markers and was diagnosed as HGPIN with adjacent atypical small acinar proliferation, suggestive of PCas. In this case, all 3 markers demonstrated patchy staining in HGPIN glands and in the adjacent small atypical glands. For the 21 cases that were favored morphologically to be benign, all 3 markers were variably positive in the atypical focus, supporting the benign diagnosis.

Overall basal cell staining intensity and the percentage of basal cells staining, in general, were strongest with the cocktail; however, they frequently were patchy or sparsely distributed, especially in lesions of PAH and AAH and some HGPIN lesions. In the majority of cases, the cocktail did not offer clinically significant differences over p63 and 34βE12 used alone, except in 1 (3%) of 37 atypical cases, use of the cocktail or p63 changed the atypical diagnosis to benign (Table 1).

34βE12, p63, and Cocktail Staining in Morphologically Benign Prostate Glands of 37 Atypical Cases

In addition to the staining pattern of atypical foci, basal cell staining also was evaluated in morphologically benign glands of all atypical cases. Scattered circumferential loss of basal cell staining in morphologically benign glands (average, 2-3 glands) was seen in 12 (32%), 5 (14%), and 5 (14%) of 37 atypical cases stained with 34βE12, p63, and the cocktail, respectively. p63 showed overall higher basal cell staining and staining intensity in benign glands compared with 34βE12 when both markers were used alone (Figure 1).

Discussion

The high-molecular-weight cytokeratin antibody, 34βE12, is a widely used basal cell marker to support the diagnosis of PCas. Recently, p63, a p53 homologue, has

| Table 1 |
| Resolution of 37 Atypical and 18 Prostate Carcinoma Cases: Comparison of Basal Cell Markers 34βE12, p63, and Cocktail* |

<table>
<thead>
<tr>
<th>Diagnosis Before Immunohistochemical Analysis</th>
<th>Final Diagnosis After</th>
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<tbody>
<tr>
<td></td>
<td>34βE12</td>
</tr>
<tr>
<td>Cancer (n = 18)</td>
<td>18</td>
</tr>
<tr>
<td>Atypical, favor benign (n = 21)</td>
<td>0</td>
</tr>
<tr>
<td>Atypical, favor PCa (n = 16)</td>
<td>0</td>
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</tbody>
</table>

PCa, prostate carcinoma.

* 34βE12 and p63.
Image I (Case 1) A, Prostate needle biopsy specimen with minute focus of small atypical glands (arrows), highly suggestive of prostate carcinoma (H&E). B-D, Same focus as A. 34βE12 stained basal cell cytoplasm (B). p63 stained basal cell nuclei (C), and the 34βE12-p63 cocktail demonstrated a combination of cytoplasmic and nuclear basal cell staining in benign glands (D). Atypical glands were negative with all 3 markers, in contrast with strong basal cell staining in adjacent benign glands. (A-D, ×200). (Case 2) A, Circumscribed focus of atypical small glands in needle biopsy specimen; morphologically, a diagnosis of partial atrophy is favored (H&E). B-D, Atypical glands demonstrated variable patchy basal cell staining with all 3 markers: 34βE12 (B), p63 (C), and the 34βE12-p63 cocktail (D); however, overall basal cell staining (staining intensity and percentage of basal cell staining) was highest with the cocktail (D) (A-D, ×200). (Case 3) A, Another specimen from a transurethral resection of the prostate containing a cluster of crowded small and large glands with similar cytologic features. Morphologically, a diagnosis of atypical adenomatous hyperplasia was favored (H&E). B-D, Same focus as A. 34βE12 demonstrated very patchy staining of atypical small glands (B); p63 demonstrated patchy but stronger staining (C). However, the 34βE12-p63 cocktail demonstrated the strongest basal cell staining compared with both markers used alone (D) (A-D, ×200).
been shown to be a very useful basal cell–specific marker, and it frequently is used in addition to 34βE12 in difficult cases.8-10 Interestingly, p63 is expressed in the nuclei of basal cells, whereas 34βE12 stains their cytoplasm. The usefulness of 34βE12 and p63 relies on the fact that PCa lacks basal cells; therefore, lack of basal cell staining in an atypical lesion lends support to the diagnosis of PCa, whereas the presence of basal cell staining in general rules out cancer, with the exception of rare cases of basal cell staining in typical PCa by reactivity to 34βE12.13

Basal cell immunohistochemical analysis, however, has some limitations. A patchy staining pattern seen in certain benign atypical lesions might create diagnostic confusion with PCa. In addition, technical factors also might contribute to staining variability. It is well demonstrated that 34βE12 is highly susceptible to the effects of formalin fixation and immunohistochemical techniques such as antigen retrieval pretreatments, resulting in staining variability with frequent loss of staining in benign glands or patchy staining patterns, which might be difficult to interpret.14-16

Varma et al16 studied the deleterious effects of prolonged formalin fixation on the 34βE12 staining pattern of benign prostate glands and its effects on different antigen pretreatments. They concluded that 34βE12 immunoreactivity is dependent on optimal fixation and the immunohistochemical protocol.16 Multhaupt et al14 found the critical role of antigen retrieval in 34βE12 immunoreactivity; 88% of benign glands from the transition zone lost 34βE12 immunoreactivity if no antigen pretreatment was used. When evaluating immunohistochemical results, optimal staining of the benign glands is critical; otherwise a negative stain in atypical glands might cast doubt on the validity of the immunohistochemical reaction.

A previous study comparing both basal cell markers demonstrated that p63 showed less variability in staining consistency for basal cell detection compared with 34βE12, particularly in the transition zone of benign prostate glands.10 In that study, none of the 51 unequivocal PCa prostate needle biopsy specimens demonstrated immunoreactivity for p63 or 34βE12. When using robust antigen retrieval methods, scattered loss (average 2-3 glands) of basal cell staining in morphologically benign glands occurred in 23% of prostate needle biopsy specimens and 100% of TURP specimens stained with 34βE12 and 9% of prostate needle biopsy specimens and 17% of TURP specimens stained with p63. These results demonstrate that even though p63 improved basal cell detection with less variability than 34βE12, it still failed to detect basal cells in a subset of benign glands and might contribute to diagnostic inaccuracy.

Table 2
Benign Prostate Glands Immunoreactivity for 34βE12, p63, and Basal Cell Cocktail Antibodies* in 37 Atypical Cases

<table>
<thead>
<tr>
<th>Staining intensity</th>
<th>Mean</th>
<th>SEM</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>34βE12</td>
<td>2.95</td>
<td>0.65</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>p63</td>
<td>3.14</td>
<td>0.42</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Cocktail</td>
<td>4.00</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Percentage of basal cells staining</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34βE12</td>
<td>92</td>
<td>9.4</td>
<td>.0002</td>
</tr>
<tr>
<td>p63</td>
<td>95</td>
<td>4.5</td>
<td>.003</td>
</tr>
<tr>
<td>Cocktail</td>
<td>97</td>
<td>3.8</td>
<td></td>
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</table>

* 34βE12 and p63.
† P values represent the comparison of the individual stain with the cocktail.
‡ Intensity was scored according to the following scale: 1, negative; 2, weak; 3, moderate; or 4, strong.
The present study showed similar results when p63 and 34βE12 markers were compared: 12 (32%) and 5 (14%) prostate needle biopsy specimens stained with 34βE12 and p63, respectively, showed scattered loss (average, 2-3 glands) of circumferential staining in morphologically benign glands. p63 showed overall stronger staining of basal cells with less staining variability than 34βE12 when compared alone (Figure 1; Table 2). However, in 4 (11%) of 37 prostate needle biopsy specimens, 34βE12 showed higher basal cell staining than p63, suggesting that in these cases, 34βE12 might be superior to p63. Therefore, in some cases, it might be prudent to perform stains for both markers or to combine both markers in the same immunohistochemical analysis (cocktail) to overcome such variation in basal cell staining and maximize the detection of basal cells.

A previous study by Zhou et al12 demonstrated that inclusion of 34βE12 and p63 (cocktail) in the same immunohistochemical reaction was feasible and improved the detection of basal cells over the use of either marker alone. These observations were made in benign glands from TURP specimens consisting mainly of the transition zone of the prostate, which usually is most susceptible to fixation artifacts. 34βE12 staining was found most susceptible and the cocktail least susceptible to variability in the basal cell detection in benign glands. There are several possible reasons for superior staining with the cocktail. 34βE12 and p63 target different antigens, the former for basal cell cytoplasm and latter for basal cell nucleus, and, therefore, combining 2 markers produces stronger final staining (Image 1) Image 2. 34βE12 and p63 might be subject to different technical variability; therefore, combining them in the same reaction might minimize technical variability. The present study focused on the diagnostic usefulness of a basal cell cocktail in prostate needle biopsy and TURP specimens containing atypical lesions because it would be of practical value in day-to-day practice.

In the vast majority of atypical cases, the basal cell cocktail was not superior to either marker used alone in resolving an atypical diagnosis. Of 37 atypical cases, 1 (3%) could be resolved as benign by using the cocktail and p63 compared to using 34βE12. In this case, the cocktail demonstrated strong staining in atypical glands and adjacent benign glands, whereas 34βE12 failed to stain optimally in this critical area. Even though the direct advantage of the cocktail for resolving atypical cases was limited, the cocktail demonstrated consistently strong staining intensity (mean staining intensity, 4.00) and improved basal cell detection compared with each used separately (Figure 1; Table 2). Staining variance also was smallest with the cocktail compared with p63 (F = 0.31) and 34βE12 (F < 0.0001) used alone.

Most important, combining 2 markers in the same reaction did not demonstrate aberrant basal cell expression in the PCa cases. However, even with the cocktail that improved basal cell detection, scattered loss of staining in benign glands still was seen in 3 (8%) of cases with all 3 markers, suggesting that these glands truly might lack basal cells, and further improvement of immunohistochemical analysis might not necessarily increase their detection. A similarly patchy staining pattern was seen in 4 atypical lesions (11%) stained with all markers, particularly in cases of AAH, PAH, and HGPIN. This expression pattern indirectly supports the theory that these lesions truly lack some basal cells and might, in fact, be in transition to PCa. This observation again emphasizes that even with robust basal cell immunohistochemical analysis, careful attention to morphologic features remains critically important to avoid misinterpretation as PCa in such cases. Of 13 atypical cases suggestive of PCa, 1 could not be resolved despite use of the cocktail (Table 1). It will be interesting to see whether inclusion of additional PCa-specific markers such as α-methylacyl coenzyme A racemase improves diagnosis in these circumstances.17-20

One could argue against use of the cocktail when it does not offer clinically significant advantages in resolving atypical cases over either marker used alone (Table 1). One also could argue for the use of p63 alone in place of 34βE12 because p63 demonstrated higher sensitivity for basal cell detection and less variability than 34βE12.

In our view, using the cocktail rather than either marker alone has several indirect advantages. The cocktail in general still improves basal cell detection with less staining variability.
Improved basal cell detection in benign atypical lesions, such as AAH or PAH, might help avoid their misinterpretation as PCa. Performing one combined reaction is more economical than performing 2 separate reactions in diagnostically difficult cases. As we increasingly encounter smaller atypical lesions in the prostate-specific antigen screening era, the chances of losing the atypical focus on 2 different slides are significantly greater. Therefore, combining 2 separate reactions into 1 reaction will improve the yield, compared with performing 2 separate reactions. Immunohistochemical analysis using the cocktail can be automated easily using conventional stainers without increasing technical complexity. Furthermore, as shown in the present study and in a previous study,12 the combined reaction does not increase the detection of aberrant basal cell expression in PCa.

In our view, achieving the highest possible basal cell sensitivity ensures that negative staining in atypical glands is not a false-negative result. Because the diagnosis of PCa is substantiated by lack of basal cell staining, it is critical that basal cell staining be performed with the highest possible sensitivity and lowest possible variability. Therefore, on the basis of this work, we recommend the routine use of the basal cell cocktail as a simple and cost-effective way to improve basal cell immunohistochemical analysis in the workup of difficult prostate lesions.

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References


