p63/AMACR Antibody Cocktail Restaining of Prostate Needle Biopsy Tissues After Transfer to Charged Slides

A Viable Approach in the Diagnosis of Small Atypical Foci That Are Lost on Block Sectioning

Omar Hameed, MBChB, and Peter A. Humphrey, MD, PhD

Abstract

We assessed the utility of using a p63/α-methylacyl-coenzyme-A racemase (AMACR) antibody cocktail on destained H&E-stained sections. We transferred 61 stored (7-11 months old) and 10 recent (<1 month old) H&E-stained sections of prostate needle biopsy tissues to charged slides and subsequently stained them with a p63/AMACR immunohistochemical antibody cocktail. The AMACR and p63 staining intensities were compared with those obtained with the same antibody cocktail performed on sections recut directly from the paraffin block. Transfer of sections and subsequent immunohistochemical staining was successful in 69 (97%) of 71 cases. For stored cases, there were similar AMACR and p63 staining intensities in destained and recut sections in 55 (90%) and 11 (18%) of 61 cases, respectively. In recent sections, AMACR and p63 staining intensities were almost identical by both methods. We conclude that p63/AMACR cocktail immunostaining of destained H&E-stained sections is a viable approach in the workup of small “suspicious” foci in recently sectioned prostate needle biopsy tissues. This approach is best used when 2 or more H&E-stained sections harbor the suspicious focus, as we always recommended preservation of at least 1 H&E-stained section.

Although most prostate needle biopsy specimens are diagnosed as benign or malignant based solely on routine H&E-stained sections, immunohistochemical analysis is not infrequently used as an adjunct in the interpretation of these specimens, especially to confirm the diagnosis of minimal (limited; <1 mm) and unusual variants of prostate carcinoma and to exclude benign mimickers of carcinoma. Lack of staining with basal cell markers in the appropriate histologic context supports a diagnosis of carcinoma in such cases.1 In addition, positive staining for α-methylacyl-coenzyme-A racemase (AMACR) seems to be of diagnostic value beyond that of a negative basal cell immunostain by adding further support to the diagnosis of malignancy.2-4 Because the use of a p63/AMACR antibody cocktail on a single slide has been found to produce a staining profile identical to using each antibody individually,5 use of such a cocktail would be most useful in the evaluation of small, diagnostically difficult foci in prostate needle biopsy tissue when only limited tissue is available for immunohistochemical evaluation.5 However, not infrequently, these foci are cut through and lost with subsequent leveling of the block for immunohistochemical evaluation.2,6,7 In these situations, we hypothesized that immunohistochemical staining of a destained H&E-stained section that contains the focus or foci in question would be a viable approach.

The aims of this study were to assess the usefulness of using a p63/AMACR antibody cocktail on destained H&E-stained sections and compare the staining produced with that on unstained recut sections immunohistochemically stained with the same cocktail.

Materials and Methods

We studied 7- to 11-month-old, stored H&E-stained sections of prostate needle biopsies from 61 cases (randomly
selected from cases of prostatic carcinoma in a previous retrospective study) and similar sections from 10 recent cases (<1 month old) from the files of the Lauren V. Ackerman Laboratory of Surgical Pathology, Washington University Medical Center, St Louis, MO. The study was approved by the Washington University School of Medicine Human Studies Committee (St Louis).

Preparation of H&E-Stained Sections for Immunohistochemical Analysis

The undersides of the H&E-stained sections first were scored with a diamond pen to delineate the area on the slide that contained the needle core biopsy tissue. The coverslips then were removed by soaking in xylene (Allegiance, McGaw Park, IL) at room temperature for approximately 2 to 3 days for the stored sections and 1 to 2 days for the recent sections. The slides then were immersed in fresh xylene to remove any residual mounting medium.

A commercially available tissue transfer mounting medium (Mount-Quick, Newcomer Supply, Middleton, WI) was used to transfer the sections to charged slides, as described previously. Briefly, a Pasteur pipette was used to spread the mounting medium, ensuring use of sufficient medium to form a slight meniscus over the tissue and cover the entire area of the needle cores. The slides then were placed in a 60°C oven for 2 hours until the coating material hardened. The latter was scored with a diamond pen to reproduce the regions previously marked on the undersurface of the glass slides. Slides were immersed in warm water for at least 2 hours to ensure that the mounting medium could easily be pried slowly off the slides with a scalpel blade. Once removed, the media slivers were trimmed around the needle core tissue with a scalpel blade along the previously scored lines. Charged slides then were moistened with a small amount of water to ensure adhesion. The trimmed media slivers were placed on the charged slides in their original orientation (Mount-Quick–coated surface face up). The slides were dried again in a 60°C oven for 2 hours and immersed in four 3-minute xylene changes until all of the medium was removed. The sections were rehydrated through 95% alcohol to distilled water.

Immunohistochemical Analysis

Transferred sections and deparaffinized and rehydrated newly cut sections from the recent cases were stained with a p63/AMACR antibody cocktail (PIN Cocktail, clones BC4A4/13H4, prediluted, Biocare Medical, Walnut Creek, CA) by immunohistochemical methods identical to those performed in a previous study. Briefly, the sections were placed in a 6% hydrogen peroxide solution in absolute methanol for 20 minutes to block endogenous peroxidase activity. This process also ensured destaining of the transferred sections. This was followed by washing 3 times with buffer (Wash Buffer ×10, 1:10 dilution, DakoCytomation, Carpinteria, CA) for 5 minutes each time. They then were placed in EDTA buffer (pH 8) in a decloaking chamber (Biocare Medical) for 3 minutes of heat-induced antigen retrieval, followed by cooling in an ice bath for 20 minutes. After rinsing, sections were loaded onto an autostainer (DakoCytomation) where the primary antibodies were applied for 30 minutes, followed by the secondary detection antibodies for another 30 minutes. Sections were visualized by diaminobenzidine chromogen for 10 minutes, rinsed, counterstained with hematoxylin for 30 seconds, and dehydrated, and a coverslip was applied.

Morphologic Evaluation

A circumferential, diffuse or apical, granular cytoplasmic staining in an entire glandular structure was considered positive for AMACR, whereas nuclear staining was considered positive for p63. The intensity of staining for both was graded semiquantiatively as negative, weak, or moderate-strong. The AMACR and p63 staining intensities in destained sections were compared with those in recut sections from the paraffin block. The χ² analysis was used to measure the differences between the AMACR and p63 staining intensities in the 2 methods. A P value of .05 was considered statistically significant. Statistical analysis was performed using SPSS 11.0 for Windows software (SPSS, Chicago, IL).

Results

Transfer of sections to charged slides and subsequent immunohistochemical analysis was successful in most cases, as tissue fell off the slide in only 2 (3%) of 71 cases.

For stored sections, there was no statistically significant difference in AMACR staining intensity in foci of prostatic carcinoma in destained sections and recut, unstained sections (P = .574) Table I. Similar staining intensities were seen in 55 cases (90%) Image 18 and Image 21, a decreased staining

<table>
<thead>
<tr>
<th>Table I</th>
<th>Comparison of AMACR and p63 Staining Intensities in 61 Recut vs Destained H&amp;E-Stained Prostate Needle Biopsy Sections Stored for 7 to 11 Months*</th>
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<tbody>
<tr>
<td>Antigen</td>
<td>Staining Intensity</td>
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<tr>
<td>---------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>AMACR</td>
<td>Recut</td>
</tr>
<tr>
<td></td>
<td>Destained</td>
</tr>
<tr>
<td>p63</td>
<td>Recut</td>
</tr>
<tr>
<td></td>
<td>Destained</td>
</tr>
</tbody>
</table>

AMACR, α-methylacyl-CoA racemase.

* Data are given as number (percentage).
intensity in destained sections in 2 cases (3%) [Image 3], and completely negative staining of positive foci on recut sections in 4 destained sections (7%). For p63, there was a significantly decreased staining intensity of basal cells in destained sections and recut sections ($P < .0001$) (Table 1). Similar staining intensities were seen in only 11 cases (18%) (Image 1), a decreased staining intensity in destained sections in 32 cases (52%) (Images 2 and 3), and completely negative staining of positive foci on recut sections in 18 destained sections (30%). The differences in AMACR and p63 staining intensities in destained and recut stored sections are summarized in Table 2.

In sections less than 1 month old, the AMACR staining intensity of foci of prostatic carcinoma and the p63 staining intensity of basal cells were almost identical between destained sections and recut unstained sections [Table 3], with only 1 case with weak p63 staining in the destained section compared with strong staining on the recut section [Image 4].

**Discussion**

In this study, we showed that transfer of prostate needle biopsy tissue sections to charged slides and subsequent immunohistochemical staining with a p63/AMACR antibody cocktail is feasible with minimal tissue loss. Such a procedure performed on 7- to 11- month-old H&E-stained sections produced...
slightly decreased AMACR and markedly decreased p63 staining intensities compared with those seen on sections cut directly from the block. These staining differences seemed to be related to storage times because they were less likely to be seen in sections less than 1 month old. The use of a p63/AMACR cocktail on destained H&E-stained sections is, therefore, useful for recently prepared slides but not for slides stored for many months.

The diagnosis of prostatic carcinoma on needle biopsy specimens can be a straightforward task in the presence of the classic architectural and cytologic features of malignancy, namely, the finding of infiltrative glands lined by luminal cells with nuclear and especially nucleolar enlargement with no basal cell presence. However, there are situations when the architectural features might suggest malignancy but the nuclear features and the presence or absence of basal cells are obscured by distortion, thick sectioning, or poor preservation. In these situations, as well as in cases of minimal (limited; <1 mm) carcinoma, immunohistochemical analysis is being used increasingly to confirm the lack of basal cells or to document staining for AMACR and, accordingly, substantiate the H&E-based diagnosis of carcinoma.

Recently, several studies have documented that positive staining for AMACR seems to be of diagnostic value beyond that of a negative basal cell immunostain by adding further support to the diagnosis of malignancy in atypical or “challenging” prostate needle biopsy specimens. Because immunohistochemical staining of prostate needle biopsy specimens with

**Image 2** A focus of minimal prostatic adenocarcinoma and adjacent benign prostatic epithelium with similar α-methylacyl-coenzyme-A racemase (AMACR) and reduced p63 staining intensity in the transferred, destained section (B and D) compared with the section recut directly from the block (A and C) (A-D, p63/AMACR cocktail immunostain, A and B, ×100; C and D, ×400).
A focus of prostatic adenocarcinoma and adjacent benign prostatic epithelium with markedly reduced α-methylacyl-coenzyme-A racemase (AMACR) and p63 staining intensities in the transferred, destained section (B) compared with the section recut directly from the block (A) (A and B, p63/AMACR cocktail immunostain, ×100).

Table 2
Summary of Differences Between AMACR and p63 Staining Intensities in 61 Recut vs Destained H&E-Stained Prostate Needle Biopsy Sections Stored for 7 to 11 Months*

<table>
<thead>
<tr>
<th>Staining Intensity</th>
<th>AMACR</th>
<th>p63</th>
</tr>
</thead>
<tbody>
<tr>
<td>Similar to recut sections</td>
<td>55 (90)</td>
<td>11 (18)</td>
</tr>
<tr>
<td>Decreased compared with recut sections</td>
<td>2 (3)</td>
<td>32 (52)</td>
</tr>
<tr>
<td>Negative (originally positive on recut sections)</td>
<td>4 (7)</td>
<td>18 (30)</td>
</tr>
</tbody>
</table>

AMACR, α-methylacyl-coenzyme-A racemase. * Data are given as number (percentage).

Table 3
Side-by-Side Comparison of AMACR and p63 Staining Intensities in 10 Recut vs Destained H&E-Stained Prostate Needle Biopsy Sections Less Than 1 Month Old

<table>
<thead>
<tr>
<th>Case No.</th>
<th>AMACR Intensity</th>
<th>p63 Intensity</th>
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<tbody>
<tr>
<td></td>
<td>Recut</td>
<td>Destained</td>
</tr>
<tr>
<td>1</td>
<td>Strong</td>
<td>Strong</td>
</tr>
<tr>
<td>2</td>
<td>Strong</td>
<td>Strong</td>
</tr>
<tr>
<td>3</td>
<td>Strong</td>
<td>Moderate</td>
</tr>
<tr>
<td>4</td>
<td>Moderate</td>
<td>Moderate</td>
</tr>
<tr>
<td>5</td>
<td>Moderate</td>
<td>Moderate</td>
</tr>
<tr>
<td>6</td>
<td>Moderate</td>
<td>Moderate</td>
</tr>
<tr>
<td>7</td>
<td>Moderate</td>
<td>Moderate</td>
</tr>
<tr>
<td>8</td>
<td>Moderate</td>
<td>Moderate</td>
</tr>
<tr>
<td>9</td>
<td>Moderate</td>
<td>Moderate</td>
</tr>
<tr>
<td>10</td>
<td>Moderate</td>
<td>Moderate</td>
</tr>
</tbody>
</table>

AMACR, α-methylacyl-coenzyme-A racemase.

Reduced p63 staining intensity of benign prostatic glands in the transferred, destained section (B) compared with the section recut directly from the block (A) in a section stored for 2 weeks (A and B, p63/AMACR cocktail immunostain, ×100). AMACR, α-methylacyl-coenzyme-A racemase.
a p63/AMACR\textsuperscript{5,10,11} or a p63/34βE12/AMACR\textsuperscript{12} antibody cocktail on a single slide is feasible, diagnostically useful, and, at least for the p63/AMACR antibody cocktail, produces results equivalent to those using each antibody individually,\textsuperscript{5} the use of such cocktails has been recommended in the evaluation of small, diagnostically difficult foci in prostate needle biopsy tissue when only limited tissue is available for immunohistochemical studies.\textsuperscript{2,5,10-12} Despite these advantages, however, a common problem with these often small foci is that they might be present on only one or a few sections and, as seen in 9\% to 33\% of cases in different series,\textsuperscript{2,6,7} are not infrequently cut through with subsequent leveling of the paraffin block to obtain additional sections for the performance of immunohistochemical analysis.

Several solutions have been proposed to deal with this problem in prostate needle biopsy specimens. One approach is to cut unstained intervening sections on all prostate needle biopsy specimens for potential immunohistochemical staining. Green and Epstein,\textsuperscript{7} describing such a practice in their institution, reported their findings based on a study of prostate needle biopsy specimens during a 2-year period. Of 94 cases requiring high-molecular-weight cytokeratin (HMWCK) staining of the intervening sections for diagnosis, the authors identified 74 cases for which they were able to repeat the immunohistochemical staining on sections recut from the block. In 31\% (33\%) of these cases, the lesion was no longer present on sections taken from the paraffin block. In other words, HMWCK staining of intervening unstained slides was integral to the diagnosis of these 31 cases, representing 2.8\% of the 1,105 biopsies performed in this time frame.\textsuperscript{7} This certainly is an attractive approach; however, the main disadvantage is the extra laboratory time and cost (estimated by the authors of the aforementioned study\textsuperscript{7} to be an extra $13.20 per case, close to $15,000 in a 2-year period) incurred by generating these extra sections. Although this approach would save 2.8\% of patients a repeated biopsy, the increased time and costs are still important considerations.

An alternative approach is destaining the H&E-stained sections followed by immunohistochemical staining. Dardick and Epstein\textsuperscript{13} destained and then reainted 105 needle biopsy specimens with an anti-HMWCK antibody, allowing a definitive diagnosis to be reached in 58\% of cases. The major problems of such an approach were technical—tissue fell off the slide in 9\% of cases, 19\% of cases did not stain, and another 21\% of cases had suboptimal staining.\textsuperscript{13} Although there was no correlation between the use of charged and noncharged slides and the staining intensity, the use of noncharged slides was much more likely to be associated with tissue falling off the slides.\textsuperscript{13} Because heat-induced epitope retrieval (HIER), required for p63 and AMACR immunostaining\textsuperscript{5} and recommended for 34βE12\textsuperscript{14} immunostaining, was not performed in the aforementioned study,\textsuperscript{13} it is unknown whether such a procedure would have resulted in additional technical problems, especially considering that HIER is a major cause of tissue falling off the slide in routine immunohistochemical analysis.\textsuperscript{15} It should be noted that the study by Dardick and Epstein\textsuperscript{13} was performed on referred needle biopsy specimens, and although a formal cost analysis was not provided, we propose that restaining H&E-stained sections might be less costly than cutting and saving intervening sections on all prostate needle biopsy specimens.

A third approach, termed tissue protection immunohistochemistry,\textsuperscript{16} involves performance of immunohistochemical stains on H&E-stained sections without the need for destaining. Although 34βE12 immunostaining of 50 prostate needle biopsy specimens by this method produced slightly weaker staining intensity compared with immunostains performed on sections recut from the paraffin block, apparently interpretation of the results was not affected.\textsuperscript{16} This approach seems to be quite appealing because it obviates the need to obtain and save unstained intervening sections and reportedly is not associated with the technical problems seen with destaining and restaining sections as described by Dardick and Epstein.\textsuperscript{13} We also agree with the authors\textsuperscript{16} that their technique probably is less costly than cutting and saving intervening sections on every prostate needle biopsy specimen. The only requirement for this technique is that the original H&E-stained sections be mounted on charged slides, which probably is the main reason that technical issues were not a problem.

The final approach, used in the present study, is somewhat similar to the last 2 approaches described in the preceding paragraphs—the main difference is that H&E-stained sections were transferred from noncharged to charged slides before immunohistochemical staining to prevent tissue falling off the slides during HIER or immunohistochemical staining. Although originally described for cytology specimens to facilitate performance of multiple immunohistochemical stains on a single smear,\textsuperscript{8,17} its use in the evaluation of prostate needle biopsy specimens, including a comparison of the diagnostic usefulness of immunohistochemical staining of such sections with that performed on sections recut from the paraffin block, has not been described in detail.\textsuperscript{16}

In the present study, we demonstrated that transfer of prostate needle biopsy sections to charged slides and subsequent immunohistochemical staining with a p63/AMACR antibody cocktail is feasible and diagnostically useful. The low incidence of tissue loss (2/71 [3\%]) compares favorably with other methods and certainly is a viable approach when one encounters “suspicious” foci on prostate needle biopsy specimens that are cut through and no longer present on additional sections obtained from the block. Indeed, this has been the approach we follow in our institution for in-house and consultation cases. It is important to note that we always retain 1 H&E-stained section with the focus of concern and use this
technique on additional section(s) harboring the atypical focus. We also have used this technique for subsequent immunohistochemical staining with p34βE12, as well as anti-p63 and anti-AMACR antibodies individually with similar reliability (data not shown). In the presence of such limited tissue, however, the diagnostic value of using a p63/AMACR antibody cocktail is greatly amplified.

At this point, we should note that this technique is neither overtly cumbersome nor so time-intensive as to require a devoted person, and most of our histotechnicians can perform it during their routine daily workload. In fact, the actual hands-on time is less than 10 minutes. As for cost, it would also seem that this technique, considering that it is being performed on selected cases, probably is cheaper than cutting and saving intervening sections on every single case, especially when one also considers the minimal additional technician time and the fact that the tissue transfer medium we used is quite inexpensive ($12 for a 30-mL tube that is enough for transfer of more than 50 sections).

In this study, we found no statistically significant difference (P = .574) in the intensity of AMACR immunostaining performed on destained, approximately 1-year-old, H&E-stained sections and those performed on sections cut immediately from the block. However, this P value might be misleading. Although the decreased AMACR staining seen in 10% (6/61) of the destained sections, including 7% (4/61) of originally positive cases that were negative after destaining, might seem statistically nonsignificant, there are several possible reasons, other than chance alone (as the high P value might suggest), that would explain these differences. Because our findings contrast with those of Vanguri et al.18 who found no difference in the sensitivity of AMACR immunostaining between 63 freshly cut and stored prostate needle biopsy sections and who did not subject the stored sections to xylene soaking, tissue transfer, or destaining before immunohistochemical staining, it is unknown whether these and possibly other unknown technical factors (such as storage temperature) might have contributed to the loss of AMACR staining intensity in our study. It also seems reasonable to conclude that increased storage time has some bearing on the intensity of AMACR immunostaining because we found an almost identical AMACR staining intensity in destained and recut sections that were both less than 1 month old. This also might explain the higher AMACR sensitivity in the study by Vanguri et al.18 because their storage time was shorter (range, 1.6-9.2 months). Also in support of this conclusion are studies that found time-dependant decay in immunoreactivity for different markers in prostate needle biopsy sections,19 as well as in other tissues.20,21

In contrast with AMACR, the effect of “slide aging” was very apparent with p63 immunostaining because there was a decreased intensity of staining in most cases (50/61 [82%]) of destained sections, including a high proportion (18/61 [30%]) of cases that were positive for p63 on recut sections and were negative in destained sections. Such striking differences and negative p63 staining were not seen in sections less than 1 month old, with only 1 case (10%) of 10 that showed strong p63 staining on the recut section and weak staining on the destained section.

It is interesting that loss of immunohistochemical staining intensity with time for p53, the tumor suppressor gene product closely related to p63, is well characterized in the literature,22-24 including a significant deterioration of staining that is apparent as early as 2 weeks of storage.24 To our knowledge, this is the first report of such deterioration of p63 staining intensity with time.

Although slide aging might have significant implications for retrospective immunohistochemical studies,19,21 our data suggest that loss of p63 immunostaining intensity in stored slides is less likely to be a problem with most time frames used in diagnostic surgical pathology. Nevertheless, further investigation of this phenomenon, possibly including a side-by-side, time course comparison of p63 immunostaining on freshly cut and stored sections, is warranted because this problem might have more bearing on consultation cases, especially when one considers that, at least for prostate needle biopsy cases sent to one of us (P.A.H.) for a second opinion, prolonged intervals (the time the biopsy is obtained at an outside institution and the case being signed out) are not uncommon. In fact, in a review of consecutive consultation cases submitted during a 16-month period, we found that 64% and 16% of cases had an interval equal to or greater than 15 and 30 days, respectively. On the other hand, as discussed, our data and those of Vanguri et al.18 would suggest that slide aging has much less of an impact on AMACR immunostaining, at least for routine diagnostic use.

We have shown that transfer of prostate needle biopsy sections to charged slides and subsequent p63/AMACR immunohistochemical staining is feasible with minimal tissue loss and, for sections less than 1 month old, produces a staining intensity very similar to that seen by immunostaining sections cut from the block with the same antibody cocktail. The use of a p63/AMACR cocktail on destained H&E-stained sections would be most useful when small atypical foci in prostate needle biopsy specimens are lost on subsequent leveling of the paraffin block for immunohistochemical analysis. A prospective study to compare the efficacy and cost-effectiveness of this method compared with other previously described methods of evaluating small atypical foci in prostate needle biopsy specimens that are lost on subsequent sectioning, including cutting and saving unstained intervening sections and so-called tissue protection immunohistochemistry, would be useful to determine the optimal method for dealing with these cases.
References


