

# Reliable Identification of Endometrial Precancers Through Combined Pax2, $\beta$ -Catenin, and Pten Immunohistochemistry

Mitzi Aguilar, BS,\* Hao Chen, MD, PhD,\* Glorimar Rivera-Colon, MD,\* Shuang Niu, MD,\* Kelley Carrick, MD,\* Katja Gwin, MD,\* Ileana C. Cuevas, PhD,\* Subhransu S. Sahoo, PhD,\* Hao-Dong Li, PhD,\* Song Zhang, PhD,†‡ Wenxin Zheng, MD,\*‡ Elena Lucas, MD,\*‡ and Diego H. Castrillon, MD, PhD\*‡

**Abstract:** The diagnosis of endometrial atypical hyperplasia/endometrioid intraepithelial neoplasia (AH/EIN) remains challenging and subjective in some cases, with variable histologic criteria and differences of opinion among gynecologic pathologists, potentially leading to under/overtreatment. There has been growing interest in the use of specific immunohistochemical markers as adjuncts in AH/EIN diagnosis. For example, the World Health Organization 2020 Classification specifies that loss of Pten, Pax2, or mismatch repair proteins are desirable diagnostic criteria. Other markers, most notably  $\beta$ -catenin and Arid1a, are also aberrantly expressed in some AH/EIN. However, the performance of some markers individually—and more importantly as a group—has not been rigorously explored, raising questions as to which marker(s) or combination(s) is the most effective in practice. Formalin-fixed paraffin-embedded tissue sections from AH/EIN cases (n=111) were analyzed by immunohistochemistry for 6 markers: Pax2, Pten, Mlh1,  $\beta$ -catenin, Arid1a, and p53. Aberrant expression was tabulated for each case and marker. An additional set of normal endometria (n=79) was also analyzed to define optimal diagnostic criteria for marker aberrance. The performance characteristics of each marker, the entire panel, and subsets thereof were quantitatively and statistically analyzed. In order of number of cases detected, the most frequently aberrant markers in AH/EIN were Pax2

(81.1% of cases), Pten (50.5%),  $\beta$ -catenin (47.7%), Arid1a (7.2%), Mlh1 (4.5%), and p53 (2.7%). The majority of cases showed aberrant expression of  $\geq 2$  markers. All 6 markers together identified 92.8% of cases. Arid1a, Mlh1, and p53 were robust and readily scored markers, but all cases showing aberrant expression of these 3 markers were also detected by Pax2, Pten, or  $\beta$ -catenin. A focused panel of only 3 markers (Pax2, Pten, and  $\beta$ -catenin) showed optimal performance characteristics as a diagnostic adjunct in the histopathologic diagnosis of AH/EIN. Use of this panel is practicable and robust, with at least 1 of the 3 markers being aberrant in 92.8% of AH/EIN.

**Key Words:** atypical endometrial hyperplasia, endometrioid intraepithelial neoplasia, Pax2, Pten,  $\beta$ -catenin, Arid1a, Mlh1, p53, immunohistochemistry

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Most endometrial cancers are endometrioid and arise from precancerous histologic precursors termed atypical hyperplasia (AH) or endometrioid intraepithelial neoplasia (EIN), depending on the classification system.<sup>1,2</sup> Despite sustained efforts to refine histologic criteria for AH/EIN diagnosis, the histomorphologic diagnosis of endometrial precancers by pathologists is subjective with frequent differences of opinion.<sup>3–6</sup> The challenges in practice are several. First, endometrial samplings (biopsies or curettings) are highly fragmented, limiting the evaluation of small lesions and obscuring assessment of overall architectural features such as gland crowding. Second, dramatic variations in glandular architecture and cytology as a consequence of fluctuating levels of estrogen and progesterone during normal cycling add another layer of complexity to histologic evaluation. Third, many patients with abnormal uterine bleeding or known AH/EIN are treated with hormonal agents that obscure architectural and cytologic features of AH/EIN.<sup>7</sup> Fourth, endometrial polyps are common non-neoplastic lesions that can exhibit considerable gland crowding but conversely often harbor AH/EIN—and because of specimen fragmentation, can be difficult to reliably identify.<sup>8,9</sup> Fifth, AH/EIN can be focal or diffuse, with gradual variations in

From the Departments of \*Pathology; †Population and Data Sciences, UT Southwestern Medical School; and ‡Harold C. Simmons Comprehensive Cancer Center, Dallas, TX.

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Correspondence: Diego H. Castrillon, MD, PhD, Department of Pathology, UT Southwestern Medical School, 6000 Harry Hines Boulevard, NB6.452, Dallas, TX 75390-9072 (e-mail: diego.castrillon@utsouthwestern.edu).

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gland architecture that can make it difficult to identify or clearly demarcate definitive regions of AH/EIN. Perhaps not surprisingly, studies have shown poor interobserver reproducibility even among expert gynecologic pathologists for the AH<sup>10</sup> or for both the AH and EIN diagnostic schema.<sup>11</sup>

These findings point to a limit of further refinements in histologic criteria, leading to the search for and validation of diagnostically useful biomarkers for AH/EIN.<sup>12–17</sup> In recognition of these challenges, the 2020 World Health Organization Classification of Female Genital Tumors states that “loss of immunoreactivity for Pten, Pax2, or mismatch repair proteins is desirable” in the diagnosis of AH/EIN.<sup>18</sup> This statement implies that a panel of immunostains is desirable in the diagnosis of AH/EIN. However, there has not yet been a systematic analysis of how such multiple markers should be deployed in practice, or if other recently described markers of some AH/EIN—most notably  $\beta$ -catenin and Arid1a—would have diagnostic benefit if included. Here, we systematically analyzed the performance characteristics of 6 immunohistochemical markers (Pax2, Pten,  $\beta$ -catenin, Arid1a, Mlh1, and p53) both individually and in combinations. In addition to refining specific criteria for scoring these markers, our findings demonstrate that a panel of 3 markers has optimal performance characteristics and is practical, feasible, efficient, and of considerable utility in the diagnosis of AH/EIN.

## MATERIALS AND METHODS

### Case Selection

After approval from the UT Southwestern IRB, we retrospectively identified cases via text searches with a final diagnosis of complex atypical hyperplasia or endometrioid intraepithelial neoplasia accessioned between 2010 and 2020 at 2 UT Southwestern teaching hospitals, Clements University Hospital and Parkland Memorial Hospital. Standard histologic diagnostic criteria were used for the initial diagnosis of AH or EIN including gland:stroma ratio >1, overt nuclear atypia/cytologic demarcation from background endometrium, size  $\geq$  1 mm, and exclusion of mimics.<sup>18–20</sup> Cases that were ambiguous or subdiagnostic for AH/EIN, or harbored definitive carcinoma per the original reports were not included in this study. Cases where the patient was undergoing treatment with high-dose progestin for a prior diagnosis of AH/EIN were excluded. Two of the AH/EIN cases showed decidual-type change consistent with progestin treatment for other conditions. We also identified cases of normal (proliferative to secretory) endometrium for use as controls including 65 proliferative, 11 secretory, and 3 interval phase. For AH/EIN and normal control endometria, unstained 4  $\mu$ m sections were cut from one representative tissue block for each case. A total of 111 AH/EIN cases and 80 control cases were selected for testing. Two specialty pathologists reviewed H&E slides for each case to verify the original diagnoses. As described in the text, one of the control cases was subsequently determined to harbor AH/EIN and was censored from the selected cases, leaving n = 79 normal cases for quantitative analyses.

None of the AH/EIN cases, with the additional pathologist review in the course of this study, were reclassified.

### Immunohistochemistry

For Pax2, Pten,  $\beta$ -catenin, Mlh1, and p53 staining protocols previously validated for clinical testing were performed in the clinical pathology laboratory on a DAKO Autostainer Link 48 instrument. The following primary antibodies were used: p53 (prediluted, clone DO-7, #IR61661-2, Agilent, Santa Clara, CA), Mlh1 (prediluted, clone ES05, #IR07961-2, Agilent),  $\beta$ -catenin (prediluted, clone  $\beta$ -catenin-1, #IR70261-2, Agilent), Pax2 (prediluted, clone EP235, #BSB2567, Cancer Diagnostics, Durham, NC), and Pten (prediluted, clone 6H2.1, #PM278AA, BioCare, Pacheco, CA) with antigen retrieval performed in low pH (6.0) for  $\beta$ -catenin and high pH (9.0) Tris/EDTA solution (Agilent) for the other markers at 97°C for 20 minutes. FLEX peroxidase block was performed for 10 minutes for  $\beta$ -catenin and 5 minutes for other markers. Primary antibody incubation time was 20 minutes for  $\beta$ -catenin, 30 minutes for p53, and 40 minutes for Pax2, Pten, and Mlh1. Incubation with Mouse Linker (Agilent) for  $\beta$ -catenin and Rabbit Linker (Agilent) for Pax2 was performed for 10 minutes. Secondary antibody (Envision/HRP) incubation time was 20 minutes for Pten,  $\beta$ -catenin, and p53, 30 minutes for Pax2, and 40 minutes for Mlh1. Arid1a immunohistochemistry was performed on a DAKO Autostainer Link 48 instrument in a research core facility (1:200 dilution, clone D2A8U, #12354, Cell Signaling Technology, Danvers, MA) with low pH (6.0) Tris/EDTA solution (Agilent) for 20 minutes at 97°C. Primary and secondary incubation times were 20 minutes each. For all antibodies, the enzymatic conversion of the 3,3'-diaminobenzidine tetrahydrochloride chromogen was performed for 10 minutes at room temperature. Hematoxylin counterstaining was performed unless otherwise indicated.

### Statistical Analysis

A  $P < 0.05$  is considered to be statistically significant. Cohen  $\kappa$ , an index that considers observed agreement with respect to agreement by chance, was used to measure the co-occurrence/agreement between biomarkers and squamous differentiation.<sup>21</sup> It has a range from -1 to 1, where 1 indicates perfect agreement, 0 random, and -1 perfect disagreement. Statistical analysis was performed using SAS 9.4 (SAS Institute, Cary, NC).

## RESULTS

### Patterns of Marker Expression in AH/EIN and Normal Control Endometria

Six immunohistochemical markers (Pax2, Pten,  $\beta$ -catenin, Arid1a, Mlh1, and p53) were selected for investigation on the basis of prior literature demonstrating that their expression is aberrant in some AH/EIN and that this aberrant expression could therefore be helpful in the diagnosis of AH/EIN.<sup>9,12–14,16,17,19,22–30</sup> A total of n = 111 cases of AH/EIN and n = 79 control endometria were selected, and entire tissue sections from formalin-fixed paraffin-embedded tissue blocks

were subjected to immunohistochemistry for the 6 markers. First, overall marker patterns in AH/EIN and normal endometria will be described with a focus on criteria to reliably distinguish between the 2, followed by the quantitative and statistical analyses of the markers individually and as a group.

### Pax2

In AH/EIN, loss of Pax2 (which localizes exclusively within nuclei) occurred across large areas. Entrapped normal glands usually retained expression, highlighting the loss of Pax2 expression (Figs. 1A, B). However, in some cases, loss of expression did not occur uniformly in AH/EIN (Fig. 1C). Within the endometrium, Pax2 is expressed only in epithelial cells, and presence of Pax2 expression in any gland(s) serves as a useful internal positive control. If such controls are not present, confirmation of expression in an external control placed on the slide is necessary. Within individual glands, Pax2 loss was consistently total relative to the very strong and uniform expression in control glands, making Pax2 an easily scored marker. These patterns are consistent with prior reports.<sup>12,14,17,19,22,23</sup>

In normal endometria, Pax2 loss can occur in single (Fig. 1D) or scattered glands (Fig. 1E). Rarely, Pax2-deficient glands in normal endometrium can be more extensive (Fig. 1F). However, in all normal endometria analyzed, such loss occurred in <5% of the endometrium, pointing to  $\geq 5\%$  loss as a useful threshold distinguishing normal versus AH/EIN (detailed quantitative results for all markers together will be presented below). The presence of occasional Pax2-deficient glands in some endometria also underscores that for any AH/EIN marker, aberrant expression needs to be evaluated in the context of architectural features concerning for AH/EIN.

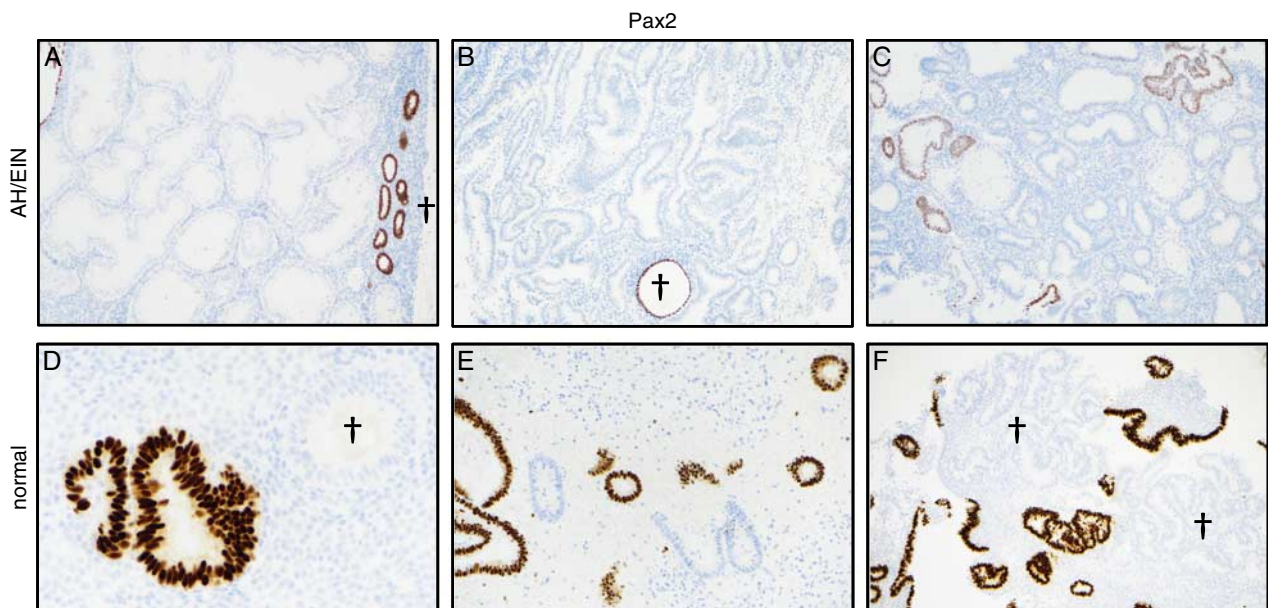
### Pten

In some AH/EIN, Pten (normally present in the nucleus, cytoplasm, and cell membrane)<sup>31</sup> can appear weak and somewhat variable; this should not be misconstrued as aberrant (Fig. 2A). True Pten loss is characterized by complete absence of nuclear and cytoplasmic expression in glands (excluding intraglandular leukocytes, which can be abundant). Pten is highly expressed in benign endometrial epithelial cells, endometrial stroma, and leukocytes, and retained stromal expression results in a “punched-out” appearance of glands when true epithelial loss is present (Fig. 2B). Pten loss can occur in some or all of the AH/EIN glands; that is, it can be heterogeneous, perhaps reflecting a second “hit” during tumor progression.<sup>31</sup> In many cases with bona fide Pten loss, some areas of definitive AH/EIN retain Pten. If the area of Pten loss comprises a significant proportion of the putative AH/EIN, Pten should be scored as lost (Fig. 2C).

In normal endometria, Pten loss in scattered glands was a common occurrence, in accordance with previous landmark studies.<sup>14,19,24,32</sup> The glands were usually single or in small clusters (Figs. 2D, E) but occasionally, larger clusters of 20 to 40 Pten-deficient glands were found (Fig. 2F, showing a cluster of ~24 null glands in a proliferative endometrium). Therefore, as with Pax2, the presence of small clusters of Pten-null glands does not by itself indicate an AH/EIN. However, in normal endometria with Pten-null glands, these constituted  $\leq 5\%$  of the endometrium.

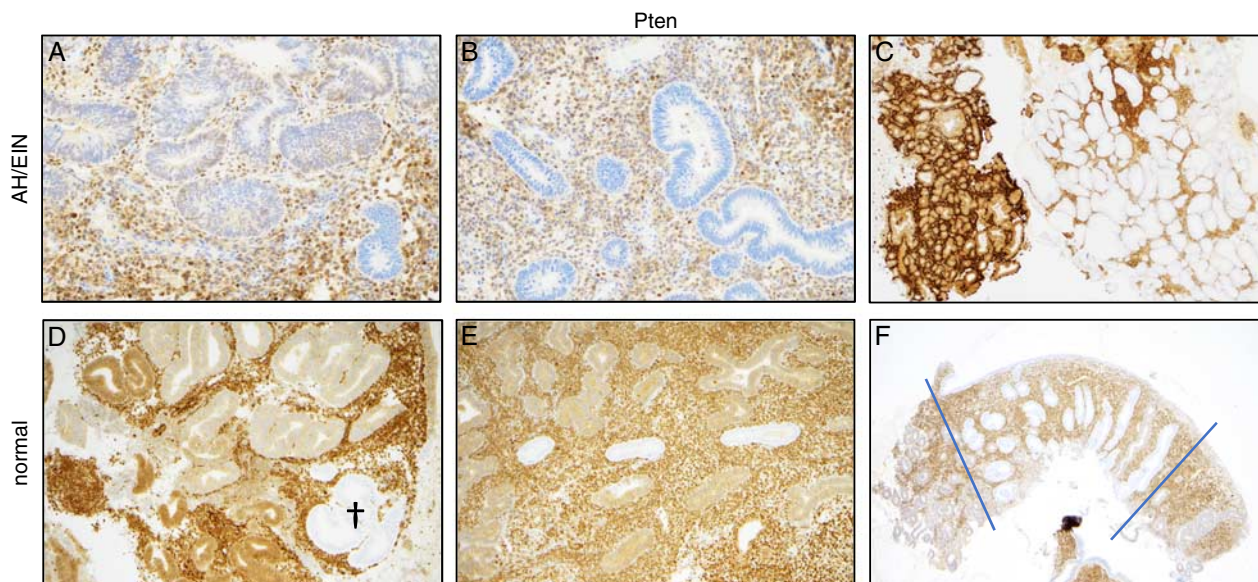
### $\beta$ -Catenin

Strong nuclear  $\beta$ -catenin localization, usually associated with overall overexpression, is a reliable indicator of  $\beta$ -catenin activation in AH/EIN or cancer.<sup>12,33,34</sup> Unlike Pten or Pax2, where loss of expression is the feature indicating aberrance,



**FIGURE 1.** Patterns of Pax2 expression in AH/EIN and normal endometrial controls. AH/EIN (A–C). Normal endometrium (D–F). Each panel corresponds to a different case. See text for interpretations. F, There is some artefactual gland crowding in areas of Pax2 loss. †, Morphologically normal glands.





**FIGURE 2.** Patterns of Pten expression in AH/EIN and normal endometrial controls. Each panel corresponds to a different case. A, Pten nonaberrant case. The single gland in the lower right corner in (A) exhibited weak staining (not complete loss). B, Pten loss in a relatively uncrowded field of glands highlights punched-out appearance of true Pten loss in a biopsy with definitive AH/EIN. C, Definitive Pten loss in extensive area of AH/EIN. D, Single Pten-deficient gland (†). E, Cluster of 3 Pten-deficient glands. F, Large cluster of Pten-deficient glands (demarcated by blue lines). See text for interpretations.

relocalization of  $\beta$ -catenin to the nucleus is the principal immunohistochemical finding indicating an underlying molecular defect. The presence of strong, distinctively nuclear expression in glands observed in many AH/EIN cases, even when focally present, makes scoring such cases straightforward (Fig. 3A). Sometimes, particularly in cases with strong cytoplasmic signal, it can be difficult to score nuclear localization. In these cases, the presence of nuclei with staining at least as intense as the cell membranes in those cells/areas is a very helpful diagnostic feature (Fig. 3B). Morular squamous metaplasia, which is associated with underlying *CTNGB1* mutations, always exhibits nuclear  $\beta$ -catenin,<sup>35</sup> and  $\beta$ -catenin should be assessed in endometrial epithelium without obvious morules. When morules are present, adjacent epithelium usually exhibits distinctive nuclear localization in some nonmorular epithelial cells (Fig. 3C). Characteristically, nuclear localization occurs in scattered cells within AH/EIN glands, and is not uniform among all cells in a gland (Figs. 3A–C).

In normal endometrium,  $\beta$ -catenin is predominantly membranous with some cytoplasmic localization and little nuclear expression (Fig. 3D).<sup>12</sup> Also, the overall pattern is usually homogenous across large areas of endometrium. We noted that several ( $n = 3$ ) interval-type (proliferative/secretory) endometria exhibited more variable expression among glands, with stronger expression in the glands with more developed secretory changes (Fig. 3E). This variability of  $\beta$ -catenin expression, typical of interval-type endometria, can give the false impression of AH/EIN. In some normal endometria, including interval-type, distinctive nuclear localization was seen. However, in such cases, the nuclear signal was less than the membranous expression in those cells (Fig. 3F), providing

a useful criterion distinguishing such expression from AH/EIN, where nuclear localization/signal is higher than the intervening cell membranes (Fig. 3B). Also, the normal gland shown in Figure 3F has relatively homogenous nuclear expression throughout the gland, whereas the nuclear localization in AH/EIN is more variable among nearby cells (Figs. 3A–C).

### Mlh1, Arid1a, and p53

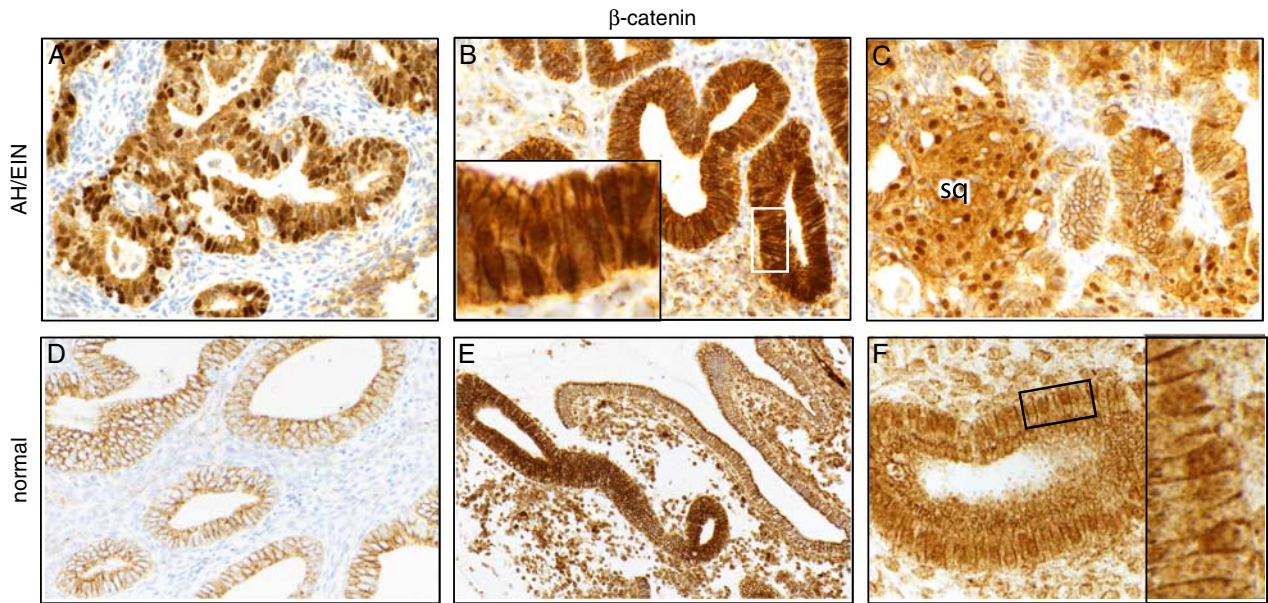
AH/EINs with Mlh1 protein deficiency were easily scored, with strong stromal nuclear staining present in endometrial stroma serving as an internal control (Figs. 4A, D).

Scoring of Arid1a loss in AH/EIN and identification of Arid1a-deficient cases was analogous to and as equally reliable and facile as Mlh1<sup>26</sup> (Figs. 4B, E), making it also a potentially useful marker. As with Mlh1, strong nuclear expression in stromal cells serves as a useful internal control highlighting Arid1a loss.

Although null patterns of p53 expression are more difficult to recognize while screening slides, the mutant overexpression pattern is readily identified, and some AH/EIN cases with foci of p53 overexpression (ie, aberrant/mutant pattern) were identified. In these cases, the p53-positive foci did not exhibit notable atypia, and thus were not suspicious for serous-type neoplasia. One (1.3%) of the 79 normal controls harbored a single gland with a mutant (overexpression) pattern; an H&E section did not show nuclear or architectural features suspicious for occult AH/EIN or serous intraepithelial carcinoma (Figs. 4C, F).

Thus, in summary, aberrant patterns of Mlh1, Arid1a, and p53 are readily scored and of potential use in the evaluation of AH/EIN.



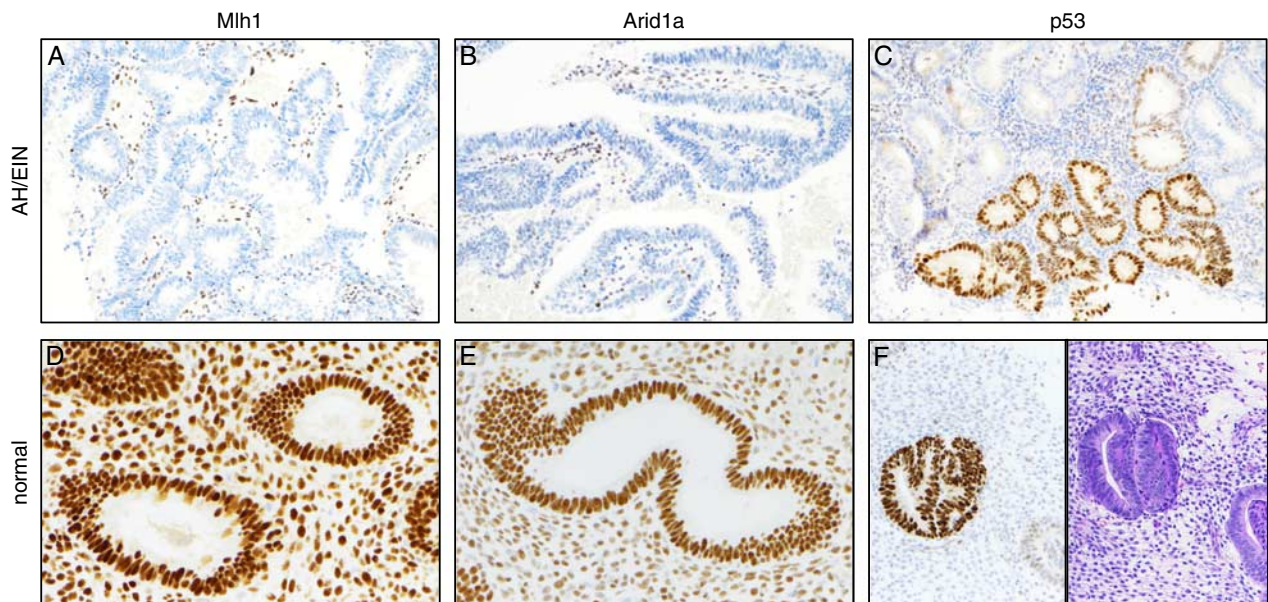


**FIGURE 3.** Patterns of  $\beta$ -catenin expression in AH/EIN and normal endometrial controls. Each panel corresponds to a different case. See text for interpretations, insets correspond to higher-magnification views of smaller boxed areas, sq = squamous differentiation (A–E). E, interval-type endometrium. F, the section was not counterstained following immunohistochemistry, to avoid nuclear hematoxylin staining that can obscure fine assessment of  $\beta$ -catenin protein localization, inset corresponds to small black rectangle.

**Reclassification of a Case on the Basis of EIN Markers**

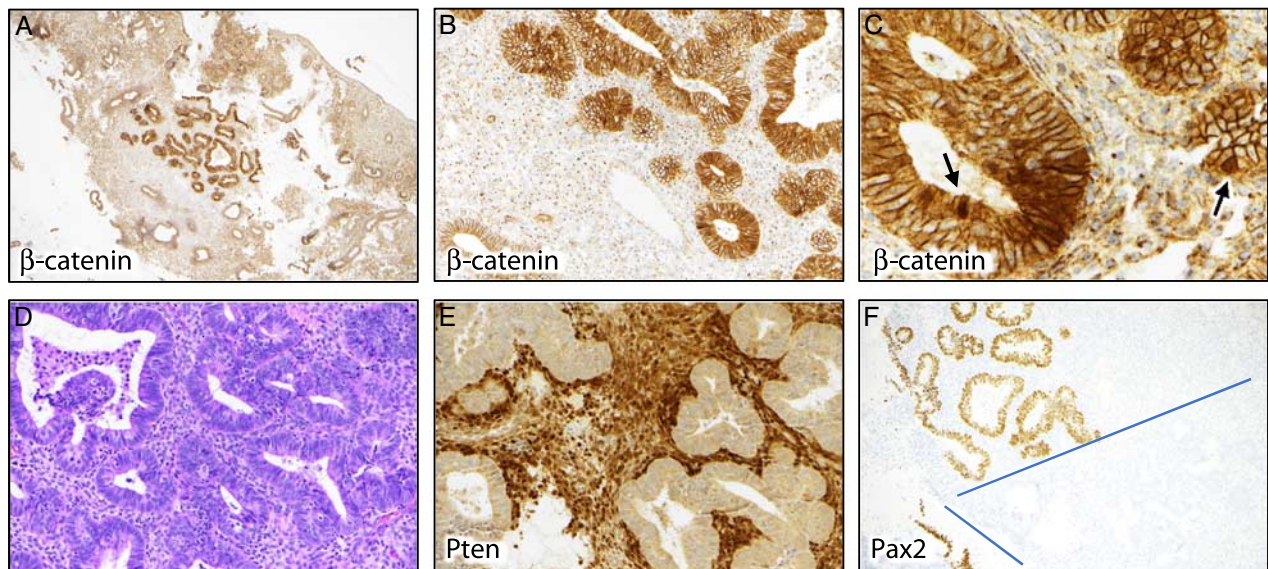
The assignment of cases in this study to the AH/EIN versus normal control groups was based on the original diagnoses. With the AH/EIN marker scoring criteria described above, one of the endometrial controls subjected to the panel exhibited an aberrant marker

pattern consistent with AH/EIN. In this case,  $\beta$ -catenin at low power showed a minute cluster of crowded glands with stronger staining than the surrounding endometrium (Fig. 5A). Intermediate magnification showed increased cytoplasmic and membranous staining in the crowded focus (Fig. 5B), while high magnification revealed scattered nuclei with very strong  $\beta$ -catenin localization,



**FIGURE 4.** Patterns of Mlh1, Arid1a, and p53 expression in AH/EIN and normal endometrial controls. Each panel corresponds to a different case (A–F). An H&E step section (F, right half of panel) shows lack of significant atypia in the p53+ gland. See text for additional interpretations.





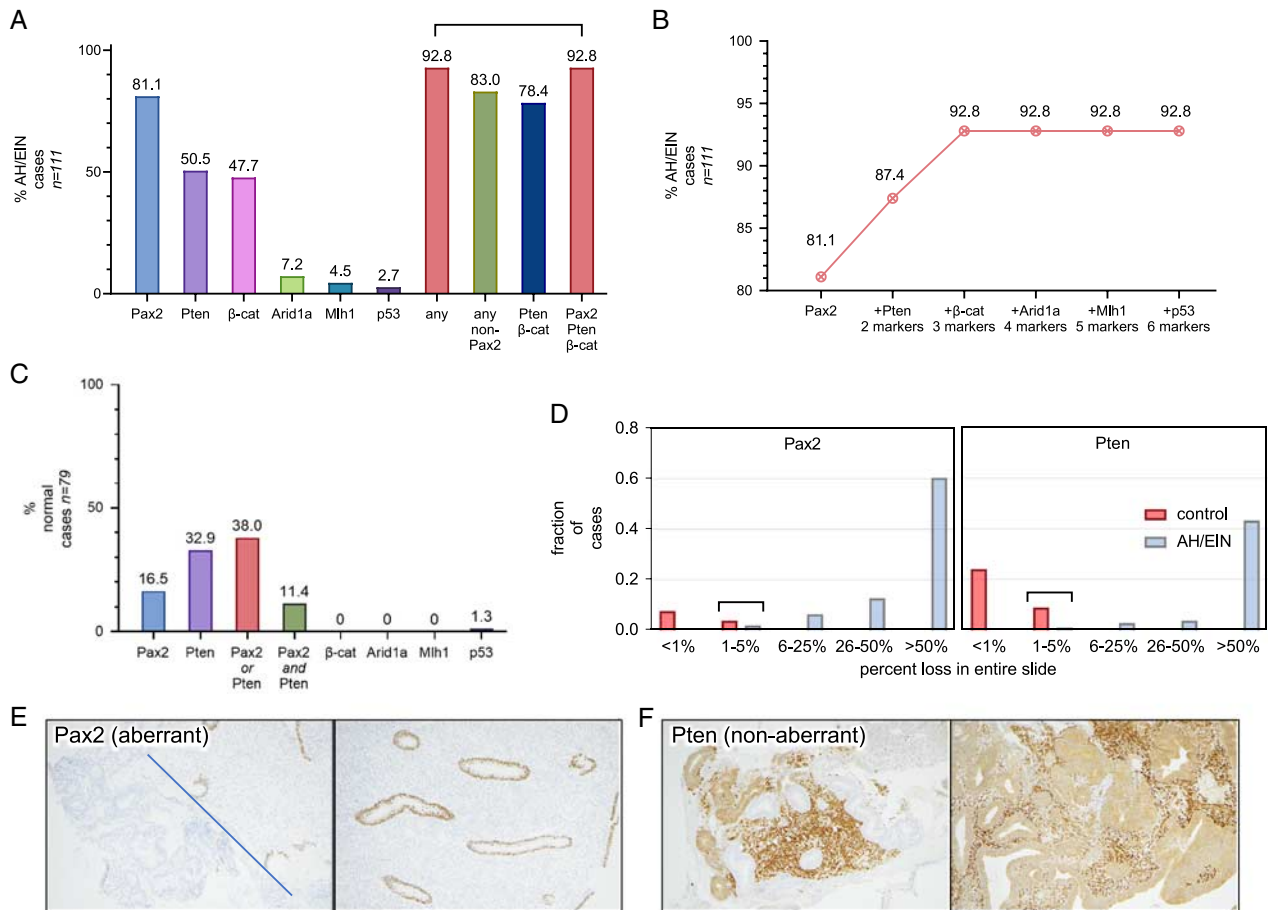
**FIGURE 5.** Endometrial biopsy originally diagnosed as normal endometrium reclassified as AH/EIN on the basis of  $\beta$ -catenin expression. A, Low magnification. B, Intermediate magnification. C, High magnification showing focal but strong nuclear localization (arrows). D, H&E. E, Retention of Pten. F, Partial loss of Pax2 within the small AH/EIN. A minute focus of Pax2 deficiency involving only a few endometrial glands is not normally sufficient for the diagnosis of AH/EIN, but in the context of the morphologic findings and aberrant  $\beta$ -catenin localization, was further supportive of the diagnosis of AH/EIN. Blue lines demarcate area of Pax2-negative glands.

indicating a mutant/aberrant pattern (Fig. 5C). Upon review of an H&E section, there was unanimous agreement that this focus, which exhibited gland crowding, cribriforming, and cytologic distinctiveness consistent with nuclear atypia, constituted a bona fide AH/EIN by standard histologic criteria despite its small (1.0 mm) size (Fig. 5D).<sup>18,19</sup> Within this focus, Pten was retained, while Pax2 was partially lost in the AH/EIN but retained in the surrounding normal endometrium, further supporting reclassification to AH/EIN (Figs. 5E, F). The case is presented here for illustration purposes but was censored from the tabulated cases.

### Quantitative Analyses of the 6 Markers in AH/EIN and Normal Endometria

As single markers, Pax2, Pten, or  $\beta$ -catenin were aberrant in a high percentage of AH/EIN cases (Pax2, 81.1%; Pten, 50.5%;  $\beta$ -catenin, 47.7%). Arid1a, Mlh1, or p53 were aberrant in a significant, but much smaller percentage of cases (7.2%, 4.5%, and 2.7%, respectively) (Fig. 6A). The potential use of each marker as part of a larger panel was then further considered. With a hypothetical panel consisting of all 6 markers, at least one of the markers would be aberrant in 92.8% of AH/EIN cases (Fig. 6A). The 5 non-Pax2 markers identified 83.0% of cases, while Pten and  $\beta$ -catenin combined identified 78.4% of cases (Fig. 6A). The additive effects of each marker in order of aberrancy in AH/EIN is shown in Figure 6B. A panel consisting of only Pax2, Pten, and  $\beta$ -catenin identified 92.8% of cases. Inclusion of the other 3 markers (Arid1a, Mlh1, and p53) did not increase the diagnostic yield further because all of the cases detected by any of these 3 markers was already scored as aberrant by Pax2, Pten, or  $\beta$ -catenin (Figs. 6A, B).

Optimal interpretation of any immunohistochemical marker for AH/EIN requires an understanding of its patterns of expression among normal endometria. To this end, entire sections of  $n=79$  endometria (not counting the case shown in Fig. 5) including proliferative to secretory endometria were analyzed by the 6 markers. Aberrancy in even a single gland was scored (Fig. 6C) and the percentage of null glands on the entire slide used to categorize the cases into 5 groups (<1%, 1% to 5%, 6% to 25%, 26% to 50%, >50%). Pax2 and Pten were definitively lost in at least one gland in a significant percentage of normal endometria, 16.5% and 32.9%, respectively (Figs. 1–5), comparable to previous studies.<sup>22,36</sup> For Pax2, the number of deficient glands numbered from 3 to 47. In most cases this constituted <1% of the total biopsy, but in a few cases the Pax2-deficient glands comprised 1% to 5% of the endometrium (Fig. 6D). For Pten, the number of deficient glands in different samples ranged from 1 to 40. As with Pax2, Pten-deficient glands comprised <1% in most cases and not >1% to 5% of the endometrium (Fig. 6D). 38.0% of control endometria were focally deficient for Pax2 or Pten, and 11.4% for Pax2 and Pten (Fig. 6C) with little overlap/concordance of Pax2 and Pten deficiency within individual glands (ie, the Pax2 and Pten-deficient glands were distinct and separate) as previously reported.<sup>14</sup> Illustrative examples of AH/EIN cases with borderline loss of Pax2 or Pten that can be difficult to interpret are shown in Figures 6E and F. In the Pax2 example, the area of Pax2 loss was 1% to 5%, but this occurred in the crowded but sparse focus suspicious for AH/EIN, whereas the majority of the biopsy consisted of Pax2<sup>+</sup> unremarkable endometrium. This case was scored as Pax2 aberrant (Fig. 6E). In the Pten example, scattered but rare

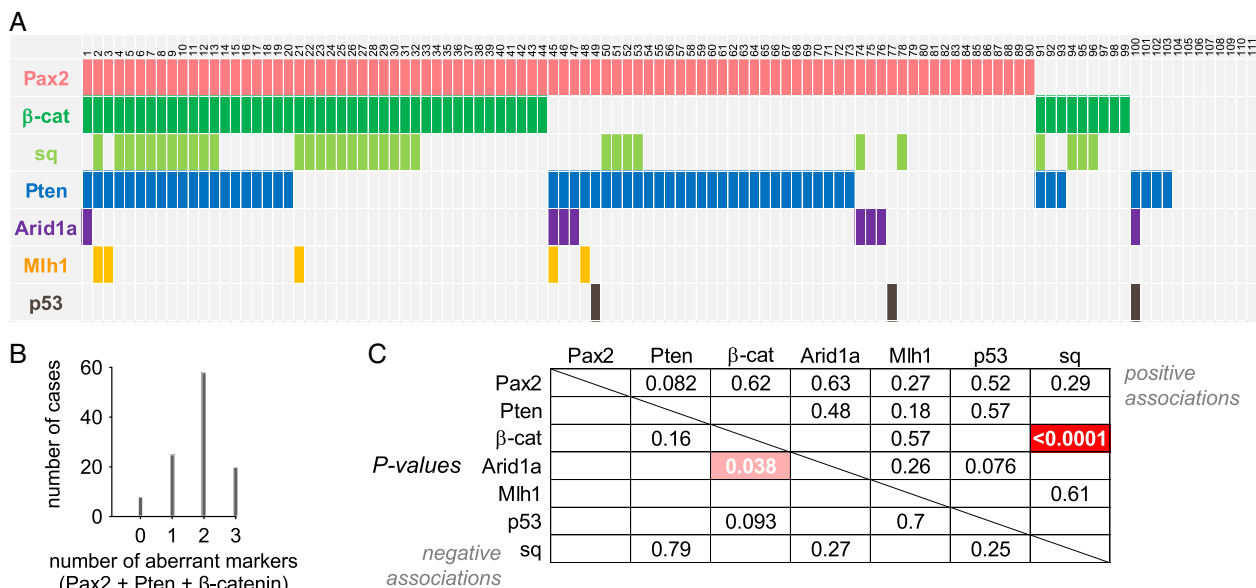


**FIGURE 6.** Quantitative analyses of the 6 candidate AH/EIN markers including group performance. **A,** Percent of AH/EIN cases showing aberrancy for individual markers, or any one marker or marker combinations as shown. **B,** Diagnostic yields following addition of individual markers, in order of greatest to least likelihood of aberrancy. Arid1a, Mlh1, or p53 did not lead to the identification of any additional AH/EIN cases, pointing to Pax2/Pten/β-catenin as the most effective and compact panel. **C,** Percent of normal endometrial controls showing aberrancy for each marker. The numbers are not entirely comparable to (A), because aberrancy within a single gland of normal endometrium was scored as aberrant, whereas aberrancy within AH/EIN was characterized by large areas involving many glands. Nonetheless, the findings indicate that evaluation of markers must always occur in the context of histologic features. **D,** Fraction (%) of control (orange) or AH/EIN (blue) cases exhibiting loss of Pax2 or Pten across different categories based on estimated overall loss on slide (<1%, 1% to 5%, 6% to 25%, 26% to 50%, or >50%). **E,** Example of borderline case where Pax2 scoring was challenging due to focal loss (1% to 5%), but interpreted as aberrant; panels show different fields with AH/EIN (left of blue line) and morphologically normal endometrium (right). **F,** Example of borderline case where Pten loss was <1% and interpreted as nonaberrant; panels show different fields (left = uncrowded endometrium; right = AH/EIN).

glands were Pten-deficient (<1% of the endometrium), but these were compatible with normal residual glands, whereas the more crowded areas suspicious for AH/EIN retained Pten. In this case, the areas of Pten loss were comparable in extent to and not readily distinguishable from the range documented in normal control endometria (sporadic loss). This case was scored as Pten nonaberrant (Fig. 6F).

In contrast, β-catenin, Arid1a, Mlh1 were not aberrant in even a single gland in any of the n = 79 normal endometria (Fig. 6C). p53 showed strong diffuse overexpression consistent with a mutant pattern in a single gland in 1 case. The biological significance of this very focal p53 mutant pattern is unclear, as this case, including the p53-overexpressing gland, did not exhibit atypia or other overt indications of neoplasia (Figs. 6C, 4F).

For the AH/EIN cases, aberrant patterns of expression for the 6 markers and squamous differentiation were arranged in a case matrix (Fig. 7A). With a panel consisting of Pax2, Pten, and β-catenin, most cases (70%) would be aberrant for 2 (52%) or 3 (18%) markers (Fig. 7B), adding further to diagnostic confidence. All pairwise associations were then formally investigated among the 7 observations by κ statistics. The strongest positive association was between β-catenin and squamous differentiation (κ = 0.41, P < 0.0001). There was a positive association between Pax2 and Pten trending towards, but not achieving, statistical significance (κ = 0.13, P = 0.082). The only statistically significant negative association (albeit among relatively few cases) was between Arid1a and β-catenin; only 1 of 8 Arid1a cases was also aberrant for β-catenin (κ = -0.11, P = 0.038, Fig. 7B).



**FIGURE 7.** Marker aberrancy matrix among AH/EIN. sq = squamous differentiation, independently scored on the H&E section for each case. A, Filled-in rectangles indicate aberrance for that marker in individual cases of AH/EIN. B, Distribution of number of cases (among n = 111 AH/EIN) aberrant for 0, 1, 2, or 3 markers with a panel consisting of Pax2, Pten, and β-catenin. C, Determination of associations among all pairwise combinations of markers and squamous differentiation (sq). Positive associations are positioned on the upper right; negative associations on the lower left. Numbers within the cells correspond to P values, with relevant κ values provided in the text. P < 0.05 highlighted in light red; P < 0.01 highlighted in bright red.

### DISCUSSION

The purpose of this study was to define the performance characteristics of potential AH/EIN markers individually and in combinations in definitive AH/EIN as a framework for clinical use and future investigations. Pax2 was the single most useful marker in the diagnosis of AH/EIN, followed by Pten and β-catenin. However, because of nonoverlapping patterns of aberrancy of these 3 markers among AH/EIN, Pten and β-catenin immunohistochemistry significantly enhanced the diagnostic yield over Pax2 alone. Pten and β-catenin alone (without Pax2) were aberrant in 78.4% of cases, and most Pax2-deficient cases were either Pten or β-catenin aberrant. Aberrancy for 2 or more markers can further improve diagnostic confidence while evaluating a particular case, and most AH/EIN with the 3-marker panel were aberrant for at least 2 markers. Together, a panel consisting of Pax2, Pten, and β-catenin can help identify a high percentage of cases (92.8%), potentially making such a panel useful in practice. This number is comparable to a recent serial genomic analysis of endometrial cancer progression, where next generation sequencing-directed immunohistochemistry found that 86% of endometrial cancers were aberrant for at least 1 of the 5 non-Pax2 markers, with persistence of AH/EIN marker patterns in samples from each patient.<sup>37</sup> Conversely, since 7.2% of bona fide AH/EIN were not aberrant for any of these markers, lack of aberrancy of all 3 should not dissuade from a diagnosis of AH/EIN when definitive histologic features are present.

We found, in accordance to previous studies, that both Arid1a and Mlh1 were lost in a minority of AH/EIN, and furthermore, that these markers were reliably scored. However, in this study of > 100 cases, no AH/EIN was uniquely diagnosed on the basis of Arid1a or Mlh1. While it seems likely that some AH/EIN could be identified only through the inclusion of Arid1a or Mlh1 (on top of the 3-marker panel), this would be a rare occurrence. Because Arid1a and Mlh1 are rarely lost in normal endometria, their inclusion should not decrease specificity. Thus, Arid1a or Mlh1 could be routinely performed if desired, or as reflex assays in triple-negative cases. Some investigators have suggested that the standard 4-marker panel for mismatch repair deficiency (Mlh1, Msh2, Msh6, and Pms2) should be performed upon an initial diagnosis of AH/EIN (in addition to endometrial cancer, as is current standard-of-care) as such screening could lead to earlier diagnosis of Lynch syndrome, prompting surveillance for colon cancer and potentially saving lives.<sup>13</sup> If this were to become standard practice, then there might be added justification to perform the 4- or 2-factor (eg, Pms2/Msh6) MMR panel in cases where the diagnosis of AH/EIN was in question.<sup>38</sup> We studied only Mlh1, as it is much more commonly inactivated (due to promoter hypermethylation) than the other 3 markers in endometrial neoplasia,<sup>12,38</sup> including AH/EIN, suggesting that inclusion of other MMR factors would have minimal additional impact in increasing diagnostic yield. Finally, our findings argue against the use of p53 in the diagnosis of AH/EIN. Although p53 is a marker of serous endometrial cancer, it is also mutated in



endometrioid adenocarcinomas, albeit typically late in disease progression. Some studies have identified aberrant p53 expression in at least rare AH/EIN cases,<sup>39</sup> and p53 was included in this study to permit a comprehensive investigation of potential AH/EIN markers. Only 3 AH/EIN cases (2.7%) showed aberrant p53 patterns, while one normal control showed a mutant pattern, albeit very focally, suggesting that specificity and sensitivity are low with possibly no benefit.

Our findings confirm previous studies showing that sporadic Pax2 and/or Pten-deficient glands are common in normal endometria.<sup>14,23</sup> While it is essential to understand this and the patterns thereof, this does not significantly limit the utility of Pax2 and Pten as practicable AH/EIN markers. In normal endometria, the deficient glands were sporadic and even if present in clusters, accounted for only a small percentage of the sample ( $\leq 5\%$ ). In contrast, in AH/EIN, Pax2 or Pten were typically lost over much more extensive areas (Fig. 6D). Very focal loss ( $< 5\%$ ) of Pax2 or Pten in an endometrial sampling should be considered with caution, and in the context of the extent of focus in question and the severity of morphologic features suspicious for AH/EIN. We do not advocate for strict percentile cutoffs for Pax2 and Pten in AH/EIN because of variations in sampling and the extent of suspected AH/EIN versus noninvolved endometrium. However, the extent of loss is rather different in normal versus AH/EIN, making the percentiles as shown in Figures 6A versus C not directly comparable.

Our studies argue that  $\beta$ -catenin is an attractive marker with considerable value. First, with the use of appropriate criteria as summarized above, very few if any normal endometria exhibit aberrant  $\beta$ -catenin localization (Fig. 5), in contrast with Pax2 and Pten. In addition,  $\beta$ -catenin immunohistochemistry detects a large number of cases (47.7%) consistent with the 52% *CTNNB1* mutation rate in mismatch repair-proficient, copy number-low endometrioid cancers based on the TCGA endometrial cancer molecular classification<sup>40</sup> and other studies.<sup>41</sup> Squamous differentiation/morular squamous metaplasia is a common finding in AH/EIN, facilitating the diagnosis of AH/EIN since morular squamous metaplasia by itself is strongly associated with or a harbinger of AH/EIN.<sup>35</sup> Thus, while squamous differentiation is a de facto “biomarker” aiding the diagnosis of AH/EIN, and squamous differentiation and morular squamous metaplasia are strongly associated (Fig. 7C and previous studies), the incorporation of  $\beta$ -catenin as an immunohistochemical stain nonetheless added considerable value because many cases with aberrant  $\beta$ -catenin did not exhibit overt squamous differentiation (Fig. 7A).

The incidence and patterns of marker aberrancy in AH/EIN generally conform to a current molecular genetic understanding of endometrial neoplasia, including early endometrial neoplasia.<sup>42,43</sup> *PTEN* is among the most frequently mutated tumor suppressor genes in endometrial cancer, usually as an early driver event resulting in loss-of-function.<sup>40,43</sup> Mutations in the

*CTNNB1* gene encoding  $\beta$ -catenin are also frequent early events,<sup>44,45</sup> occurring in  $\sim 50\%$  of endometrial cancers.<sup>40,41</sup> Most *CTNNB1* mutations alter specific residues within exon 3 that are part of a  $\beta$ -catenin protein degradation motif. These mutations inhibit degradation and lead to stabilization of  $\beta$ -catenin, resulting in protein overexpression and abnormal relocalization from the membrane/cytoplasm to the nucleus,<sup>34,41,46</sup> helping to rationalize our findings. In endometrial cancers with defective MMR, the MMR defect (most commonly *MLH1* hypermethylation) is the initial defect.<sup>13,42,47,48</sup> This and the familiarity of pathologists with MMR screening made *Mlh1* a potentially attractive AH/EIN marker, although our study shows that it would have limited utility as an additional marker in the 3-marker panel.<sup>13</sup> *ARID1A* is frequently mutated in endometrioid adenocarcinomas, leading to complete loss of the protein in many mutant cases<sup>26,49,50</sup> although it is even more frequently mutated in clear cell adenocarcinomas.<sup>43,51</sup> Unlike the above loci, Pax2 loss does not appear to be associated with *PAX2* mutations<sup>40</sup> and the mechanisms underlying its loss in endometrial precancers remain poorly understood. One notable aspect of marker aberrancy in AH/EIN is that patterns of aberrancy are usually nonoverlapping (ie, markers are aberrant in different regions of the AH/EIN).<sup>14</sup> This likely reflects a sequential acquisition of mutations that characterize clonal outgrowth in early endometrial neoplasia.<sup>19,37</sup>

Most cases of AH/EIN can be confidently diagnosed without the use of immunostains. Nonetheless, there should be considerable benefit to the routine use of an AH/EIN biomarker panel. First, we believe that routine use of the panel will help pathologists refine their diagnostic accuracy and skills.<sup>16</sup> Second, and more importantly, many women with AH/EIN undergo conservative management with long-term progestin administration. This necessitates routine surveillance with repeat endometrial samplings, and yet, progestin markedly masks the histologic features of AH/EIN, making surveillance difficult in practice.<sup>7,52</sup> A large recent longitudinal investigation of Pax2 and Pten expression patterns in serial biopsies from women treated with progestin found that expression patterns in pretreatment AH/EIN were consistently recapitulated by AH/EIN present following treatment.<sup>53</sup>  $\beta$ -Catenin patterns are also likely to be recapitulated following treatment.<sup>37</sup> Thus, in addition to facilitating the initial diagnosis of AH/EIN, establishment of baseline expression patterns should be useful diagnostically in follow-up biopsies in the setting of progestin treatment. Although this study provides critical information regarding patterns of marker aberrance and panel performance in definitive AH/EIN, additional investigations will be needed to determine the incidence and patterns of marker aberrance in mimics of AH/EIN, including endometrial polyps, disordered proliferative endometrium, or non-AH.

In conclusion, our study, which systematically evaluated markers currently known to detect some AH/EIN and thus most likely to be diagnostically useful, supports the combined use of Pax2, Pten, and  $\beta$ -catenin in the diagnosis of AH/EIN.

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