Joint Loss of PAX2 and PTEN Expression in Endometrial Precancers and Cancer

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Abstract

Latent endometrial carcinoma precancers are normal-appearing endometrial glands with sporadic loss of tumor suppressor gene function such as PTEN. Progression to carcinoma is inefficient and requires additional genetic damage that creates a histologic precursor lesion called endometrial intraepithelial neoplasia (EIN). In this study, we examined loss of PAX2 expression, a gene required for embryonic uterine development, during endometrial carcinogenesis. Normal proliferative, EIN, and malignant (endometrial adenocarcinoma) endometrial tissues were immunostained for PTEN and PAX2. Proliferative samples with loss of protein in at least one gland were scored as latent precancers. EIN and cancer lesions were scored by the majority pattern. Overall prevalence and topography of joint PAX2-PTEN expression loss was examined. The prevalence of PAX2 protein loss in the sequence of normal to precursor to cancer was 36%, 71%, and 77%, respectively, and for PTEN, it was 49%, 44%, and 68%, respectively. The normal endometrial prevalence of PAX2- or PTEN-deficient latent precancers was unaffected by biopsy indication, but increased significantly with age. Coincident loss of PAX2 and PTEN expression in an individual normal endometrium was seen in 21% of patients, but usually involved different glands. Coincident loss was more common in precancers (31%) and carcinoma (55%), in which case, both markers were protein null in an overlapping clonal distribution. PAX2 and PTEN protein loss occurs independently and accumulates with increasing age in latent precancers of normal premenopausal endometrium. Loss of function of both genes in an overlapping distribution characterizes the clinical emergence of a premalignant lesion which is carried forward to carcinoma.

Introduction

The most common endometrial malignancy, endometrioid type endometrial adenocarcinoma, is often preceded 3 to 4 years earlier by a monoclonal genetically mutated precursor called endometrial intraepithelial neoplasia (EIN; ref. 1). These precursors have a distinctive histopathologic appearance which allows them to be diagnosed by pathologists (2), but carcinogenesis begins long before any specific lesion such as EIN develops. Following the cessation of known endometrial cancer risk—increasing (unopposed estrogens) and risk-reducing [such as the use of hormonally inert intrauterine devices (ref. 3) or administration of progesterin-containing oral contraceptives (refs. 4, 5)] exposures, the endometrium usually reverts to normal histology within a few menstrual cycles, but the interval of altered risk could last for years (6). This has led to the prediction that there are stable long-term alterations within the histologically unremarkable endometrium which occur in response to nongenetic risk modifiers. Biomarkers which are informative in disclosing the earliest stages of disease offer the possibility of directly observing these events in normal tissues long before clinical detection.

Inactivation of the tumor suppressor gene PTEN has been associated with the development of endometrial carcinoma in mouse knockout (7) and human observational studies (8), a role entirely consistent with its antitumorigenic role as a mediator of cell division and enabler of apoptosis (9). More surprising was the discovery that very small burdens of PTEN protein–deficient glands caused by somatic PTEN gene mutation and/or deletion are frequently found in the endometria of otherwise normal cycling premenopausal women (10). These may be retained for years, through many menstrual cycles, during which mutant cells are continuously exposed to changing systemic hormonal conditions (11). The affected glands are undetectable by routine examination. These occult lesions are appropriately designated “latent precancers” to indicate their hidden nature and requirement for additional hits before they could be recognized clinically.

PTEN mutation alone, however, is insufficient to cause endometrial cancer. The 35% latent precancer rate defined by loss of PTEN function is counterbalanced by a lifetime endometrial cancer risk of only 2.5% (12). Emergence from latency to overt clinical disease (whether EIN or carcinoma) is so...
Some data suggest that loss of constitutive PAX2 expression correlates with endometrial and cervical (18) malignant transformation. Quantitative RNA expression studies of PAX2 in human endometrial tissues show high levels of expression in benign proliferative endometrium with 2-fold reduction upon tamoxifen therapy, and a 5-fold reduction in the cancers (19). These findings present the unique possibility that in the proliferating and self-renewing endometrial epithelial cells, PAX2 acts as a tumor suppressor. In this study, we further examine the idea that loss of PAX2 function occurs in endometrial carcinogenesis, and relate changes in PAX2 to those which occur in parallel with the endometrial tumor suppressor gene, PTEN.

Materials and Methods

Case selection

Pathology reports at Brigham and Women’s Hospital were screened, with institutional review board approval, for a diagnosis of proliferative endometrium, EIN, or endometrial adenocarcinoma, and associated endometrial biopsies or curettage specimens retrieved as paraffin tissue blocks from the diagnostic archive. All diagnoses were confirmed by unblinded slide re-review (by G.L. Mutter), and cases were rejected if the expected diagnosis was not confirmed. Additional case details are described below. Each patient contributed only a single sample to the study, which was included in only one diagnostic group.

Malignant endometrial tissues received between July 1 and December 31, 2008 were retrieved based on a reported diagnosis of “endometrial intraepithelial neoplasia.” Of the 127 candidate cases, 23 were excluded due to concurrent adenocarcinoma, 12 because they were repeat biopsies in patients already represented (the first was retained as eligible), 19 due to unavailable slides or blocks, 1 due to cautery artifact, 9 due to insufficient material (minimum tissue requirement was defined by tissue area of ∼0.5 cm² of tissue, mostly functionals, in the slide), 7 due to the absence of lesions in the slides available, and 4 due to immunohistochemistry failures. This resulted in successful PAX2 and PTEN immunohistochemistry results from 52 EIN-bearing biopsies.

Normal proliferative endometria from premenopausal women (age <50 years) received between July 1 and December 31, 2008 were retrieved based on a reported diagnosis of “proliferative endometrium.” Of the 389 candidate cases, we excluded 3 because of missing slides, 102 because of coexisting pathologic endometrial conditions (59 with anovulation or coexisting neoplasm, 24 polyps, 5 with active gestation, 13 endometritis, and 1 IUD bearing), 21 because of fragmentation or menstrual breakdown, 10 due to insufficient tissue, 10 because the patients were represented by an eligible prior biopsy, 4 because of immunohistochemistry failures, and 32 random cases unneeded to achieve desired study power. Patients with any of the following were then excluded: current history of sex steroid hormone use (n = 6), previous known endometrial disease (n = 7), or prior history of tamoxifen use (n = 3). This left 191 proliferative endometrial biopsies, which yielded both PAX2 and PTEN immunohistochemistry results.

Clinical indications for biopsy within the normal proliferative group

Indications for biopsy were retrieved from the pathology requisition and classified into one of four general classes as follows: (a) extrinsic: endometrial biopsy performed as part of workup of known nonendometrial disease (e.g., uterine fibroids, known nonendometrial pathology such as cervical disease); (b) intrinsic: endometrial biopsy performed because of known endometrial diagnosis documented prior to biopsy (e.g., prior endometritis), or symptoms (bleeding) directly referable to the endometrium itself; (c) screen: endometrial sampling performed incidental to endometrial unrelated procedure (e.g., tubal ligation), in reflex to a nonspecific screening test (endometrial cells on pap smear or thick endometrium on ultrasound), or in response to nonspecific symptoms or signs (infertility, pelvic pain); or (d) unknown: no indication for biopsy provided by the clinician.

PAX2 and PTEN immunohistochemistry

One representative paraffin tissue block was obtained from each pathology specimen, and stained for PTEN (murine monoclonal antibody 6h2.1 from Dako, used at
by comparing the number of defective glands to the total null glands in normal proliferative endometria was obtained. Affected endometrial gland quantitation in proliferative endometria

polyclonal antibodies (ZRX2 versus 928), or purified IgG and expressing glands when using different lots of crude pattern of staining, and identification of individual null normal proliferative, EIN, and adenocarcinoma with PAX2-directed antibody 928, another rabbit polyclonal antibody Z-RX2 from Immunovision. Four representative cases each

The number of PAX2- and PTEN-defective glands in affected proliferative endometria was counted in replicate by two observers from immunostained sections using a hand counter. Replicate counts were plotted as a linear regression and 90% confidence interval outliers identified. The average of the two observations was rounded to the nearest whole number for nonoutlier cases. Outlier specimens underwent a third gland count, and the two closest measurements were then averaged.

Joint loss of PAX2 and PTEN expression

Specimens containing both PAX2- and PTEN protein-defective glands were evaluated for overlap within a shared population of individual glands. For EIN and carcinoma specimens, histologic lesions occupying most or all of the specimen, side-by-side comparison of immunostain results of adjacent serial sections allowed scoring as overlapping or not. A modified approach was necessary with the normal proliferative endometria, both because the number of affected glands was very small, and localization required a fine degree of spatial resolution. Proliferative samples in which both PAX2- and PTEN-deficient endometrial glands had been detected were reexamined and each tissue region containing protein null glands highlighted on the coverslip with an ink pen. The marked-up glass slides for PTEN and PAX2 were then aligned to identify those specimens in which both stain markups colocalized to the same tissue fragments. Slides with PAX2- and PTEN protein–null glands within the same tissue fragments were then examined together and the number of individual glands deficient in both proteins enumerated with a hand counter.

Statistical methods

Categorical comparisons were assessed using a Pearson χ² test; categorical comparison across categories of an ordered variable were assessed using the Kruskal-Wallis test. Comparisons of quantitative variables were assessed using the Student’s t test with a pooled estimate of variance when between two groups of cases, and with ANOVA when among more than two groups. Pearson χ² tests were used to evaluate whether loss of PAX2 and PTEN proteins was significantly associated within each diagnostic group. The McNemar test was used to assess whether a case was more likely to have PTEN- or PAX2-null glands when only one of the genes was protein-deficient.

Affected endometrial gland quantitation in proliferative endometria

An estimate of the burden of PAX2- and PTEN protein–null glands in normal proliferative endometria was obtained by comparing the number of defective glands to the total number of glands present. The total number of endometrial glands seen in an average specimen from this study was estimated in 21 randomly selected proliferative endometria stained for pan-keratin (anti-human pankeratin cocktail of AE1 and AE3 murine monoclonal antibody, Dako, used at 1:100 primary antibody incubation for 2 hours at room temperature) to clearly accent gland contours. Keratin-stained slides were scanned in an iScan virtual microscopy device (Biologic), and the total number of endometrial glands in each slide was counted using the counter function in Image Viewer (v1.6.0, Biologic). Incidental endocervical and lower uterine segment glands were excluded by reference to a serial H&E-stained section. The number of PAX2- and PTEN-defective glands in affected proliferative endometria was counted in replicate by two observers from immunostained sections using a hand counter. Replicate counts were plotted as a linear regression and 90% confidence interval outliers identified. The average of the two observations was rounded to the nearest whole number for nonoutlier cases. Outlier specimens underwent a third gland count, and the two closest measurements were then averaged.

PAX2 immunohistochemistry validation by multiple PAX2 antibodies

The PAX2 polyclonal antibody used throughout this study, Z-RX2, had been produced by immunizing a rabbit with the COOH-terminal domain (amino acids 188–385) of the murine Pax-2 protein, and used by other laboratories to detect PAX2 protein in mice and humans (17, 20, 21). To further validate the performance of this reagent within our study material, we compared PAX2 protein detection by Z-RX2 with results obtained in the same tissues using alternative PAX2 antibodies. Four representative cases each from the proliferative, EIN, and carcinoma groups were selected based on the presence of localized foci of PAX2 protein–null glands. Serial sections of tissue were immunostained with (a) crude Z-RX2 used as above, (b) column affinity purified Z-RX2 (designated ZAP-AP, used at 1:1,000 dilution), and (c) antibody 928, another rabbit polyclonal antibody directed against the same peptide used to create Z-RX2 (used at 1:500 dilution). All selected cases of normal proliferative, EIN, and adenocarcinoma with PAX2-null glands detected by antibody ZRX2 showed the same pattern of staining, and identification of individual null and expressing glands when using different lots of crude polyclonal antibodies (ZRX2 versus 928), or purified IgG fraction (ZRX2 versus PAXAP; data not shown).
Subject ages varied significantly among the groups (ANOVA, $P < 0.001$) averaging 41.8 (SD, 6.1) years for normal, 50.3 (SD, 10.1) years for premalignant, and 60.5 (SD, 12.3) years for malignant endometria.

Latent precancer prevalence in the normal proliferative endometria was not associated with clinical indications for biopsy (Supplementary Table S2, $\chi^2 P = 0.146$ for PAX2, and $P = 0.715$ for PTEN), or sampling device used (curette versus biopsy; Supplementary Table S2, $\chi^2 P = 0.282$ for PAX2, and $P = 0.588$ for PTEN). Clinical indications for
endometrial sampling did not vary significantly (Supplementary Table S2, \( \chi^2 P = 0.373 \)) among diagnostic groups of normal, premalignant, and malignant endometria. The sampling instrument used (curette versus biopsy device) was more frequently (55%) a curette in cancer patients, compared with 25% and 42% in normal and premalignant patients, respectively (Supplementary Table S2, \( \chi^2 P < 0.001 \)).

Joint loss of PAX2 and PTEN expression in EIN and cancer specimens occurred in a clonal distribution, involving most or all neoplastic glands (Fig. 1). When protein products of both genes were lost, there was extensive or complete geographic overlap of PAX2 and PTEN loss across the lesional field in all (34 of 34) of the cancers and most (94%, 15 of 16) of the EIN lesions. In proliferative endometrium, 21% of cases were both PAX2 and PTEN null, and an additional 43% were either PAX2 null or PTEN null (Table 1). Proliferative cases which were null for only a single gene were more likely to be PTEN null (\( P = 0.004 \)). In EIN, 31% of cases were both PAX2 and PTEN null, and an additional 54% were either PAX2 null or PTEN null. EIN cases which were null for only a single gene were more likely to be PAX2 null (\( P = 0.008 \)). In the cancers, 55% were both PAX2 and PTEN null, and an additional 35% were null for only one of the two genes. There were no significant differences in the prevalence of PAX2 and PTEN null cases among those with cancer (\( P = 0.201 \)).

The histologic presentation of PAX2- and PTEN-deficient glands in proliferative endometrium was distinctive in two regards (Fig. 2), and contrasted greatly with the extensive or complete overlap of joint protein losses seen in EIN and cancers (Fig. 1). First, the number of protein-deficient glands in normal tissues was very small, only involving a few glands of the hundreds present in an average specimen (Table 1). A random sample of 21 normal endometria averaged 784 total glands per specimen (SD, 525; median, 691; range, 91–1,902), identified by a keratin stain that highlighted all glandular epithelium irrespective of PAX2 or PTEN status. The total number of glands per specimen did not vary significantly (\( t \) test, \( P = 0.668 \)) between sample formats, comparing endometrial curettage to endometrial biopsy. Protein-deficient glands were a minor component of those present in the normal proliferative endometria, with only 0.46% of all glands lacking PAX2, and 1.34% null for PTEN. Second, when PAX2 and PTEN protein loss did occur in the same normal endometrial specimen, it usually involved mutually exclusive subsets of glands (Fig. 2). Only 15 individual glands (0.01% of all examined) had loss of

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**Table 1.** PAX2 and PTEN proteins are rarely lost in the same normal proliferative endometrial glands

<table>
<thead>
<tr>
<th>Proportion of glands with loss of protein expression</th>
<th>PAX2 only</th>
<th>PTEN only</th>
<th>PAX2 and PTEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.46% (694/149,744)*</td>
<td>1.34% (2,003/149,744)*</td>
<td>0.01% (15/149,744)*</td>
<td></td>
</tr>
</tbody>
</table>

*Number of affected glands in all samples divided by estimated total number of glands in all samples.

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**Table 2.** Proportion of endometrial tissue samples showing loss of PAX2 and PTEN protein expression, by diagnosis

<table>
<thead>
<tr>
<th></th>
<th>Normal proliferative, ( N = 191 )</th>
<th>Intraepithelial neoplasia (EIN), ( N = 52 )</th>
<th>Cancer, ( N = 62 )</th>
<th>Kruskal-Wallis ( P^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAX2 null</td>
<td>35.6%</td>
<td>71.2%</td>
<td>77.4%</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PTEN null</td>
<td>49.2%</td>
<td>44.2%</td>
<td>67.7%</td>
<td>0.064</td>
</tr>
<tr>
<td>Joint PAX2 and PTEN null</td>
<td>20.9%</td>
<td>30.8%</td>
<td>54.8%</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Joint PAX2 and PTEN Expression</td>
<td>36.1%</td>
<td>15.4%</td>
<td>9.7%</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>McNemar ( \chi^2 ) test of agreement of PAX2 and PTEN loss (^\dagger)</td>
<td>( P = 0.004 )</td>
<td>( P = 0.008 )</td>
<td>( P = 0.201 )</td>
<td></td>
</tr>
<tr>
<td>Pearson’s ( \chi^2 )</td>
<td>( P = 0.048 )</td>
<td>( P = 0.822 )</td>
<td>( P = 0.335 )</td>
<td></td>
</tr>
</tbody>
</table>

*Nonparametric test of equality of proportions across diagnosis groups.

\(^\dagger\)McNemar \( \chi^2 \) test measuring whether PAX2 compared with PTEN protein loss was equally frequent among cases with loss of a single protein within each diagnostic group.

\(^\ddagger\)Pearson \( \chi^2 \) tests measuring whether PAX2 and PTEN loss was significantly associated in individual patients within each diagnostic group.
The prevalence of PAX2 and PTEN protein loss varied significantly within normal proliferative and within premalignant EIN tissues, but not within cancers (Table 2; McNemar test within each diagnostic group). One of our hypotheses was that accumulation of increased genetic damage in the sequence of normal→premalignant→malignant tissues. Using a Kruskal-Wallis test (Table 2), we found this to be significant with PAX2 (P < 0.0001) and a nonsignificant trend (P = 0.064) with PTEN. Interestingly, the greatest stepwise change in the prevalence of loss of protein expression occurred at different junctures for the two genes: for PAX2 at the normal-premalignant transition (increase from 36% to 71%), and for PTEN at the EIN-cancer transition (increase from 44% to 68%).

The proportion of endometria containing both PAX2- and PTEN-deficient glands increased in the normal→premalignant→malignant sequence, from 21% to 31% to 55%, respectively (Table 2; Supplementary Table S1). This was paralleled by a decline in the proportion of patients free of both PAX2- and PTEN-null glands, from 36% in normal to 15% in premalignant and only 10% in malignant endometria.

Figure 3. Somatically acquired sporadic loss of PAX2 and PTEN protein function is age-dependent in normal proliferative endometrial tissues (ANOVA, P = 0.002). Notched box plot of patient age at the time of endometrial biopsy with the specified PAX2 and PTEN immunostain findings. Each solid circle is one patient, boxes indicate central 50% of values, notches are confidence intervals, and whiskers represent 1.5 times the interquartile range. "wt," wild-type, protein expressed in all glands; "null," latent precancer with isolated protein-deficient glands.
(24), and in breast cancers, downregulates a poor prognostic indicator, the growth factor ERB-2 (25). PAX2 expression increases upon cell division, a pattern shown previously for some tumor suppressor genes that act to control the rate of proliferation (26). At this time, we do not know the mechanism of PAX2 protein loss in endometrial carcinogenesis, but it may be due to a primary PAX2 gene—inactivating event. Loss of PAX2 protein on the basis of PAX2 gene mutation (27) or deletion (28) has been described in the hereditary renal-coloboma syndrome, and epigenetic silencing is seen in melanomas (29). The incidence of endometrial carcinoma has not been studied in patients with renal-coloboma syndrome, in which PAX2 gene inactivation causes malformations of the kidneys and eyes (30).

The pattern of PAX2 protein loss across the range of tissues examined is similar to that of PTEN, supporting its application as a biomarker for latent precancers. Latent endometrial precancers identified through the use of PTEN are common in normal tissues (10), contain mutations carried forward years later to adenocarcinoma (11), and involve when exposed to cancer-protective factors such as oral contraceptives or intrauterine device use (13). This has led to the theory that some exposures modulate endometrial cancer risk by their effect on latent precancers, and these can be detected directly within the target tissue with markers such as PTEN. This report presents PAX2 as an additional marker for this effect.

This study shows that the phenomenon of sporadic loss of gene function within normal endometrial tissues, including PTEN in 49% and PAX2 in 36% of normal cycling premenopausal endometria, is a process that may occur independently and in parallel with multiple genes. Combining these two markers in a single patient series, 64% of all women already bear small subpopulations of protein-null endometrial glands before menopause. Such a high frequency of occurrence challenges the notions of normality, as in this study population, the majority state is one in which normal tissues have already lost their genetic homogeneity through mutations and other stable gene-inactivating events that are likely to occur in regenerative tissues. We do not believe this effect to be unique to our patient population, as it has been seen (using PTEN) in other institutions at comparable rates (14). Its consistent occurrence across different types of patients (Supplementary Table S2) suggests that it is not a peculiarity of a specific diagnosis, symptom, or indication for physician visit. These normal tissues with sporadically acquired gene-specific defects are appropriately described as “latent precancers” to reflect both a very low efficiency of progression to carcinoma and a need for accumulation of additional genetic damage before they display any histopathologic alterations. These events are among the earliest ever shown in a human tumorigenic pathway, and provide insights into preclinical events of carcinogenesis, from an initial phase of expansile malignant behavior (EIN-cancer interface).

The notion of latent precancers, in which acquired sporadic loss of gene function in normal tissues represents early stages of carcinogenesis, began with data from a single gene, PTEN. Many of the model predictions, such as clonal continuity with cancer and reduced prevalence being associated with reduced cancer risk states have been experimentally confirmed with this single marker. This article extends the model by showing that latent precancers may involve multiple genes; these frequently co-occur in individual tissues and yield a phenotype that is highly dependent on the overlapping versus nonoverlapping topography of affected glands. In the case of endometrium, PAX2 and PTEN seem to be inactivated independently, but when this does occur in the same glands promotes neoplastic transformation. This cross-sectional study is not without its limitations, which include a sample size that does not enable adjustment for age differences, and lack of prospective follow-up of early-stage lesions in individual patients over time. More work is needed.
to define a constructive role, if any, for latent precancer diagnosis in routine clinical practice, or use of PAX2 as a diagnostic tumor marker.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References
