

Emergence, Involution, and Progression to Carcinoma of Mutant Clones in Normal Endometrial Tissues

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Abstract

Sporadic somatic inactivation of genes such as PTEN within histologically normal endometrium (latent precancers) is an early step in endometrial carcinogenesis. We have used clone-specific mutations of PTEN to determine the fate of latent precancers over time in women who do (high risk) and do not (low risk) develop endometrial neoplasia. PTEN immunohistochemistry was performed on 45 occurrences of endometrial neoplasia and their paired antecedent benign biopsies, along with age matched sample pairs from 167 patients who did not develop a neoplasm. When PTEN-deficient cells were present at both time points, DNA sequencing was performed to determine whether they were single or multiple independent events. Loss of PTEN protein in isolated glands was common in the initial normal biopsies of high- and low-risk groups (42% and 27%, respectively, $P = 0.066$). Protein-deficient glands have a tendency to disappear over time in low-risk women ($P = 0.047$) and, even when "persistent," are infrequently (19%, 3/16) confirmed to be the same clone. Similarly, only a small proportion (6.7%, 1/15) of latent precancers seen in high-risk women are the direct progenitors of subsequent neoplasia. There is a high rate of latent precancer turnover in both low- and high-risk patients, with rare long-term persistence of unique clones, which may or may not progress to a histologic lesion. The temporal dynamics of clonal emergence, persistence, and involution are sufficiently complex that in the individual patient, the presence of a latent precancer has an unknown contribution to long-term cancer risk. *Cancer Res*; 74(10); 2796–802. ©2014 AACR.

Introduction

Starting with a first mutation in normal glandular cells, it is the sequential accumulation of genetic damage within a continuous lineage that drives an evolving phenotype to endometrial cancer. PTEN is capable of participating in these events from their inception, as evidenced by somatic PTEN inactivation in small numbers of normal appearing endometrial glands in almost half of endogenously cycling premenopausal women (1). This high prevalence of observed PTEN inactivation in "normal" tissues is counterpoised by a 2.5% lifetime risk of endometrial cancer (2). These mutated but otherwise unremarkable normal glands have been dubbed "latent precancers" in recognition of their normal histologic appearance, and to

emphasize that other events must transpire to bring them to clinical attention.

Loss of function of the tumor suppressor gene *PTEN* through somatic mutation and/or deletion is the most common genetic change in endometrioid endometrial carcinoma, being present in 83% of sporadic cases (3). Further evidence for a causal role of PTEN inactivation in endometrial carcinogenesis is that when inactivated in genetically modified mice, a high frequency of endometrial malignancies results (4, 5). PTEN, however, does not act alone, as isolated inactivation of PTEN is insufficient in humans to cause endometrial cancer (1). Rather, sporadic endometrial cancers, and their immediate histologic progenitor called endometrial intraepithelial neoplasia (EIN; ref. 6), already demonstrate a broad spectrum of coincident genetic events, or multiple "hits," at the time of clinical presentation. Other genes that are frequently abnormal in endometrial carcinoma include *KRAS* (10%–30%; ref. 7), *CTNNB1* (β -catenin, 25%–38%; ref. 8), *PIK3CA* (30%; ref. 8), *PAX2* (77%; ref. 9), and microsatellite repair factors (13%–24%; refs. 7, 8).

By the time EIN is visible to a diagnostic pathologist within routinely stained tissue sections, the genetically altered clone has expanded to encompass millions of morphologically abnormal individual cells with multiple mutations (10, 11). Coinactivation of *PAX2* and PTEN is seen in a clonal distribution in 31% of EIN (9). *KRAS* mutation and microsatellite instability are also frequent in this stage, and observed specific mutations are carried forward to subsequent cancers, confirming direct lineage continuity (12, 13). At the time of initial

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presentation with EIN, 37% of women already have an occult concurrent endometrial adenocarcinoma, and those that are cancer free have a 45-fold increased risk for future cancer (14, 15). By the time an EIN lesion appears, the cancer risk is sufficiently elevated that the standard of care in the United States is hysterectomy, as would be undertaken for carcinoma itself (16).

Although latent precancers have a demonstrably inefficient progression to malignancy, factors that influence their fate are poorly understood. One testable hypothesis is that nongenetic risk modifiers act as positive or negative selection factors for latent precancers already present in normal tissues at the time of exposure. There is now data showing this to be the case with the cancer-protective hormone progesterone, which seems to selectively ablate PTEN-null endometrial glands in normal endometrium (17). In women treated with other specific interventions known to reduce endometrial cancer risk, such as oral contraceptives (18, 19) or intrauterine device placement (20), there is a decline in the prevalence of endometrial latent precancers in proportion to the magnitude of reduced cancer incidence shown in epidemiologic studies (21). In summary, one possible mechanism of risk reduction below the general population is intervention to "kill off" latent precancers before they even come to clinical attention. This possibility presents a novel therapeutic target for true cancer prevention, that of erasing the burden of latent precancers with initial genetic hits.

In a proof-of-principle experiment, lineage continuity of clone-specific mutations has previously been shown between latent precancers and subsequent endometrial carcinoma in individual patients, separated by up to 13 years (22). PTEN is an informative marker for such studies, because the underlying mechanism of PTEN inactivation is primarily due to irreversible structural changes in the *PTEN* gene itself. Inactivation is a stable one-way event within affected glands, and the particular observed mutations are informative markers for unique clones (1). This prior report concentrated exclusively on a few selected patients who actually developed carcinoma, lacking comparable multi-time point studies on the fate of latent precancers in women who remain cancer free.

The current study systematically examines the frequency and fate of incident latent endometrial precancers over time in individual patients who develop cancer during follow-up ("high risk"), in comparison with women who do not ("low risk") develop cancer during a comparable follow-up interval. In doing so, we sought to address the following hypotheses: (i) Are latent precancers more frequent in women who ultimately develop cancer? (ii) Is the high prevalence of latent precancers at a single observation point due to a high frequency of short-lived phenomena, or infrequent events that persist for a long time? and (iii) How often do antecedent latent precancers have demonstrable lineage continuity with subsequent cancer?

Patients and Methods

We assembled a sequential series of patients who had an endometrioid neoplasm (EIN or carcinoma) at endometrial biopsy or curetting, and an available antecedent benign biopsy (high risk), and matched these with samples from neoplasm-

free women. Paired samples from each patient within a 5-year window were subjected to PTEN immunohistochemistry. When PTEN protein production was defective on multiple occasions in one patient, the gene was sequenced to classify underlying mutations as concordant (mutation carried forward over time in a common lineage) or discordant (different mutations indicating independent events).

Case selection

Endometrial biopsies and curettings were retrieved by diagnostic review of the pathology files at Brigham and Women's Hospital (Boston, MA). Paired "index" (the later, second sample) and prior endometrial samples separated by 28 to 1820 days were identified for individual patients (ages 30–90 years at the index biopsy) to study the fate and clonal continuity of PTEN-defective glands over time. High-risk patients, those with EIN or endometrial carcinoma in the second biopsy and a prior benign sample, were compared with low-risk patients with paired samples and no history or diagnosis of EIN or carcinoma (Fig. 1).

Patients in the high-risk category were defined as follows. An index biopsy containing an initial occurrence of EIN or carcinoma between January 1, 2001 and June 30, 2006 was the first requirement. Patients having prior endometrial samples with a benign histology were accepted as candidate cases.

Candidates for the low risk, or "control" group were initially identified by availability of at least two benign endometrial biopsies within the period 1995 and 2007, separated by 28 to 1,820 days. The medical record was reviewed and any woman with a history of EIN or endometrial carcinoma was excluded. Three low-risk candidates' age and biopsy-interval matched to each high-risk case were then selected for PTEN analysis.

Diagnostic review

Original hematoxylin and eosin (H&E)-stained slides selected by pathology report diagnosis were re-reviewed by a pathologist (G.L. Mutter) using published criteria (6). In brief, areas diagnosed as EIN were required to meet four criteria (23):

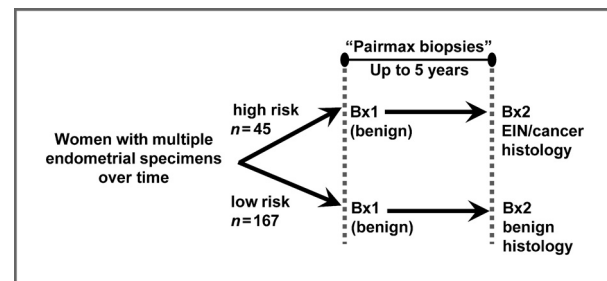


Figure 1. Experimental design. Women with an index (Bx2) benign or neoplastic (either EIN or cancer) endometrial biopsy were stratified, respectively, into low-risk or high-risk groups, and those with prior benign biopsies (Bx1) within a 5-year window retrieved for comparison. First (prior) and second (index) endometrial samples were then immunostained for PTEN, and the *PTEN* gene sequenced in those cases where both biopsies had PTEN null glands. Formal statistical analysis was performed for maximally separated sample pairs within a 5-year sample interval ("Pairmax" samples; Tables 1 and 2). When available, additional ancillary samples outside the 5-year window, or intermediate to the Pairmax samples, are reported separately (Table 3).

Table 1. Trends in emergence, involution, and persistence of PTEN protein-deficient (null by immunohistochemistry) glands over time in paired biopsies of women who do (high risk) or do not (low risk) develop endometrial neoplasms (EIN, cancer)

Pattern	First-second PTEN status	High risk % (n)	Low risk % (n)	Total % (n)
Stable wt	NL-NL	37.8% (17/45)	64.1% (107/167)	58.5% (124/212)
Null emergence	NL-null	20.0% (9/45)	9.0% (15/167)	11.3% (24/212)
Null involution	Null-NL	6.7% (3/45)	16.8% (28/167)	14.6% (31/212)
Null persistence	Null-null	35.6% (16/45)	10.2% (17/167)	15.6% (33/212)
	Total	100% (45/45)	100% (167/167)	100 (212/212)
	N	45	167	

NOTE: Median interval between first and second biopsies is 2.4 years.

Abbreviation: NL, normal, protein expressed.

(i) area of glands exceeds that of stroma; (ii) cytology is altered in the crowded focus; (iii) minimum focus size of 1 mm; and (iv) exclusion of mimics and carcinoma. Carcinoma was diagnosed when one of the following features was present in neoplastic epithelium: (i) "rambling" or mazelike glands; (ii) solid areas of epithelium; (iii) significant cribriforming; or (iv) thread-like intervening fibrous tissue with polygonal distortion of contiguous glands. Benign tissues, those lacking EIN or carcinoma, included cycling or noncycling (inactive or atrophic pattern was common in older patients) endometrial tissue. One tissue block representative of the diagnosis was retrieved and used for PTEN immunohistochemistry, and as indicated below, DNA isolation by laser capture microdissection (LCM) and Sanger sequencing of *PTEN*.

PTEN immunohistochemistry

One representative paraffin tissue block from each pathology specimen was stained for PTEN (murine monoclonal antibody 6h2.1 from Dako, catalog number M362729-2, used at 1:100 dilution overnight primary antibody incubation at 4°C) as described previously (9). In brief, paraffin sections were rehydrated and underwent microwave antigen retrieval before adding primary antibody overnight at 4°C. Slides were washed, incubated with appropriate secondary biotinylated immuno-

globulin (Vectastain ABC Kit, Vector Laboratories, Inc.) and signal detected by sequential addition of avidin peroxidase and 3,3'-diaminobenzidine. Endometrial glandular epithelial staining of independent replicate experiments was scored on two separate occasions by reviewers (G.L. Mutter and N.M. Monte) blinded to the patient group. Typically, PTEN-defective glands are sharply offset at high contrast from endometrial stroma (1), which serves as an internal positive control. Discordant interpretations were resolved by consensus review at a multiheaded microscope. Low-risk samples with discrete loss of protein in at least one gland were scored as PTEN-null latent precancers. EIN and cancer lesions were scored by the majority pattern.

Pairing of index and prior specimens for PTEN analysis

Antecedent paired samples were identified within 28 to 1,820 days of the index Bx. For those cases with multiple prior biopsies having successful PTEN analysis, prior biopsy with the maximum interval up to 1,820 days to index biopsy was selected to define a pair of prior+index specimens ("Pairmax" samples). The PTEN protein status of sequential paired prior-index samples from each patient as determined by immunohistochemistry was classified as (i) expressing-expressing; (ii) null-null; (iii) null-expressing; or (iv) expressing-null.

Table 2. PTEN mutation conservation over time (same mutation in first and second biopsies) occurs with similar frequency between high and low cancer risk patients (6.7% and 18.8%, respectively, Fisher exact, $P = 0.575$)

Mutation pattern	First Bx	Second Bx	High risk	Low risk	Total
None	wt	wt	26.7% (4/15)	12.5% (2/16)	19.4% (6/31)
Nonconserved	wt	Mutant	20.0% (3/15)	18.8% (3/16)	19.4% (6/31)
Nonconserved	Mutant	wt	0% (0/15)	12.5% (2/16)	6.5% (2/31)
Conserved	Same mutation	Same mutation	6.7% (1/15)	18.8% (3/16)	12.9% (4/31)
Nonconserved	Different mutation	Different mutation	46.7% (7/15)	37.5% (6/16)	41.9% (13/31)
Total			15	16	31

NOTE: Most commonly, protein deficiencies seen at two time points are due to independent events (different mutations in first and second biopsies).

Table 3. PTEN mutation patterns of ancillary third samples not selected as part of formal sample pairs (Pairmax) reported in Tables 1–3

Case	Risk group	Paired (Pairmax) sample Genotype	Ancillary 3rd sample			
			Timing relative to Pairmax window	Timing before final specimen (index)	Histology	Seq result
P0001	High	Nonconserved, wt-mutant	Outside 5-year window	5.2 y	Normal	Mutation same as EIN
P0018	High	Nonconserved, different mutations	Intermediate	1.9 y	Normal	Mutation same as EIN
P0488	High	Nonconserved, different mutations	Outside 5-year window	7.1 y	Normal	Unique nonconserved (third mutation)
P2942	Low	Nonconserved, different mutations	Intermediate	1.6 y	Normal	Unique nonconserved (third mutation)

DNA extraction and *PTEN* Sanger sequencing

Cases with *PTEN* null glands in both the prior and index biopsies were candidates for *PTEN* sequencing to determine lineage relationships between the two time points. Blocks were resectioned to produce eight serial slides, of which the first and last were restained for *PTEN* to confirm availability of *PTEN* null glands within the recut material. The intervening polyethylene naphthoate membrane slides (P.A.L.M. Microlaser Technologies AG) were stained with H&E, dried, and under direction of the flanking immunostains, *PTEN* protein-defective glands were mapped and microdissected using a PALM microbeam LCM instrument (P.A.L.M. Microlaser Technologies AG). DNA was isolated by proteinase digestion at 60°C for 48 hours, removal of electrolytes by addition of chelex-100 beads (Bio-Rad), inactivation of proteinase by boiling, and removal of all solid materials by centrifugation. This typically yielded 50 µL of DNA solubilized in supernatant, which was used as input for PCR reactions.

DNA from *PTEN* protein-deficient endometrial epithelial cells were Sanger sequenced looking for somatic mutations. Approximately 10 to 50 ng of DNA per sample was PCR amplified using primers that define the coding region and flanking introns of all nine *PTEN* gene exons (3). PCR products were also subjected to denaturing gradient gel electrophoresis (DGGE), which in our hands is virtually 100% sensitive and specific in detecting sequence-confirmed *PTEN* mutations (24). DNA samples showing DGGE variants are resubjected to PCR and semiautomated direct sequencing using an ABI3730xl.

Results

Successful *PTEN* immunohistochemistry was performed in paired endometrial samples of 45 high-risk and 167 low-risk patients (Fig. 1). High-risk patients were defined by a later (second, or "index") specimen containing either carcinoma ($n = 9$) or EIN ($n = 36$). Low-risk patients had only benign findings on all specimens. The average follow-up interval time between biopsies studied was 2.4 years (individual case details are available in Supplementary Table S1). The high- and low-risk patient groups did not differ significantly by age (respective years median 52.5 vs. 52.7, range 31–82 vs. 32–84, $P = 0.626$)

or follow-up interval in days (respective average days 837 vs. 872, median 771 vs. 856, range 29–1,767 vs. 37–1,784, $P = 0.682$).

PTEN immunohistochemistry results are shown in Table 1 organized by risk group and pattern of change within individual patients over time. There was a significant ($P < 0.001$) difference in lack of *PTEN* expression in the second specimens, defined as the sum of *PTEN* null emergence and *PTEN* null persistence, of the high-risk (55.6%) compared with low-risk (19.2%) groups. This was expected based upon prior work showing loss of *PTEN* expression in pathologic specimens showing precancerous and malignant histologic patterns (9). There was a nonsignificant trend ($P = 0.07$) for a higher prevalence of latent precancers in the first endometrial specimen of the high-risk (42.22%) compared with low-risk (26.95%) patients.

The likelihood of conservation (either null–null or expressing–expressing) of *PTEN* status between first and second biopsies was similar for high (73.33%) and low (74.25%) risk groups ($P = 1.000$). Remaining patients demonstrated a change in *PTEN* immunophenotype over time, either due to involution of preexisting null glands (null–expressing) or emergence of new ones (expressing–null). First and second biopsies were thus separately analyzed by risk group for tendency to gain (emergence) or lose (involution) *PTEN* null glands. The high-risk group had a nonsignificant (McNemar test of symmetry of discordant categories $P = 0.083$) tendency to acquire *PTEN* null glands over time, whereas the low-risk group had a significant (McNemar test of symmetry $P = 0.047$) tendency to lose *PTEN* null glands over time.

The proportion of women with a persistent *PTEN* protein null phenotype across two paired endometrial samples was significantly ($P < 0.001$) higher for those who developed an endometrial neoplasm (16/45 or 35.6% of high-risk patients) compared with those who remained neoplasm free (17/167 or 10.2% of low-risk patients) throughout. Although this is described as "persistence" of an immunophenotype, it might be caused either by long-term survival of one *PTEN* null clone over time, or an increased rate of genesis/turnover in the high-risk patients.

We next used clone-specific mutations of the *PTEN* gene to distinguish between persistent single and multiple independent clonogenic events in women with *PTEN* protein null glands at

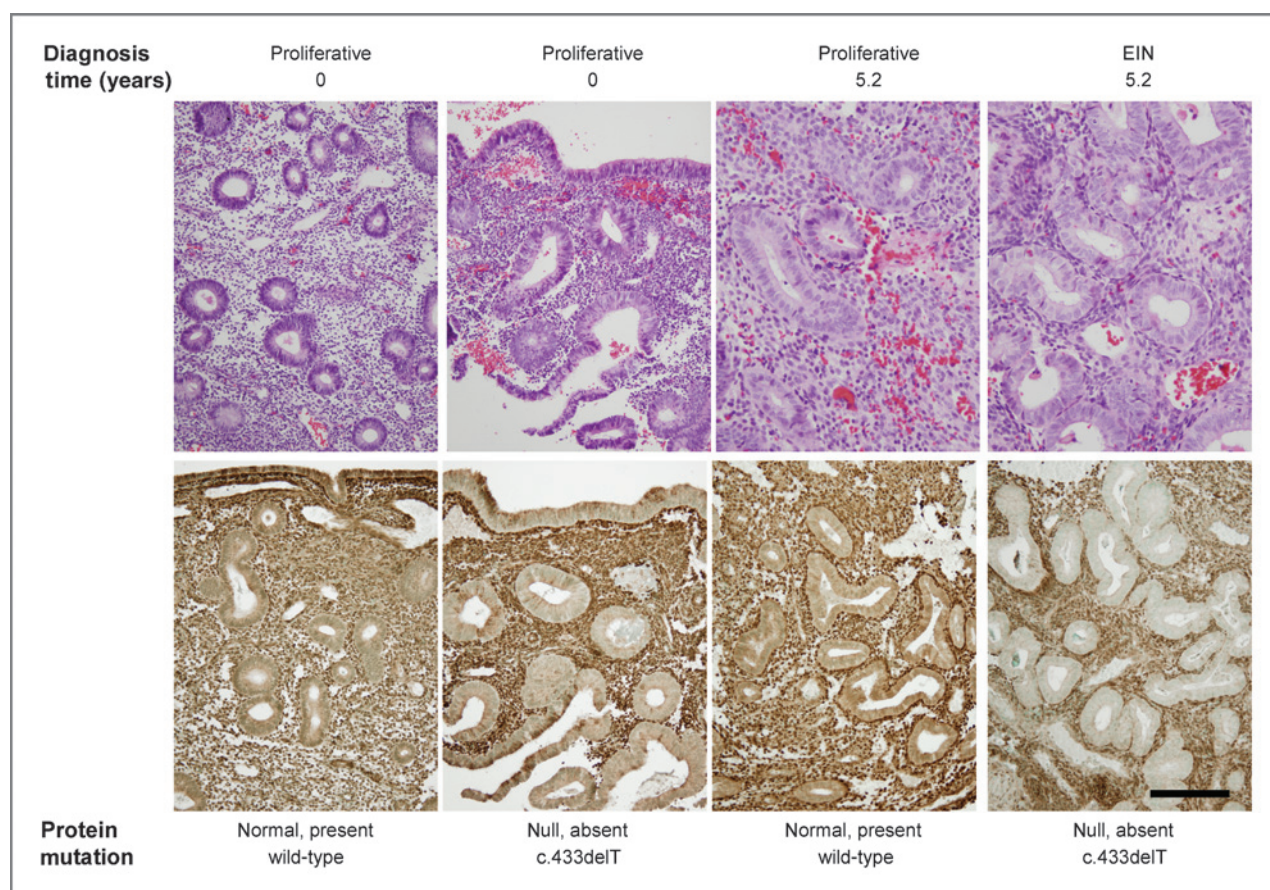


Figure 2. Representative example of progression from latent precancer to EIN over a 5.2-year interval shown by conservation of clone-specific *PTEN* mutation. Top row, H&E stain; bottom row, *PTEN* immunohistochemistry. Scale bar, 200 μ m (case P0001).

two time points. Microdissected *PTEN* null glands from 31 patients with null glands in two biopsies separated by a median interval of 2.1 years were sequenced for the *PTEN* gene. The results (Table 2) prove the general principle that *PTEN*-defective clones may persist for several years in both high- and low-risk patients, but at a low overall rate of only 13% (4/31) of all studied patients. The frequency of conserved mutations was not significantly different by risk group, being seen in 7% (1/15, 1.4 years apart) of high-risk and 19% (3/16, 4.3, 0.5, and 1.1 years apart) of low-risk patients. An apparent "simplification" of the repertoire of mutations over time, seen in some paired samples, is explained either by sampling of separate populations of *PTEN*-mutant clones concurrently present within the specimen, or progressive deletion of previously mutated segments of DNA (such as P1179 in Supplementary Table S1).

The most frequent result of sequencing was demonstration of independent somatic *PTEN* mutations within *PTEN* protein-deficient glands sampled at different time points, found in 47% (7/15) of high-risk and 38% (6/16) of low-risk patients (Table 2). This demonstrates turnover of clones over time, with an existing mutant clone undergoing involution, only to be replaced later by a new one.

Sampling of tissue pairs in Tables 1 and 2 was strictly defined ("Pairmax" specimens maximally separated within 5 years of

the index, but at least a month or last specimen used to define the risk group) to avoid selection bias and allow statistical intercomparison of results between patient categories. In some patients, however, the number of available tissue samples exceeded these formal selections, being either outside the 5-year prior window, or intervening between those chosen. Descriptive results of these ancillary third samples, which appear in Table 3, can be summarized as follows. Two patients (Table 3; P0488 and P2942) were shown to have a third unique (independent) mutation at an additional time point, thereby documenting up to three *de novo* mutagenic events separated by time in individual women. Two high-risk patients (Table 3; patients P0001 and P0018) showed previous benign tissues with mutations identical to those seen 5.2 (Fig. 2) and 1.9 years later in EIN. In both cases, the earliest biopsy studied by the formal selection algorithm (Pairmax) lacked the mutation of the EIN.

Discussion

Progression from an initial mutation (latent precancer) in normal tissues to malignant carcinoma is a process that requires long-term persistence of a continuous lineage of mutated cells within the endometrial glandular compartment.

Such cells are potential targets for hormonal and nonhormonal selective pressures that may have the ability to alter the ultimate cancer outcome. "Latent" is indeed an appropriate term for these isolated mutated glands in an unremarkable histologic context, as something additional must happen to significantly stratify individual patient risk.

The current study confirms that a high prevalence of latent precancers is seen both in women who develop endometrial cancer (42%) or remain cancer free (27%; $P = 0.07$). This high frequency, independent of clinical outcome, means that discovery of a latent precancer in otherwise normal appearing tissue has little or no specific predictive value in determining future cancer risk in the individual patient. This can be explained, in part, by inefficient and unpredictable progression. A prior study of an unselected general population of premenopausal women showed a 43% latent precancer rate, which contrasts with a very low overall population lifetime endometrial cancer risk of 2.5% (2).

The high prevalence of latent precancers seen in a single benign endometrial sample is largely due to a high turnover of clones over short periods, rather than long-term persistence of few clones. This is the case in both high and low cancer risk patients, in which only 2.4% and 1.9%, respectively, show a continuous lineage of a unique sequenced clone between repeat samples taken on average 2.4 years apart. Much more commonly protein-deficient glands are seen at only one time point (26% overall). Of the patients with "persistent" protein null glands at two time points, the vast majority (87%) are not confirmed to be a single clone. Thus, although it is sometimes possible to retrospectively identify a mutated progenitor cell in normal tissue years before clinical disease (22), it is an infrequent event of low prospective predictive value.

Group trends in changing latent precancer prevalence within defined populations of exposed women may, however, provide general insights about tissue-based mechanisms of cancer prevention. We have shown previously that there is a reduced prevalence of latent endometrial precancers in women exposed to endometrial cancer risk reducing factors such as progestins, oral contraceptives, and intrauterine devices (17, 21, 25). This suggests that these factors act directly upon the histologically unremarkable endometrium to promote involution, and/or reduce emergence, of latent precancers. Measurement of latent precancer prevalence before and after particular exposures might provide a short term marker of protective response.

We know from prior studies that loss of PTEN protein is more frequent (68%–83%; refs. 1, 9) in endometrial neoplasia than in normal tissues (43%–49%; refs. 1, 9), consistent with acquisition of increased genetic damage during progression. This was generally confirmed in the current study, in which an increase in the prevalence of PTEN protein loss from the first (benign) to second (neoplastic) samples of the high-risk group was seen, with 20% of high-risk patients showing new loss of PTEN function (emergence of null) between time points. A protein null phenotype requires inactivation of both PTEN alleles, and these may include any combination of mutation, deletion, and epigenetic inactivation. We only studied mutation in the current study because these are most informative as

lineage markers, and acknowledge that companion alleles might be inactivated by other mechanisms.

In addition to the two Pairmax samples studied in the high-risk group, there were several additional available samples outside the qualifying criteria for specimen selection (Table 3). Some were more than 5 years distant from the index specimen ("outside pre-prior"), whereas others were intermediate to the extreme Pairmax time points (intermediate). We included these in our sequencing, and found three additional cases with mutations conserved between the latent precancer and neoplasm. This indicates that there is a significant sampling error in detection of latent precancers that are genotypically matched to subsequent neoplasia. The basis of sampling error is probably a combination of factors, which may include spatial (incomplete sample at one time point), temporal (sampled at a time when the clone was not present), and technical (sequencing) elements.

The emergence of latent precancers, as PTEN-mutant normal appearing cells, is common, and takes place repeatedly as independent events over time in the individual patient. Although 27% of normal women demonstrate latent precancers at any one moment, these persist as unique continuous clones for long periods of time in only 2% of women.

A dynamic process of clonal emergence and involution changes the repertoire of mutations seen at any single time point sampling.

The finding of histologically unremarkable mutated epithelium in normal tissues is not unique to the endometrium. A similar phenomenon of latent precancers occurs in the fallopian tube with mutation of the *p53* gene to form "P53 signatures" in up to a third of sequential hysterectomies removed for benign indications (26). These p53-mutant cells are devoid of cytologic or architectural changes, but with further mutation can progress to a clinically premalignant histologically altered serous tubal intraepithelial carcinoma prone to metastasize to the ovary as high-grade serous cancers (27).

Latent precancers as detected by PTEN immunohistochemistry within normal endometrium are approximately equally frequent between women who eventually will develop endometrial cancer and those who do not. Thus, there is currently no basis to use detection of a latent precancer at one time point in the individual woman as an indicator of future cancer risk. A high likelihood of latent precancer spontaneous involution and low efficiency of progression are intermediate events that distance the observation of a latent precancer from subsequent cancer occurrence. With additional genetic damage, and progression from histologic normalcy to an overtly abnormal histotype incorporating both cytologic and architectural alterations, EIN can be diagnosed. EIN is the proximate precursor lesion of endometrioid endometrial carcinoma, with a 37% likelihood of concurrent cancer and 45-fold prospective risk elevation (6).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: G.L. Mutter, D. Neuberg, A. Ferenczy, C. Eng
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G.L. Mutter, N.M. Monte, D. Neuberg, C. Eng

Writing, review, and/or revision of the manuscript: G.L. Mutter, N.M. Monte, D. Neuberg, A. Ferenczy, C. Eng

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G.L. Mutter, N.M. Monte

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