Altered PTEN Expression as a Diagnostic Marker for the Earliest Endometrial Precancers

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Background: **PTEN tumor suppressor gene mutations are the most frequent genetic lesions in endometrial adenocarcinomas of the endometrioid subtype. Testing the hypothesis that altered PTEN function precedes the appearance of endometrial adenocarcinoma has been difficult, however, partly because of uncertainties in precancer diagnosis.** ***Methods:*** **Two series of endometrial cancer and precancer (endometrial intraepithelial neoplasia, as diagnosed by computerized morphometric analysis) tissue samples were studied, one for PTEN mutations by the use of denaturing gradient gel electrophoresis and another for PTEN protein expression by immunohistochemistry. Endometria altered by high estrogen levels that are unopposed by progestins—conditions known to increase cancer risk—were also studied by immunohistochemistry. Fisher's exact test was used for statistical analysis.** ***Results:*** **The PTEN mutation rate was 83% (25 of 30) in endometrioid endometrial adenocarcinomas and 55% (16 of 29) in precancers, and the difference in number of mutations was statistically significant (two-sided $P = .025$). No normal endometria showed PTEN mutations. Although most precancers and cancers had a mutation in only one PTEN allele, endometrioid endometrial adenocarcinomas showed complete loss of PTEN protein expression in 61% (20 of 33) of cases, and 97% (32 of 33) showed at least some diminution in expression. Cancers and most precancers exhibited contiguous groups of PTEN-negative glands, while endometria altered by unopposed estrogens showed isolated PTEN-negative glands.** ***Conclusions:*** **Loss of PTEN function by mutational or other mechanisms is an early event in endometrial tumorigenesis that may occur in response to known endocrine risk factors and offers an informative immunohistochemical biomarker for premalignant disease. Individual PTEN-negative glands in estrogen-exposed endometria are the earliest recognizable stage of endometrial carcinogenesis. Proliferation into dense clusters that form discrete premalignant lesions follows. [J Natl Cancer Inst 2000;92:924–31]**

Somatic mutation or deletion of the PTEN tumor suppressor gene has been reported in approximately 40% *(1,2)* and 40%–76% *(3,4),* respectively, of endometrial adenocarcinomas. Further evidence for PTEN function within the female reproductive tract is evident in pten knockout (null mutant) mice that develop complex proliferative endometrial lesions *(5).* In humans, familial inheritance of mutant PTEN alleles in Cowden syndrome causes multiorgan development of benign hamartomatous and malignant epithelial tumors *(6–8),* including an elevated incidence of endometrial adenocarcinoma (Eng C, Peacocke M: unpublished observations).

Patients with endometrioid endometrial adenocarcinoma *(1,2)* account for 80% of endometrial cancer patients in the United States *(9,10).* Among all histologic subtypes of endometrial adenocarcinomas, the endometrioid subtype appears to have the highest rate of somatic PTEN mutations *(1,2).* Routine histopathology readily discriminates endometrioid endometrial adenocarcinomas from nonendometrioid tumors, such as the papillary serous and clear-cell adenocarcinomas that also occur in the endometrium. Risk for endometrioid endometrial adenocarcinomas is increased in patients with high estrogen levels that are unopposed by progestins *(11)* and in patients with a physically distinctive precancerous lesion *(12).* Interaction between genetic and hormonal events during the premalignant phases of endometrial tumorigenesis has been hypothesized, yet it has never been precisely elucidated.

The inaccessibility of premalignant tissues, the controversy concerning their interpretation, and the paucity of high-yield candidate genes are long-standing—but now fast-disappearing—barriers to productive exploration of the biology of endometrial precancers. Polymerase chain reaction (PCR)-based methods, including detailed mutational *(13),* clonal *(14),* and even lineage reconstruction *(15)* analyses, have improved the analytic repertoire suited to physically small precancers. Accurate diagnosis of the precancers themselves, typically termed "hyperplasias" in the widely used World Health Organization nomenclature *(16),* has been difficult to standardize *(17).* Even when criteria are agreed upon, reproducibility *(18)* is suboptimal. Previous reports of PTEN mutations in putative endometrial precancers *(19–21)* have used subjective diagnostic criteria. Objective computerized morphometry *(12,22,23),* which uses image analysis algorithms that have excellent ability to predict concurrent *(23)* or future *(12)* carcinoma, has been shown to improve the reproducibility of histopathologic diagnoses. We have previously validated computerized morphometric analysis as an accurate means of precancer identification by showing that most of the lesions that it classifies as precancers are, in fact, monoclonal neoplasms *(24),* albeit benign ones prone to malignant transformation.

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See "Note" following "References."

We have performed PTEN mutation analysis and protein expression studies in a spectrum of precisely classified endometrial tissues to test our hypothesis that changes in PTEN structure and function are among the earliest events in the pathway to endometrioid endometrial cancer. A series of unopposed estrogen-exposed endometria was included to determine if altered PTEN function might precede the appearance of endometrial intraepithelial neoplasia (EIN), which we define as a precancer diagnosed by morphometry.

Materials and Methods

Tissue Samples

Two separate series of paraffin-embedded endometrial tissue samples were selected from the pathology files of Brigham and Women's Hospital (Boston, MA) by report review for diagnoses of endometrial adenocarcinoma and/or anovulatory–hyperplastic endometrium after approval was received from the Human Studies Committee at that institution. The first series of samples, hysterectomy specimens from 30 patients with endometrioid endometrial adenocarcinoma and premalignant lesions ("precancers") that were diagnosed objectively by computerized morphometric analysis, was used for PTEN mutational analysis. A single region representative of each tissue diagnosis was selected in each sample. Ten of these hysterectomy specimens also contained histologically normal endometrium suitable for analysis, and all 30 contained normal myometrial tissue for use as a DNA control. Endometrial polyps were excluded from the analysis. The second series of samples, endometrial tissues from 54 patients (34 hysterectomy specimens and 20 curetting/biopsy specimens), was used for PTEN immunohistochemistry. The samples used for immunohistochemistry were all less than 1 year old, whereas most of the first series of hysterectomy specimens (used for mutational analysis) were from surgeries performed more than 2 years earlier.

Histologic Classification by Use of Computerized Morphometric Analysis

Diagnostic classification was accomplished by a combination of review by a pathologist (G. L. Mutter) and computerized morphometry. First, carcinomas were distinguished from premalignant lesions by the presence of at least one of three diagnostic features: 1) myometrial invasion, 2) solid areas of neoplastic epithelium, or 3) extensively meandering, interconnected glandular structures. Endometrial tissues that were judged not to constitute carcinomas were circumscribed with ink on the glass slide. Computerized morphometric analysis of corresponding delineated regions on hematoxylin–eosin-stained sections was performed (by J. P. A. Baak) by use of the QProdit 6.1 system (Leica, Cambridge, U.K.) as described previously *(12,23,25).* For each lesion, the D score was calculated from the volume percentage stroma (VPS), standard deviation of shortest nuclear axis (SDSNA), and gland outer surface density (OUTSD) [$D = 0.6229 + (0.0439 \times VPS) - (3.9934 \times \ln[SDSNA]) - (0.1592 \times OUTSD)$ *(12,23)*] and was then classified as precancerous (EIN) ($D<0$), indeterminate ($0 \leq D \leq 1$), or benign ($D>1$) based on the previously developed *(12,23)* outcome-predictive formula. Endometrial areas scored as benign were subclassified by pathologist (G. L. Mutter) review. Atrophic, cycling, or reactive endometrium was identified and grouped as "normal." Unopposed estrogen-exposed endometria were diagnosed by the appearance of occasional glandular cysts in a disordered proliferative field without sufficient glandular crowding or atypia to qualify as a precancer. The source of unopposed estrogen was either endogenous (anovulatory cycles) or exogenous (pharmacologic estrogens).

DNA Isolation and Amplification

Genomic DNA from endometrial tissues (normal, precancer, or cancer) obtained at hysterectomy was isolated by selective UV irradiation *(14)* of areas of the paraffin sections that were typically 3 mm in diameter and contained dozens of individual glands. PTEN-coding sequences were amplified by PCR by use of target-specific oligodeoxynucleotide primers. Intron-based PCR primers were used to minimize coamplification of the processed (intronless) PTEN pseudogene on chromosome 9 *(26).* In the following list of the primers that we used, each like-numbered pair comprises the forward ("FGC") and reverse ("RGC") primers for the correspondingly numbered PTEN exon: 1FGC (5′-CGT CTG CCA TCT CTC TCC TCC T-3′), 1RGC (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GAA ATA ATA AAT CCG TCT ACT CCC ACG TTC T-3′), 2FGC (5′-CGT CCC GCG TTT GAT TGC TGC ATA TTT CAG-3′), 2RGC (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GTC TAA ATG AAA ACA CAA CAT G-3′), 3FGC (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GTA AAT GGT ATT TGA GAT TAG-3′), 3RGC (5′-GCG CGA AGA TAT TTG CAA GCA TAC A-3′), 4FGC (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GAA ATA ATA AAC ATT ATA AAG ATT CAG GCA ATG-3′), 4RGC (5′-GAC AGT AAG ATA CAG TCT ATC-3′), 5.1FGC (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GTT TTT TCT TAT TCT GAG GTT ATC-3′), 5.1RGC (5′-TCA TTA CAC CAG TTC GTC C-3′), 5.2FGC (5′-TCA TGT TGC AGC AAT TCA C-3′), 5.2RGC (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GGAA GAG GAA AGG AAA AAC ATC-3′), 6FGC (5′-GCG CGT TTC AAT TTG GCT TCT CTT T-3′), 6RGC (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GAA ATA ATA AAT AAG AAA ACT GTT CCA ATA C-3′), 7FGC (5′-CGT CCC GCA ATA CTG GTA TGT ATT TAA C-3′), 7RGC (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GGA TAT TTC TCC CAA TGA AAG-3′), 8FGC (5′-CGG TTT CAC TTT TGG GTA AAT A-3′), 8RGC (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GAC CCC CAC AAA ATG TTT AAT-3′), 9FGC (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GTC ACT AAA TAG TTT AAG ATG-3′), and 9RGC (5′-TTC ATT CTC TGG ATC AGA GT-3′). Since each sense primer had a 30- to 45-base-pair GC clamp at its 5′ end (e.g., primer 1RGC), each amplicon (PCR product) included a domain with a lower melting temperature (the sequence to be analyzed for mutations) and a domain with a higher melting temperature (the GC clamp).

Detection and Sequence Analysis of PTEN Mutations

Denaturing gradient gel electrophoresis (DGGE) separates amplicons on the basis of melting temperature, which varies with nucleotide composition. We used a 10% polyacrylamide gel containing 5% glycerol and a linear 15%–50% urea–formamide gradient, which simulates a temperature gradient, and subjected our samples to electrophoresis at 100 V for 16 hours at 60 °C *(27).* Under these conditions, a double-stranded PCR product moves through the gel until it reaches the level corresponding to the melting temperature of its lower melting domain, whereupon that domain melts instantly. Since the high-melting GC clamp holds the amplicon together, migration ceases. PCR products of mutant DNAs exhibit altered migration during DGGE and usually appear as doublets of mutant PTEN products admixed with wild-type DNA that was contributed by contaminating normal tissues or the companion allele.

PCR products were visualized by UV transillumination of ethidium bromide-stained gels. DNA was isolated from bands identified as aberrant and was further amplified, and a nested sequencing primer was used to generate fluorescence-labeled sequencing products that were analyzed on a semiautomated DNA sequencer (ABI377; Perkin-Elmer Corp., Norwalk, CT) as described previously *(6).* DGGE gels and sequencing chromatograms were independently read by J. B. Kum, C. Eng, and J. T. Fitzgerald or G. L. Mutter.

Analysis of Loss of Heterozygosity

DNA from carcinoma and adjacent normal myometrium was amplified in the presence of [α-^{32}P]thymidine 5′-triphosphate with primers that define D10S541 and D10S215 (MapPairs; Research Genetics, Huntsville, AL), which are polymorphic microsatellite loci at the 3′ and 5′ ends, respectively, of PTEN. PCR products of these polymorphic microsatellites were separated on nondenaturing polyacrylamide gels *(28,29).* The intensities of bands representing PCR products of tumor alleles were visually compared with those on a reference set of calibrated autoradiographs *(30)* of normal myometrium and scored as positive for loss of heterozygosity (LOH) when there was at least a 50% reduction in the intensity of the band corresponding to one allele.

Immunohistochemistry

Monoclonal antibody 6H2.1, raised against a 100-amino acid oligopeptide identical to the C-terminal end of human PTEN protein *(31),* was used in all of the immunocytochemical analyses. Specificity has been demonstrated previously by western blot analysis of wild-type and PTEN-null cell lines *(31).* Furthermore, when the PTEN-specific antibody was incubated with competing synthetic PTEN peptide (the native antigen) and used to immunostain paraffin-embedded sections of known PTEN-expressing tissues, no immunostaining was observed *(31).*

Since PTEN immunohistochemistry by use of the 6H2.1 antibody requires freshly cut paraffin sections from recently embedded (within 6–12 months) tissues to maximize the signal, we used our second

series of endometrial tissue samples, which met this requirement, for immunohistochemistry. Formalin-fixed tissue samples were embedded in paraffin by standard histologic procedures. Immunostaining was performed by use of a microwave antigen-retrieval protocol as described previously *(31)*. Sections were incubated with monoclonal antibody 6H2.1 (dilution 1 : 100 in phosphate buffer) for 1 hour at room temperature, washed, and incubated with a secondary biotinylated horse anti-mouse immunoglobulin G (Vecstatin ABC kit; Vector Laboratories, Inc., Burlingame, CA). PTEN expression, as reflected by immunostaining, was detected by sequential addition of avidin peroxidase (Vector Laboratories, Inc.) and 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, MO), which gives a brown reaction product. The intensity of the epithelial staining was scored (by G. L. Mutter and J. T. Fitzgerald) in methyl green-counterstained slides from 0 (absent) to 3 (intense). Endometrial stroma and/or normal endometrial epithelium provided an internal positive control, and negative controls without addition of primary antibody showed low background staining in all cases.

Statistical Analysis

Fisher's exact tests were performed by use of SYSTAT v. 9.0 (Statistical Package for Social Sciences, Chicago, IL). All *P* values are two-sided.

RESULTS

To determine the earliest stage of endometrial neoplasia in which PTEN mutation occurs, we examined 30 hysterectomy specimens containing endometrioid endometrial adenocarcinomas as well as coexisting computerized morphometry-diagnosed benign or premalignant endometrial tissue for the presence of mutations. Somatic (occurring in tumor only) PTEN mutations were found in 25 (83%) of 30 endometrial cancers and in 16 (55%) of 29 precancers (Table 1). Fisher's exact test of diagnosis (endometrioid cancer versus precancer) by PTEN mutation (present versus absent) showed that cancers had a statistically significant ($P = .025$) increased number of PTEN mutations compared with their precursors.

None of the 10 samples of normal endometria that we examined showed mutations in PTEN. It is interesting that, among both cancers and precancers, the majority (73% [22 of 30] and 52% [15 of 29], respectively) harbored a mutation in only one exon, but intragenic mutations affecting at least two exons were also observed (Table 1). Fig. 1 shows the number of mutant PTEN exons in 39 nonmalignant tissues that were clearly segregated, by computerized morphometric analysis, into the precancerous (EIN) (D score <0) or benign (D score >1) groups and shows how the D scores of mutations in the two groups are distributed.

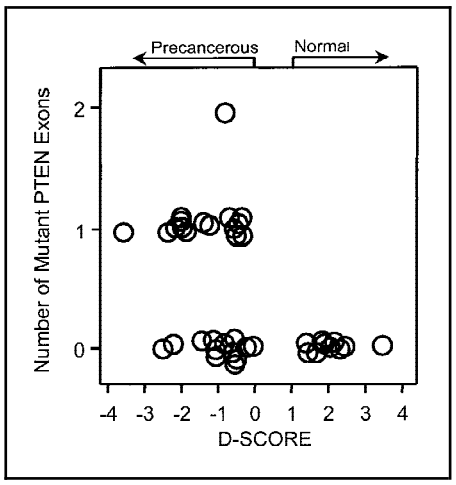

Fig. 1. PTEN mutation and lesion classification by computerized morphometry. Thirty-nine nonmalignant tissues were classified as precancerous or benign on the basis of computerized morphometric D scores, as defined in the text *(12,23)*. Each **circle** shows the D score and the number of mutant PTEN exons detected in a single tissue sample. Symbols are slightly randomly jittered to improve visibility of overlapping symbols.

We found three mutations deep within

Table 1. Number of PTEN mutations and immunohistochemical assessment of PTEN protein expression in endometrial tissue samples

	PTEN mutations*			PTEN protein expression†				
		No. (%) of PTEN mutations			Immunohistochemical assessment of staining, No. (%)			
Endometrial tissue diagnosis	No. of samples	Any mutation	Mutations in ⩾2 exons	No. of samples	Absent	Mild	Moderate	Intense
Endometrioid cancer	30	25 (83)	3 (10)	33	20 (61)	4 (12)	8 (24)	1 (3)
Precancer (EIN)‡	29	16 (55)	1 (3)	12	9 (75)	1 (8)	2 (17)	0
Indeterminate§	ND	ND	ND	9	5 (56)	1 (11)	3 (33)	0
Unopposed estrogen effect‖	ND	ND	ND	7	2 (29)	0	2 (29)	3 (43)
Normal¶	10	0	0	20#	1 (5)	9 (45)	9 (45)	1 (5)
Nonendometrioid cancer	ND	ND	ND	8**	2 (25)	2 (25)	2 (25)	2 (25)

*Genomic DNA from 30 hysterectomy specimens containing endometrioid endometrial adenocarcinoma and premalignant lesions ("precancers") was amplified by the polymerase chain reaction by use of primers for nine PTEN exon mutations, and mutations detected by denaturing gradient gel electrophoresis were confirmed by direct sequencing. One precancer area failed to amplify, and PTEN mutations were also analyzed in an additional 10 regions of histologically normal endometrium in these same hysterectomy specimens. ND = no data.

†Formalin-fixed endometrial tissues from 54 patients (34 hysterectomy specimens and 20 curetting/biopsy specimens) were embedded in paraffin and imunostained with antibody 6H2.1, which detects PTEN protein, and the epithelial/glandular cells were scored.

‡Diagnosed as precancerous (D [defined in text] <0) by computerized morphometry. All samples were independently confirmed as endometrial intraepithelial neoplasia (EIN) by the pathologist (G. L. Mutter).

§Diagnosed as indeterminate (0⩽D⩽1) by computerized morphometry. Diagnosed as EIN (six of nine), unopposed estrogen (one of nine), secretory endometrium (one of nine), or unknown (one of nine) by the pathologist (G. L. Mutter).

‖Diagnosed as benign (D>1) by computerized morphometry, with stigmata of unopposed estrogen.

¶Diagnosed as benign by computerized morphometry; included atrophic, inactive, or cycling endometrium.

#Unstained glands were always admixed with stained glands. One severely atrophic endometrium contained no discernible PTEN protein.

**Two undifferentiated carcinomas, four papillary serous carcinomas, and two malignant mixed Müllerian tumors.

introns, but they are not included in the data shown in Table 1 or in Fig. 1 because they are unlikely to have any functional impact. A detailed listing of mutations found is available at www.jnci.oupjournals.org/content/vol92/issue11/.

PCR-based analysis to determine LOH of markers within or flanking PTEN was performed on the series of 30 endometrial carcinoma samples shown in Table 1. Overall, the LOH frequency was 23% (seven of 30) (data not shown), and all samples with LOH had PTEN mutations in the remaining allele, indicating inactivation of both PTEN alleles. Attempts to perform LOH analysis on precancers were confounded by the presence of contaminating normal stromal tissue.

The number of genetically altered PTEN alleles within individual endometrial adenocarcinomas can be estimated by combining deletion (LOH) and mutation (DGGE, Table 1) data. Ten (33%) of 30 endometrioid carcinomas had homozygous PTEN inactivation (seven with LOH of one allele and mutation of the second allele and three with mutations in two or more PTEN exons), and another 50% (15 of 30) had hemizygous PTEN genomic lesions (DGGE-detected mutation in one allele only, without LOH of second allele).

Probable biallelic inactivation of PTEN is reflected in lack of PTEN protein expression, which can be assessed by immunohistochemistry. Fig. 2 shows immunohistochemical detection of PTEN protein (brown precipitate) by antibody 6H2.1 in areas of endometrial adenocarcinoma, endometrial precancer, and benign endometrium. Although all of the tissue samples shown in Fig. 2 are from one patient, it illustrates the salient PTEN immunohistochemical findings that are typical of malignant, premalignant, and estrogen-driven endometria from the 81 (excluding nonendometrioid cancers) endometrial tissue samples that we have examined. The carcinoma in Fig. 2, A, is devoid of PTEN staining, but adjacent endometrial stromal cells and vascular endothelium contain cytoplasmic and nuclear PTEN protein. A zone of precancerous glands devoid of PTEN protein (Fig. 2, B; upper left) contrasts with abundant stromal staining and an adjacent region of normal endometrial glands (Fig. 2, B; lower right) that show both nuclear and cytoplasmic PTEN staining. The high-magnification views (Fig. 2, C and E) of the upper-right corner of Fig. 2, B, show the interface between PTEN-negative (precancerous) and PTEN-positive (benign) glands, including one transition within an individual gland (Fig. 2, E). Companion hematoxylin–eosin-stained sections (Fig. 2, D and F) are the equivalent of those immunostained with anti-PTEN antibody and show the histologic structure of the tissues.

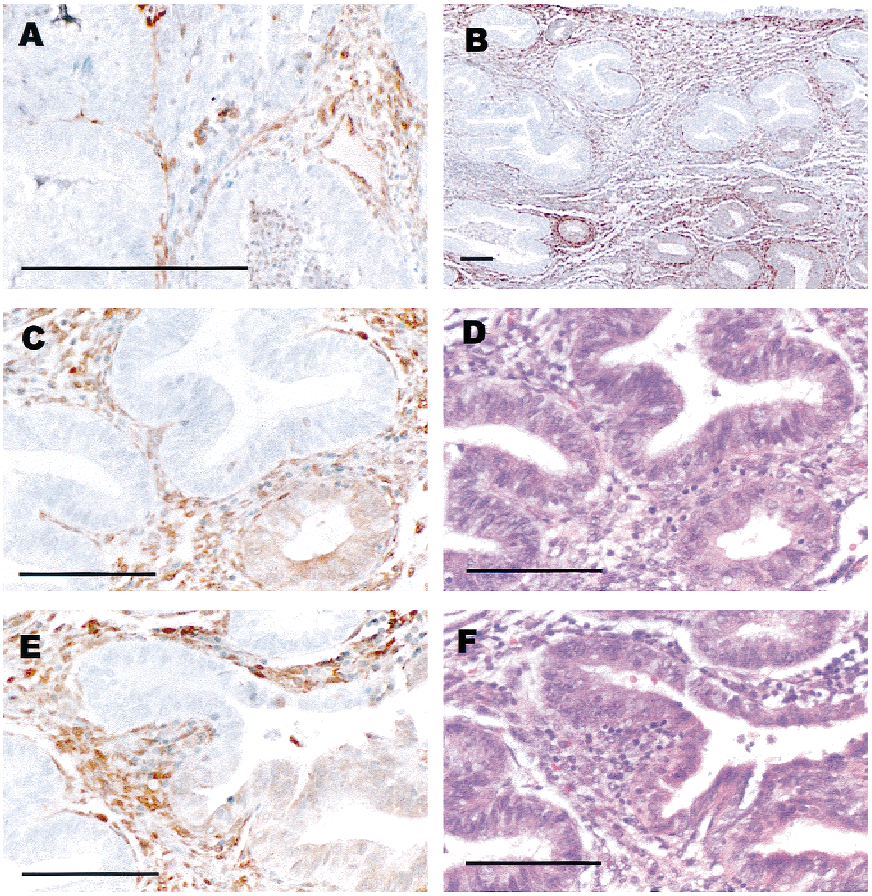

Fig. 2. PTEN protein in endometrial cancer and precancerous endometrial intraepithelial neoplasia. Immunohistochemical staining (**brown**) of PTEN protein with antibody 6H2.1 in **A**) endometrial adenocarcinoma; **B**) a geographic zone of precancerous glands (**upper half and left**) contrasting with an adjacent region of normal endometrial glands (**lower right**); **C**) higher power view of upper right region of panel B; and **E**) transition from PTEN-expressing to nonexpressing epithelium within an individual gland. Companion hematoxylin–eosin-stained serial sections of panels C and E are shown in panels **D** and **F,** respectively. **Scale bar** is 100 μm.

Whereas most unopposed estrogen-exposed endometria showed ubiquitous epithelial PTEN protein expression, 29% (two of seven) had a background of PTEN protein-positive glands punctuated by scattered negative glands. Fig. 3 shows endometria with heterogeneous PTEN protein expression. It demonstrates scattered PTEN-negative glands that are interposed among PTEN-expressing glands to present an interrupted pattern that is different from the geographic distribution within the (monoclonal) readily diagnosed precancers shown in Fig. 2. This intermittent pattern was seen at a variety of gland densities, ranging from the closely packed architecture characteristic of precancers defined by computerized morphometry (Fig. 3, A–C) to the low densities of a disordered proliferative endometrium (unopposed estrogen effect) (Figs. 3, D–F). The cytology of PTEN-nonexpressing glands may be similar to (Fig. 3, B and C) or different from (Fig. 3, E, versus Fig. 3, F) that of surrounding expressing glands. Panels G and H of Fig. 3 show a persistent estrogen-exposed endometrium characterized by cysts, which retains epithelial and stromal PTEN expression. Most areas of tubal change in estrogen-driven, disordered proliferative endometrium continue to express PTEN protein. Companion hematoxylin–eosin-stained sections (Fig. 3, C and F) are the equivalent of those in Fig. 2.

Six diagnostic classes of endometrial

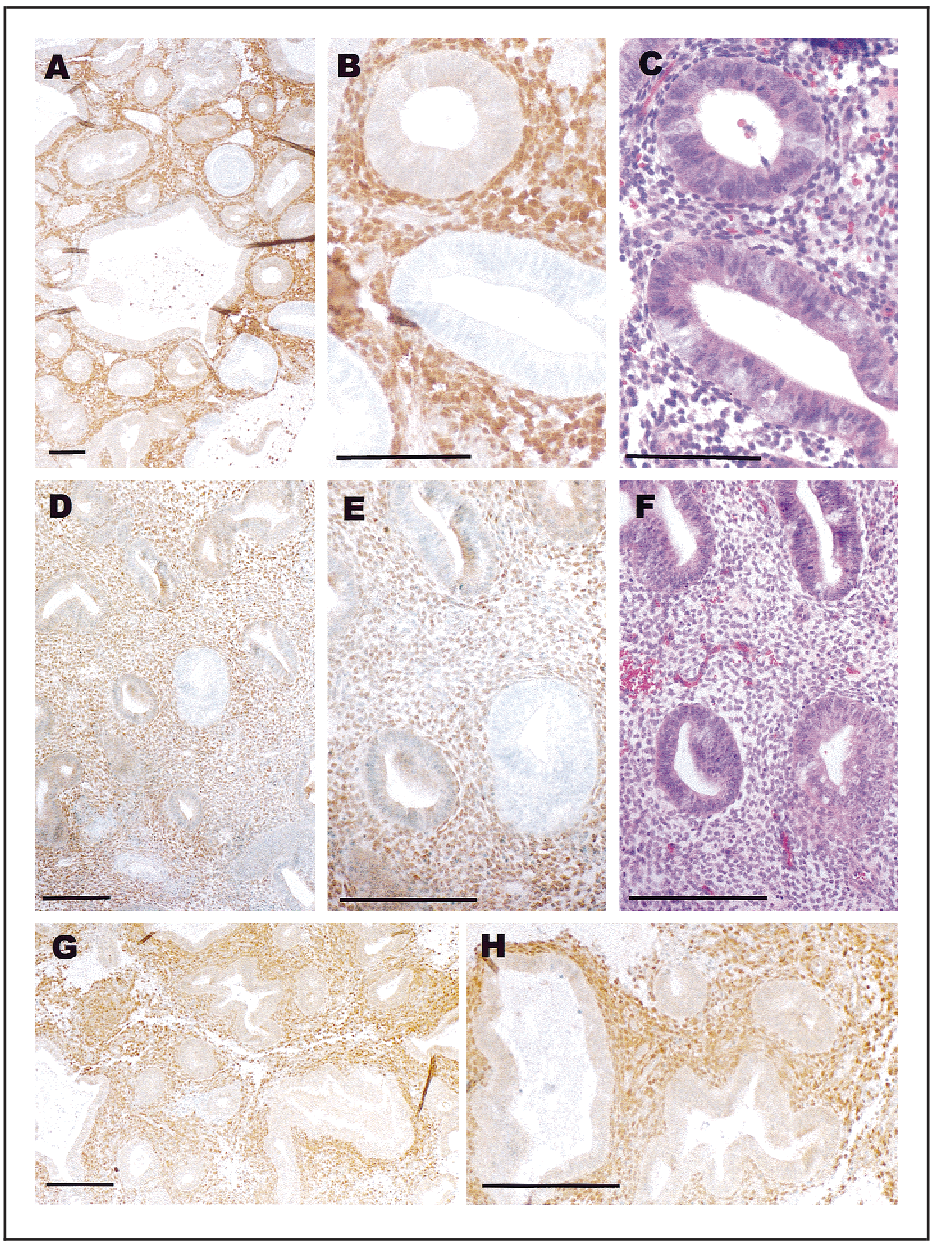

Fig. 3. PTEN protein heterogeneity in precancers and endometria with unopposed estrogen effect. Immunohistochemical staining (**brown**) of PTEN protein with antibody 6H2.1 of **A**) closely packed glands characteristic of precancers defined by computerized morphometry (isolated glands are PTEN negative); **B**) higher power view of panel A; **D**) disordered proliferative endometrium (unopposed estrogen effect) with scattered PTEN-negative glands; **E**) higher power view of panel D; **G**) persistent estrogen-exposed endometrium characterized by cysts, retaining epithelial and stromal PTEN expression throughout; and **H**) higher power view of panel G. Companion hematoxylin–eosin-stained serial sections of panels B and E are shown in panels **C** and **F,** respectively. **Scale bar** is 100 μm.

tissue samples were assessed by immunohistochemistry for PTEN protein expression (Table 1). Of all the endometrioid endometrial cancers tested, 97% (32 of 33) showed either complete absence of or reduced PTEN protein expression. PTEN protein expression was absent from endometrioid endometrial adenocarcinomas more frequently (20 [61%] of 33 samples) than it was absent from nonendometrioid carcinomas (two [25%] of eight samples). Because of the small sample size, however, the difference in PTEN expression (absent versus present at any intensity) as a function of tumor type (endometrioid versus nonendometrioid) was not statistically significant ($P = .115$).

Of nine computerized morphometry-defined indeterminate endometrial samples, five (56%) showed no PTEN protein expression. Of 20 morphometrically diagnosed normal tissues, only one had no PTEN expression. This was an atrophic endometrium.

Both adjacent endometrial stroma and endothelial cells of blood vessels in immediate proximity to the tumor were moderately PTEN protein positive. (Fig. 2, A, is a typical example.) Precancerous (EIN) lesions had no discernible PTEN protein expression in 75% (nine of 12) of the examples, most commonly in closely packed expanses of PTEN-negative glands offset by dispersed benign glands having a different cytology. (Panels B and C in Fig. 2 are examples.) A less frequent pattern of heterogeneous PTEN staining was seen in some "transitional" benign–precancer examples without cytologic changes (Fig. 3, A–C). No statistically significant difference in PTEN protein expression (absent versus present at any intensity) as a function of diagnosis (cancer versus EIN) was observed ($P = .491$), although the small sample size (33 cancers and 12 EIN lesions) limits the power of this comparison.

Discussion

Based on the current results, it is clear that loss of PTEN function begins in the earliest stages of endometrial tumorigenesis, under conditions of unopposed estrogen exposure that have long been known *(11,32,33)* to increase cancer risk. We have found some endometria with protracted estrogen stimulation unopposed by progestins in which individual glands have already ceased production of PTEN protein. Contiguous expanses of tightly packed glands may also be PTEN negative; these are precancerous lesions that have been shown previously to be monoclonal *(24).* Our results showed that the PTEN mutation rate in precancers diagnosed by computerized morphometry, which predict a high likelihood of coexisting or future endometrial carcinoma *(12,23),* is 55%; that the PTEN mutation rate in endometrioid cancers is 83%; and that the difference is statistically significant. Thus, PTEN inactivation occurs during the initiation of precancers from a normal background state, and additional PTEN damage accumulates in the transition from premalignant to malignant disease. Thus, immunohistochemically detected loss of PTEN expression is an informative biomarker for endometrial neoplasia, including precancerous lesions.

Loss of PTEN protein in nests of crowded endometrial glands follows the predictions of monoclonal growth *(14,34),* namely, that all cells in the lesion share the same PTEN status. Precancer

diagnosis by use of computerized morphometric analysis requires histologic sections with crowded groups of endometrial glands over a field of several square millimeters. It is these clusters of crowded glands that correspond to premalignant lesions usually diagnosed by pathologists as atypical endometrial hyperplasias and which we have designated as EIN by use of morphometry. In practice, diagnosis of endometrial precancers by use of hyperplasia *(16)* terminology is only modestly reproducible *(17,18,35),* especially for nonatypical hyperplasias, a category containing monoclonal putative precancers and polyclonal benign tissues. Computerized morphometry, however, appropriately classifies "nonatypical" hyperplasias into high- and low-risk subgroups *(24).* Fig. 1 shows that mutations in the computerized morphometry-defined high-risk group (D<0, precancerous) are evenly distributed across a rather broad range of D scores and are not clustered at some extreme negative D score distant from the threshold of 0. This validates the idea that all endometrial specimens with a D score below 0 have a high likelihood of having a PTEN mutation.

More troublesome to diagnose are lesions with histologic features intermediate between benign and precancerous, as represented by the "indeterminate" category in Table 1. It is this group of patients in whom PTEN immunohistochemistry may elucidate clonal loss of PTEN expression among a strongly PTEN-positive background of convoluted and "busy" glands. Immunohistochemical identification of individual isolated PTEN-negative endometrial glands in endometria with unopposed estrogen exposure pushes the limits of detection of precancers to an earlier stage of development than was previously possible. The natural history of individual PTEN-negative glands has not yet been determined experimentally, but a rich epidemiologic literature *(32,33)* showing a 3.1- to 7.3-fold increased risk of endometrial adenocarcinoma in women exposed to unopposed estrogens is consistent with the notion that, in some women, these single glands may progressively expand into histologically recognizable premalignant, and ultimately malignant, processes.

Suppression of PTEN expression in a mitotically active estrogenic environment (unopposed by progestins) may compromise growth control more than loss of PTEN protein in mitotically quiescent cells. Highly mitotic cells, such as normal estrogen-stimulated proliferative endometrial glands, contain abundant PTEN protein. Progesterone, which is known to prevent many of the tumorigenic effects of estrogens, diminishes *in vivo* endometrial epithelial PTEN protein expression over a period of 4–5 days, to a point where it is no longer detected in the glands of mid-secretory endometrium (data not shown). If these fluctuations in PTEN protein are indeed driven by changing physiologic requirements for the protein, then it is reasonable to predict that the reduced ability to make PTEN protein has a greater effect under estrogenic than under progestenic conditions.

PTEN inactivation (loss of protein) in endometrioid adenocarcinomas and in several other tumor types cannot be explained solely on the basis of observed mutations. This observation suggests that expression of PTEN is repressed at the transcriptional and translational levels by other mechanisms. Fewer than 30% of hematologic malignancies have a structural PTEN alteration, but 70% are PTEN negative as judged by western blot analysis *(36).* Forty percent of breast cancers are PTEN genetic hemizygotes, and fewer than 5% of cases have biallelic PTEN genomic lesions, yet 15% are devoid of protein that is detectable by immunohistochemistry *(31).* In this study, inactivation of both PTEN alleles, as a result of either a mutation or a deletion (LOH), was observed in only 33% of endometrial cancers, but 61% of those cancers did not express PTEN protein. More frequent is a hemizygous PTEN genotype in 50% of endometrial cancer cases. Candidate mechanisms for inactivation of the second allele include (undetected) mutation in regulatory regions, epigenetic modification of flanking DNA sequences (e.g., by methylation), or decreased translation. Increased protein degradation in a hemizygous state could also give negative PTEN immunohistochemistry results that would be indistinguishable from biallelic inactivation.

The PTEN mutation rate of 83% that we observed in our series of endometrial adenocarcinomas is about double that of most previous reports *(1,2,19,20),* probably because of the combined effects of our mutation-detection and sample-selection methods. The DGGE-screening method is very sensitive in PTEN mutation detection compared with the single-strand conformational polymorphism analysis, and its specificity has been confirmed by direct sequencing of DGGE-identified mutations *(27).* DGGE can detect variants, even at mutant-to-normal allele ratios of 1 : 100, while sequencing requires this ratio to be at least 35 : 100 [reviewed in *(37)*].

The tissue samples that we used for mutational analysis were selected for the presence of both malignant and premalignant endometrial tissues at the time of hysterectomy, thereby enriching the study population for those tumors that develop through a hierarchy of progressive events. Endometrioid adenocarcinoma and its precursors have severalfold higher PTEN mutation rates than those malignant tumors (nonendometrioid, including papillary serous type) that arise abruptly without displaying an intermediate premalignant phase. It is unlikely that the balance of microsatellite-stable and microsatellite-unstable tumors can explain the high PTEN mutation rate that we observed, since both had similar PTEN mutation rates in our series.

The mechanism of diminished PTEN protein expression was indirectly addressed in our study, in which, for technical reasons, genomic and protein expression PTEN analyses were carried out on independent tissue series. Our ability to relate changes in PTEN expression to causal genomic events is thus inferential and limited by our sample size. Simultaneous scoring of PTEN mutation and deletion against expression in individual tissues would determine whether these mechanisms alone can explain the majority of lost PTEN expression. One advantage of using this particular series of tissue samples for PTEN mutational analysis is that it was subjected previously to a number of specialized analyses. Of the 30 cancers analyzed, 10 were microsatellite unstable and 20 were microsatellite stable *(14,15,24),* with PTEN mutation rates of 90% (nine of 10) and 80% (16 of 20), respectively. In 29 precancers, 57% (12 of 21) of microsatellite-stable and 50% (four of eight) of microsatellite-unstable *(14,15,24)* lesions had at least one PTEN mutation. KRAS mutations *(13)* were observed in 21% (six of 29) of the cancers, and all (six of six) of these also had PTEN mutations in at least one exon.

Our observations have demonstrated that complete inactivation of PTEN occurs in the great majority of endometrial carcinomas, especially those of the endo-

metrioid subtype, and even in half of all precancers (EIN). Nearly all (97%) of the endometrial cancer tissue samples that we tested had either complete absence of PTEN protein expression or reduced expression of PTEN protein (Table 1). Inactivation could be a result of structural changes (mutation or LOH) or epigenetic modification of the PTEN gene itself or its regulatory elements. Although relatively few endometrial carcinomas had biallelic structural alterations (either two or more PTEN mutations affecting both alleles or PTEN mutation in one allele and LOH of the other), we found complete loss of PTEN protein expression in 61% (20 of 33) (Table 1). Although the distribution of multiple exonic hits between one or two alleles is unknown, the number of PTEN exons affected by mutation provides some indication of that fraction of cases that are candidates for biallelic mutational inactivation.

Morphometrically defined precancers are usually diagnosed as atypical endometrial hyperplasias. It is, therefore, of interest to note that, among computerized morphometrically diagnosed precancers in which approximately half had PTEN mutations, three quarters displayed complete absence of PTEN protein (Table 1). Although only nine computerized morphometry-defined indeterminate endometrial samples were available for analysis, more than half (56%) showed no PTEN protein expression. In contrast, only one of 20 morphometrically diagnosed normal tissues did not express PTEN protein.

PTEN is a major gene involved in the pathogenesis of endometrioid endometrial adenocarcinoma. Our data suggest that altered PTEN function is partly responsible for the etiology of the majority of endometrial cancers with a premalignant phase and participates in their progression to carcinoma. Thus, decreased PTEN expression or function is a marker of the earliest endometrial precancers, and we propose that use of PTEN immunostaining in a clinical setting may be informative in identifying premalignant lesions that are likely to progress to carcinoma.

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NOTE

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