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PRINCIPAL INVESTIGATOR: Charis Eng, M.D., Ph.D.

CONTRACTING ORGANIZATION: The Ohio State University Research Foundation
Columbus, Ohio 43210-1239

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Charis Eng, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Ohio State University Research Foundation
Columbus, Ohio 43210-1239

E-Mail: eng-1@medctr.osu.edu

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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Germline mutations in *PTEN* on 10q23.3 cause 80% of classic Cowden syndrome (CS) and 60% of Bannayan-Riley-Ruvalcaba syndrome (CS) as well as up to 20% of Proteus syndrome and up to 50% of unclassified Proteus-like syndromes. The major thrust of this grant was to identify and characterize PTEN's involvement in families and individuals with one ore more CS component tumors, of which prominently is breast cancer. During the funding period, the PI has found that approximately 5% of CS-like presentations have germline *PTEN* mutations, and the probability is increased by the presence of endometrial cancer. Because of this and related work by the PI, endometrial carcinoma has been added in as a true component cancer of CS and has been incorporated into the International Cowden Consotium Operational Diagnostic Criteria as well as the NCCN Guidelines. In contrast, site-specific breast cancer is not associated with germline *PTEN* mutations although 5% of apparently sporadic breast cancer presentations are associated with such mutations. Extending her work on breast and endometrial carcinomas, the PI has uncovered epigenetic mechanisms of inactivation of PTEN in progression of breast cancer and in the initiation of the earliest endometrial precancers.

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PI: Charis Eng, MD, PhD
Final Report: Genetics of PTEN in Cowden Syndrome and Sporadic Breast Cancer
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INTRODUCTION

Germline mutations in *PTEN*, encoding a dual specificity phosphatase tumor suppressor on 10q23.3, are associated with 80% of Cowden syndrome (CS) cases ascertained by the criteria of the International Cowden Consortium (1, 2). CS is an autosomal dominant disorder characterized by multiple hamartomas and a high risk of breast and thyroid cancers. Further, approximately 60% of Bannayan-Riley-Ruvalcaba syndrome cases (BRR) have germline *PTEN* mutations as well, thus making CS and BRR allelic (3). Initially felt to be unrelated, BRR is characterized by macrocephaly, lipomatosis, and speckled penis, and until the PI's analyses, was felt not to be associated with cancer. Subsequently, up to 50% of all non-BRR, non-CS, Proteus-like individuals and up to 25% of classic Proteus syndrome were found to have a germline *PTEN* mutations (4, 5). The PI had proposed to determine whether and at what frequency occult germline *PTEN* mutations occur in non-CS/BRR families. Towards these ends, the specific aims were:

1. To determine the frequency and nature of germline *PTEN* mutations in non-CS/BRR site-specific breast cancer families; and
2. To determine the frequency and nature of germline *PTEN* mutations in non-CS/BRR breast-thyroid and/or endometrial carcinoma families/individuals, so called CS-like cases.

BODY

Task 1: Mutation Analysis in Non-CS Breast Cancer Families

In the initial two years of the funding period, the PI accrued 21 *BRCA1/2* mutation negative site-specific familial breast cancer cases. All 21 probands were subjected to direct mutation analysis using a combination of PCR-based DGGE and sequence analysis as previously described (6, 7). None of the 21 probands were found to harbor germline *PTEN* mutations. Further, among the 21 families, 10 had samples from 2 or more affected individuals available to us. Genotyping using polymorphic microsatellite and SNP markers within and flanking *PTEN* was performed in these 10 families. Exclusion of linkage to 10q22-q24 was found in 7 families, with the remaining 3 uninformative (ie "consistent with linkage"). In view of these findings and two recent published reports (8, 9), we know that site specific breast cancer families are not accounted for by *PTEN* mutation. Because of this overwhelming data, the PI truncated accrual of this sub-project. Instead, in view of the interesting data obtained from Task 2 (below), the PI focused the major efforts of the final year of funding on Task 2, and tie Task 1 to Task 2 by accrual of non-CS families segregating breast and endometrial cancers or single individuals with both breast and endometrial cancers but who do not meet the diagnostic criteria of CS.

Following up on plans noted in the first Annual Report, the PI then examined a series of well characterized sporadic breast carcinomas to correlate *PTEN* somatic mutation and

PTEN expression by immunohistochemistry using a specific monoclonal antibody 6H2.1 directed against the final 100 C-terminal amino acids of human PTEN (10). These observations are detailed in a reprint enclosed in the Appendix (10). In brief, the PI examined 33 sporadic invasive adenocarcinomas of the breast for *PTEN* mutations and deletions and compared them to protein expression by immunohistochemistry. Among these 33 tumors, the PI found that somatic intragenic *PTEN* mutations rarely occur in primary breast carcinomas (none in this series). Instead, hemizygous deletion as well as epigenetic silencing are the mechanisms of inactivation of PTEN in breast carcinogenesis and they pertain in approximately 15-40% of all primary sporadic adenocarcinomas of the breast. In an effort to determine the precise mechanism of epigenetic silencing, the PI first examined for hypermethylation of the promoter. Since *PTEN* carries a TATA-less promoter, and the 5' UTR does harbor several CpG islands and sequences predicted to bind transcription factors (including the E2F's and GATA's), 9 regions rich in CpG's were examined up to and including 1000 bp upstream of the ATG. Using a series of breast cancer cell lines (all of which had ample *PTEN* transcript except one that had decreased *PTEN* transcript and no structural genomic alterations), the PI determined, using bisulfite sequencing and methylation-specific PCR (11, 12), that hypermethylation of region 7 (in the –200 to –400 region) seemed to be associated with transcriptional silencing (unpublished data). This and other mechanisms of *PTEN* inactivation are being actively pursued.

Task 2: Mutation Analysis in Non-CS Breast-Thyroid and/or Endometrial Carcinoma Families/Individuals ("CS-Like Families")

In Year 1 of the funding period, the PI reported on a study to examine germline *PTEN* mutations in families and individuals ascertained by the minimal presence of breast cancer and any anatomical thyroid disorder in a single individual or in a minimum of two first-degree relatives in a family but who did not meet the Consortium criteria for the diagnosis of CS (13). Of 64 CS-like cases ascertained, one was found to have a germline *PTEN* mutation. This family had bilateral breast cancer, follicular thyroid carcinoma and endometrial adenocarcinoma. There were only 4 other families with endometrial cancer. In the past 2 years (Years 2 and 3 of the funding period), the PI has continued to accrue CS-like families with endometrial carcinoma, usually breast and endometrium occurring in a minimum of two related individuals or in a single individual, as well as CS-like families with breast cancer, any structural thyroid disorder and endometrial carcinoma in a minimum of two related individuals or in a single individual. To date, 14 more unrelated probands have been accrued and have been mutation analyzed and 2 more have been found to carry germline *PTEN* mutations.

Because of these studies examining CS-like families (13), especially those containing endometrial carcinomas, the PI's data suggests that the presence of endometrial cancer may increase the likelihood of finding germline *PTEN* mutation, even in CS-like families. In another recent study, a nested cohort comprising 103 eligible women with multiple primary cancers within the 32 826-member Nurses' Health Study were examined for the occult presence of germline *PTEN* mutations (14). Among 103 cases, 5 (5%) were found to have germline missense mutations, all of which have been shown to cause some loss-of-function. Of these 5, 2 cases themselves had endometrial cancer. This study,

therefore, suggests that occult germline mutations of *PTEN* and by extrapolation, CS, occur with a higher frequency than previously believed. Further, these data confirm the PI's previous observations (13) that endometrial carcinoma might be an important component cancer of CS, and indeed, its presence in a case or family that is reminiscent of CS but does not meet Consortium criteria might actually help increase the prior probablity of finding *PTEN* mutation. Taken together, these molecular-based observations, together with previous clinical epidemiologic studies, (15) were felt sufficient to revise the Consortium criteria for the diagnosis of CS to include endometrial carcinoma (Table 1) (16). The inclusion of endometrial carcinoma to the Consortium operational diagnostic criteria will most likely be adopted by the National Comprehensive Cancer Center Genetics/High Risk Panel at its next revision.

Table 1. International Cowden Consortium operational criteria for the diagnosis of CS, Ver. 2000

Pathognomonic Criteria

Mucocutanous lesions:

- Trichilemmomas, facial
- Acral keratoses
- Papillomatous papules
- Mucosal lesions

Major Criteria

Breast carcinoma
Thyroid carcinoma (non-medullary), esp. follicular thyroid carcinoma
Macrocephaly (Megalencephaly) (say, ≥95%ile)
Lhermitte-Duclos disease (LDD)
Endometrial carcinoma

Minor Criteria

Other thyroid lesions (e.g adenoma or multinodular goiter)
Mental retardation (say, IQ ≤ 75)
GI hamartomas
Fibrocystic disease of the breast
Lipomas
Fibromas
GU tumors (eg, **renal cell carcinoma**, uterine fibroids) or malformation

Operational Diagnosis in an Individual:

1. Mucocutanous lesions alone if:
a) there are 6 or more facial papules, of which 3 or more must be trichilemmoma, or
b) cutaneous facial papules and oral mucosal papillomatosis, or
c) oral mucosal papillomatosis and acral keratoses, or
d) palmo plantar keratoses, 6 or more
2. 2 Major criteria but one must include macrocephaly or LDD
3. 1 Major and 3 minor criteria
4. 4 minor criteria

Operational Diagnosis in a Family where One Individual is Diagnostic for Cowden
1. The pathognomonic criterion/ia
2. Any one major criterion with or without minor criteria
3. Two minor criteria

*Operational diagnostic criteria are reviewed and revised on a continuous basis as new clinical and genetic information becomes available.

Because endometrial carcinoma occurrence per se in classic CS individuals and families are not as frequent as breast or thyroid carcinomas and yet appears to be a very important determinant of *PTEN* germline status, the PI then sought to examine the normal cycling endometrium and sporadic endometrial cancers and precancers as they relate to *PTEN* mutation and expression. As further detailed in an accompanying reprint in the Appendix (17), the PI and collaborator examined PTEN expression by immunohistochemistry with a specific monoclonal antibody 6H2.1 (10) and RT-PCR in the normal cycling endometrium under physiologic changes in sex steroid hormone levels. The PI found that under estrogenic predominance, the proliferative endometrium shows ubiquitous nuclear and cytoplasmic PTEN expression by immunohistochemistry. After 3-4 days of progestational exposure (primed with estrogen), the glandular epithelium maintains cytoplasmic PTEN expression, with waning nuclear expression (17). These observations suggest that PTEN expression might be modulated by sex steroid hormones that may have implications for neoplasia not only of the endometrium but also of the breast.

Because of PTEN's prominent etiologic role in CS and because the PI has shown that endometrial carcinoma is a true component of this syndrome, the PI and collaborator, Dr. G.L. Mutter, investigated neoplastic endometria in relationship to *PTEN* mutation and expression. These observations are detailed in an accompanying reprint (7) found in the Appendix. In brief, examining 2 series of 30 and 33 endometrial adenocarcinomas of the endometrioid histology, the PI found a mutation frequency of approximately 80% in carcinomas and 55% in endometrial precancers (7). Approximately 60% of the carcinomas showed no PTEN protein expression by immunohistochemistry and up to 97% have no or diminished expression. Indeed, there were precancers which showed diminished or no PTEN expression in the absence of somatic mutation or deletion. The data suggest that loss of PTEN expression can precede intragenic *PTEN* mutation even in the endometrial precancer stage and that either *PTEN* mutation or epigenetic PTEN silencing together play a role in >90% of sporadic endometrial carcinomas. In view of these tantalizing observations suggesting that *PTEN* inactivation, either by genetic or epigenetic means, can act as the or one of the earliest events in endometrial carcinogenesis, the PI hypothesized that *PTEN* inactivation occurs even prior to the precancer stage. To test the hypothesis, the PI and collaborator Dr. Mutter examined normal proliferative, persistent proliferative and EIN (endometrial intraepithelial neoplasia) from premenopausal women (18) (see paper in Appendix). PTEN null endometrial glands (ie, without PTEN protein expression by immunohistochemistry) were noted in 43, 56, and 63% in proliferative, persistent proliferative, and EIN diagnostic categories respectively. The occurrence of PTEN-null glands in 43% (24/56) of histologically normal proliferative endometrium (confirmed by staining two sections in

each case) was unexpectedly high. The PI then examined 19 out of 24 proliferative endometria with PTEN-null glands had sufficient material for microdissection. Matched DNA from PTEN expressing and non-expressing glands from the same patient were co-processed for direct comparison of PTEN mutation and deletion. All PTEN expressing matched control glands had a wild-type (normal) genotype whereas 84% (16/19) of non-expressing glands had a somatic mutation (n=8) and/or loss of at least one 10q23 heterozygous marker (n=13) in the region of the *PTEN* locus. Our data demonstrate for the first time that somatic *PTEN* mutation and/or epigenetic silencing occurs even in normal appearing endometrial glands and represents the earliest alterations, apart from germline mutation, that predisposes to endometrial carcinogenesis.

In view of the observations in breast and endometrial carcinomas, the PI decided to compare these data to those for two other sex steroid hormone-related carcinomas, cervical carcinoma and ovarian adenocarcinoma (19, 20) (see papers in Appendix). Until the PI's study, the literature had reported a lack of *PTEN* involvement in cervical cancers. The PI examined 20 cervical carcinomas originating from Japan (mainly the catchment area of Tokyo). Of these 20, 15 were squamous cell carcinomas, of which 15% harbored somatic intragenic mutations in *PTEN* (19) (see Appendix) accompanied by loss of the remaining wild-type allele. Three further tumors were shown to have hemizygous loss of *PTEN*, and one likely had a homozygous deletion of *PTEN*. Thus, in cervical carcinomas, a relatively high frequency of biallelic inactivating *PTEN* mutations are observed. This is in contrast to breast adenocarcinomas, where biallelic structural "hits" are extremely rare. Interestingly, among these series of cervical carcinoma cases, 1 was found to harbor an occult germline *PTEN* mutation. For ovarian carcinomas, the literature cited a lack of somatic intragenic *PTEN* mutations and a LOH frequency of 30-50%. The PI examined 177 sporadic adenocarcinomas of the ovary and found 6 with somatic intragenic mutation of *PTEN*, 1 germline mutation, and 64 (45%) with hemizygous deletion of the *PTEN* region (20) (see Appendix). Among these 177 tumors, 44 were available for analysis of PTEN expression by immunohistochemistry. In general, there seemed to be an association between *PTEN* structural defects (somatic mutation, deletion) and decreased or absent expression. There were a total of 15 tumors that had neither *PTEN* mutation nor deletion but had no or decreased PTEN protein expression. Interestingly, while functional studies, including our own in a breast cancer model, suggest that cyclin D1 and p27 are downstream effectors of PTEN-mediated growth arrest, there seemed not to be a tight correlation of PTEN expression and that of p27 and cyclin D1. While the mutation and expression data have yielded information about PTEN pertinent to the pathogenesis of women's cancers, including those of the breast, endometrium, cervix and ovary, it is interesting to note that rare presentations of apparently sporadic ovarian and cervical cancer can harbor occult germline *PTEN* mutations, with all its implications for future breast and thyroid cancer risk in the patient and her relatives.

KEY RESEARCH ACCOMPLISHMENTS

- That endometrial carcinoma occurring in a CS-like individual or family might increase the prior probability of finding a germline *PTEN* mutation.

- Because of the PI's observations, the Operational Diagnostic Criteria of the International Cowden Consortium has been revised to include endometrial carcinoma as a major component criterion.

- The revised Consortium criteria has been adopted into the next revision of the National Comprehensive Cancer Network Genetics/High Risk Panel recommendations.

- That PTEN plays a major role in sporadic endometrial precancer and cancer.

- That inactivation of PTEN can occur even in histologically normal endometrial glands and might represent very early predisposition to endometrial cancer.

- That PTEN plays an important role in sporadic breast carcinogenesis, accounting for perhaps 15-40% of all such cancers.

- That the mechanisms of inactivation of breast and other women's cancers are varied with a tissue-specific predominant mechanism of inactivation.

REPORTABLE OUTCOMES

Bibliography of Peer Reviewed Articles

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Eng C. Commentary. Will the real Cowden syndrome please stand up: new diagnostic criteria. J Med Genet 2000; 37:828-30.

Mutter GL, Lin M-C, FitzGerald JT, Kum JB, **Eng C**. Changes in endometrial *PTEN* expression throughout the human menstrual cycle. J Clin Endocrinol Metab 2000; 85:2334-8.

Mutter GL, Lin M-C, FitzGerald JT, Kum JB, Baak JPA, Lees JA, Weng LP, **Eng C**. Altered *PTEN* expression as a molecular diagnostic marker for the earliest endometrial precancers. J Natl Cancer Inst 2000; 92:924-31.

Kurose K, Zhou XP, Araki T, **Eng C**. Biallelic inactivating mutations and an occult germline mutation of *PTEN* in primary cervical carcinomas. Gene Chrom Cancer 2000; 29:166-72.

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Kurose K, Zhou XP, Araki T, Cannistra SA, Maher ER, **Eng C**. Frequent loss of PTEN expression is linked to elevated phosphorylated Akt levels, but not associated with p27 and cyclin D1 expression, in primary epithelial ovarian carcinomas. Am J Pathol 2001; 158:2097-2106.

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Eng C, Gimm O, Perren A, Zhou XP, Mutter GL. Different mechanisms of PTEN inactivation in sporadic cancers are tissue-specific. Am J Hum Genet 2000; 67S (Abstract 43, Slide Presention)

Kurose K, Zhou XP, Araki T, Cannistra SA, Maher ER, **Eng C**. Frequent loss of PTEN expression is linked to elevated levels of phosphorylated AKT levels but not associated with p27 and cyclin D1 expression in primary epithelial ovarian carcinomas. Proc Am Assoc Cancer Res 2001; 42 (Abstract 1050, Poster Presentation)

Informatics, Databases, Etc

Continued expansion of clinical cancer genetics CS/BRR/CS-like clinical-genotype database.

Funding Applied For Partially Based on Work Funded by This Award

National Institutes of Health R01 (submitted July 1, 2001 revised submission) entitled, "Individual and Age-Dependent Risk of Cancer in *PTEN* Syndromes"

American Cancer Society Research Scholar Grant (Oct. 15, 2001 deadline for revised submission) entitled, "Genetics of *PTEN* in Cowden and Related Syndromes and Familial Breast Cancer"

Honors, Awards and Significant Appointments

Elected to the American Society for Clinical Investigation, April, 2001

Appointed as Co-Director, Division of Human Genetics, Department of Internal Medicine, The Ohio State University, Columbus, Sept., 2001

Appointed as the William C. and Joan E. Davis Professor of Cancer Research, The Ohio State University, Columbus (effective Oct. 1, 2001)

CONCLUSIONS

To date, because of work related to Task 1, we know that non-syndromic, site-specific familial breast cancer that is not associated with *BRCA1* and *BRCA2* is not associated with germline *PTEN* mutations. Our findings have been corroborated by two other groups (8, 9). This is an instructive negative finding in light of the fact that the PI had

also found (unrelated to this DAMD award) that approximately 5% of unselected, apparently isolated breast cancer presentations before the age of 40 carry occult germline *PTEN* mutations (21). Further, the PI has uncovered epigenetic phenomena related to PTEN silencing in a proportion of sporadic breast adenocarcinomas.

Work related to Task 2 has yielded interesting data, which have important implications for the practice of clinical cancer genetics. Individuals or families who have some but not all features of CS, ascertained in a specific way (noted above), have a minimum 5% probability of carrying germline *PTEN* mutations. The probability of having a mutation might be increased with the presence of endometrial carcinoma either in the proband or a relative. These findings have led to a revision of the diagnostic criteria of the International Cowden Consortium as well as the National Comprehensive Cancer Network. Because of the finding that endometrial carcinoma might be a key feature indicating CS, the PI explored sporadic endometrial carcinoma and has shown that PTEN plays a major role in sporadic endometrial carcinogenesis as well. The PI has found that inactivation of PTEN, either by genetic and/or epigenetic mechanisms, occurs in the earliest precancers. More remarkably, the PI has noted silencing of PTEN and/or somatic *PTEN* mutations in a subset of normal appearing endometrial glands. This might mark the earliest non-germline predisposition to endometrial carcinoma. From the PI's study of sporadic women's cancers, it can be concluded that a small but finite proportion of such presentations actually are unrecognized CS as manifested by uncovering occult germline *PTEN* mutations. This has important implications in the practice of clinical cancer genetics.

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lower limb asymmetry, arterio-venous malformations and lipomatosis. Hum. Mol. Genet. 2000;9:765-8.

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List of Personnel Receiving Pay from the Research Effort

Charis Eng, MD, PhD, PI, Oct., 1998 – Sept., 2001
Fred A. Wright, PhD, Co-I, Oct., 1999-Jun., 2000
Xiao-Ping Zhou, MD, PhD, Postdoctoral Researcher, Mar., 1999-Sept., 2001
Anitra Hammond, BA, Research Associate and Database Officer, Jan., 2000-Sept, 2001

APPENDIX

1. REPRINTS/PRE-PRINTS

Peer Reviewed Journal Articles

Marsh DJ, Dahia PLM, Caron S, Kum JB, Frayling IM, Tomlinson IPM, Hughes KS, Hodgson SV, Murday VA, Houlston R, **Eng C**. Germline *PTEN* mutations in Cowden syndrome-like families. J Med Genet 1998; 35:881-5.

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Abstracts

Eng C, Gimm O, Perren A, Zhou XP, Mutter GL. Different mechanisms of PTEN inactivation in sporadic cancers are tissue-specific. Am J Hum Genet 2000; 67S (Abstract 43)

Kurose K, Zhou XP, Araki T, Cannistra SA, Maher ER, **Eng C**. Frequent loss of PTEN expression is linked to elevated levels of phosphorylated AKT levels but not associated with p27 and cyclin D1 expression in primary epithelial ovarian carcinomas. Proc Am Assoc Cancer Res 2001; 42 (Abstract 1050)

2. PI'S NIH-STYLE BIOSKETCH

Original articles

Germline PTEN mutations in Cowden syndrome-like families

Debbie J Marsh, Patricia L M Dahia, Stacey Caron, Jennifer B Kum, Ian M Frayling, Ian P M Tomlinson, Kevin S Hughes, Rosalind A Eeles, Shirley V Hodgson, Vicky A Murday, Richard Houlston, Charis Eng

Department of Adult Oncology and Charles A Dana Human Cancer Genetics Unit, Dana-Farber Cancer Institute, Department of Medicine, Harvard Medical School, Susan and Richard Smith Laboratories, SM822, 1 Jimmy Fund Way, Boston, MA 02115-6084, USA
D J Marsh
P L M Dahia
S Caron
J B Kum
C Eng

ICRF Colorectal Cancer Unit, St Mark's Hospital, Northwick Park, Middlesex, UK
I M Frayling

Tumour Genetics Group, Wellcome Trust Centre for Human Genetics, University of Oxford, UK
I P M Tomlinson

Department of Surgery, Lahey Clinic, Burlington, MA 02115, USA
K S Hughes

Section of Molecular Carcinogenesis, Institute of Cancer Research and Cancer Genetics Clinic, Royal Marsden Hospital, Sutton, Surrey, UK
R A Eeles
R Houlston

Department of Medical Genetics, United Medical and Dental Schools of Guy's and St Thomas's Hospital, London, UK
S V Hodgson

Department of Clinical Genetics, St George's Hospital Medical School, London, UK
V A Murday

Abstract

Cowden syndrome (CS) or multiple hamartoma syndrome (MIM 158350) is an autosomal dominant disorder with an increased risk for breast and thyroid carcinoma. The diagnosis of CS, as operationally defined by the International Cowden Consortium, is made when a patient, or family, has a combination of pathognomonic major and/or minor criteria. The CS gene has recently been identified as PTEN, which maps at 10q23.3 and encodes a dual specificity phosphatase. PTEN appears to function as a tumour suppressor in CS, with between 13-80% of CS families harbouring germline nonsense, missense, and frameshift mutations predicted to disrupt normal PTEN function. To date, only a small number of tumour suppressor genes, including BRCA1, BRCA2, and p53, have been associated with familial breast or breast/ovarian cancer families. Given the involvement of PTEN in CS, we postulated that PTEN was a likely candidate to play a role in families with a "CS-like" phenotype, but not classical CS. To answer these questions, we gathered a series of patients from families who had features reminiscent of CS but did not meet the Consortium Criteria. Using a combination of denaturing gradient gel electrophoresis (DGGE), temporal temperature gel electrophoresis (TTGE), and sequence analysis, we screened 64 unrelated CS-like subjects for germline mutations in PTEN. A single male with follicular thyroid carcinoma from one of these 64 (2%) CS-like families harboured a germline point mutation, c.209T→C. This mutation occurred at the last nucleotide of exon 3 and within a region homologous to the cytoskeletal proteins tensin and auxilin. We conclude that germline PTEN mutations play a relatively minor role in CS-like families. In addition, our data would suggest that, for the most part, the strict International Cowden Consortium operational diagnostic criteria for CS are quite robust and should remain in place.
(*J Med Genet* 1998;35:881–885)

Keywords: PTEN; Cowden syndrome; breast; thyroid

Breast and thyroid carcinoma are two frequently occurring neoplasms in the female population. Increased risks for both breast and thyroid cancer are prominent features of Cowden syndrome (CS). The hallmark phenotype of this inherited cancer syndrome is the presence of hamartomas, developmentally incorrect, benign, hyperplastic growths, in multiple organ systems including the skin, gastrointestinal tract, central nervous system, breast, and thyroid. Breast cancer will develop in 25-50% of women with CS and 3-10% of all CS patients will develop thyroid cancer.[1 2] At present, only four tumour suppressor genes have been associated with familial breast cancer, BRCA1, BRCA2, p53, and PTEN.[3–7] Initially thought to account for over 80% of hereditary breast cancer,[8 9] germline mutations in BRCA1 and BRCA2 together are now thought to account for 25-50% of all familial breast cancer,[10] thus opening up the possibility of other BRCAX genes. Along these lines, germline mutations in p53 are associated with 70% of cases of Li-Fraumeni syndrome, an autosomal dominant condition comprising breast cancer, brain tumours, sarcomas, and adrenocortical carcinomas.[3 4 11] Recently, the CS susceptibility gene has been identified as the tumour suppressor gene PTEN, also known as MMAC1 and TEP1.[7 12–14] PTEN maps to 10q23.3 and encodes a 403 amino acid dual specificity phosphatase.[12–15] Germline missense and truncating mutations have been reported in between 13-80% of patients with CS.[7 16–18] It should be noted that while initial linkage studies of 12 families with CS was highly suggestive of a single locus for CS,[19] a subsequent study proposes that genetic heterogeneity may exist in CS.[16]

At the somatic level, PTEN has been shown to be mutated or deleted in a number of human malignancies, including sporadic breast, brain, prostate, and kidney cancer cell lines, as well as in a number of primary tumours including endometrial carcinomas, glioblastomas, malignant melanoma, and thyroid and breast tumours.[20–33]

Given the role of PTEN in CS and the relatively large percentage of familial cases of breast cancer that are not caused by germline mutation of BRCA1, BRCA2, or p53, we sought to determine whether PTEN may be mutated in

Table 1 Phenotypic classification of CS-like families

Phenotype of families	*No of families*
Breast and thyroid carcinoma occurring together in at least one person	22
Breast and thyroid carcinoma occurring in different subjects	32
Breast carcinoma and thyroid disease (eg goitre)	3
Breast carcinoma/CS-like (eg trichilemmoma), no thyroid involvement	6
Thyroid carcinoma/CS-like, no breast involvement	1
Total	64

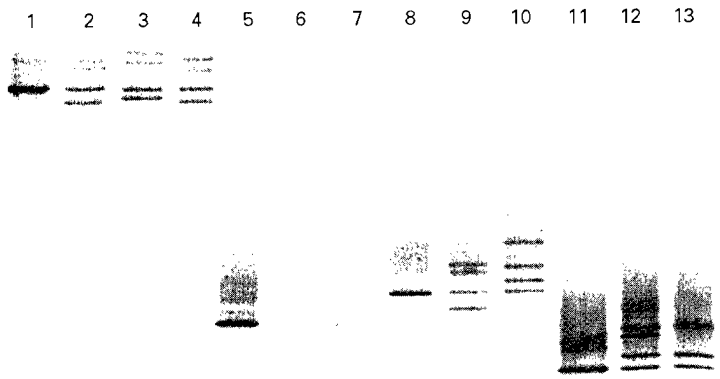

Figure 1 DGGE detection of c.209T→C in the germline of a patient from a CS-like family. Control mutations from CS and BRR families are also included to display the sensitivity of this technique for the detection of PTEN mutations. Lane 1, wild type control (exon 3); lane 2, Y68H (exon 3); lane 3, IVS2-2A→G (exon 3); lane 4, c.209T→C (exon 3); lane 5, wild type control (amplicon 5I, representing the 5' half of exon 5); lane 6, Q87X (amplicon 5I); lane 7, c.347-351delAGAAT (amplicon 5I); lane 8, wild type control (amplicon 5II, representing the 3' half of exon 5); lane 9, C124R (amplicon 5II); lane 10, E157X (amplicon 5II); lane 11, wild type control (exon 7); lane 12, R233X (exon 7); lane 13, c.791ATins (exon 7).

the germline of families that did not meet the strict diagnostic criteria for CS determined by the International Cowden Consortium.[2 19] The phenotypes of these families were, minimally, breast and non-medullary thyroid cancers, and, maximally, a sum of phenotypes falling just short of the Consortium Criteria for CS.

Material and methods

PATIENTS

Members of 64 unrelated CS-like families were collected for analysis (table 1). These CS-like families were defined as families or people that have some, but not all, of the features of CS and do not meet the operational diagnostic criteria of the International Cowden Consortium. Minimally, these CS-like families contained at least one member with both non-medullary thyroid cancer and at least one other related member with breast cancer diagnosed at any age. They also could comprise subjects with both breast cancer and non-medullary thyroid cancer. Alternatively, families could be made up of either breast or non-medullary thyroid cancer and other features of CS, such as trichilemmomas, without meeting the consortium criteria for CS.

The diagnostic criteria for classical CS used in this study has been previously described by the Consortium.[2 19] In brief, the diagnosis of CS requires that a patient or family meet a combination of pathognomonic major and minor criteria. Major criteria include breast cancer, non-medullary thyroid cancer (especially follicular thyroid carcinoma), macrocephaly (≥97th centile), and Lhermitte-Duclos disease (LDD), which is a dysplastic gangliocytoma of the cerebellum that can cause seizures, tremors, and poor coordination. Hamartomas of the skin, including trichilemmomas (benign tumours of the hair follicle infundibulum) and mucocutaneous papillomatous papules (for example, scrotal tongue), are diagnostic if there are six or more papules, with three or more being trichilemmomas. Minor criteria include benign thyroid lesions such as multinodular goitre and adenomas, fibrocystic breast disease, mental retardation (IQ≤75), gastrointestinal hamartomas, lipomas, fibromas, and genitourinary tumours or malformations. Individual people or families would be diagnosed with CS if they have two major criteria, where one is either LDD or macrocephaly, one major with three minor criteria, or four minor criteria. No patients in this study fulfilled these criteria. Constitutional DNA was extracted from blood leucocytes using standard, previously described methods.[34] Approval for the use of human subjects in this study was obtained under IRB approved protocol 94-138 (Dana-Farber Cancer Institute).

DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE) AND TEMPORAL TEMPERATURE GEL ELECTROPHORESIS (TTGE)

A combination of DGGE and TTGE was performed for all nine exons of PTEN. GC clamped primer sequences, PCR conditions, and DGGE conditions have been previously described,[35] with the exception of primers for exons 2 and 4. Exon 2 and 4 primer sequences, with GC clamps added, were as follows: exon 2, 2F, 5'-CGT CCC GCG TTT GAT TGC TGC ATA TTT CAG-3' and 2R, 5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GTC TAA ATG AAA ACA CAA CAT G-3'; exon 4, 4F, 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' and 4R, 5'-GAC AGT AAG ATA CAG TCT ATC-3'. Split exon 5 primers with GC clamps and conditions for mutation detection have been previously reported.[26]

TTGE is a mutation detection technique using the basic PCR fragment denaturation principles of DGGE. The major difference between these methods is that a temperature gradient, rather than a chemical gradient of varying urea and glycerol percentages, is used for strand separation of the GC clamped homo- and heteroduplexed PCR products by generating a linear temperature gradient over the length of the electrophoresis run (Bio-Rad Laboratories, Hercules, CA). One or 0.75 mm thick gels of 10% polyacrylamide:bis (37.5:1) (Bio-Rad Laboratories) and 7 mol/l urea (Bio-Rad Laboratories) were run using the DCode™ Universal Mutation Detection System (Bio-Rad Laboratories). Electrophoresis was performed at 130 V for six hours with a temperature gradient of 46-58°C and a ramp rate of 2°C per hour. TTGE fragments were visualised under ultraviolet transillumination after the gel was stained with ethidium bromide (Bio-Rad Laboratories).

Both DGGE and TTGE have proven high accuracy in detecting mutations in general and specifically in detecting known PTEN mutations from CS patients (fig 1).

CRC Human Cancer Genetics Research Group, University of Cambridge, Addenbrooke's Hospital, Box 238, Level 3, Laboratories Block, Hills Road, Cambridge CB2 2QQ, UK
C Eng

Correspondence to: Dr Eng, Human Cancer Genetics Program, Ohio State University Comprehensive Cancer Center, 420 W 12th Avenue, 690 MRF, Columbus, OH 43210, USA.

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SEQUENCE ANALYSIS

Exons which showed DGGE and TTGE variants underwent direct sequence analysis. The PCR primers and reaction conditions have been described elsewhere.[7 14 28 35] PCR products were gel isolated and purified using the Wizard PCR Preps DNA Purification System (Promega, Madison, WI). Direct sequencing of these products was performed using the ABI Prism dye terminator cycle sequencing ready reaction kit (Perkin-Elmer Corp. Norwalk, CT). Cycle sequencing products were electrophoresed on 6% Long ranger gels (FMC Bioproducts, Rockland, ME) and analysed on an Applied Biosystems model 373A automated DNA sequencer (Perkin-Elmer Corp).

PTEN POLYMORPHISM ANALYSIS

A previously identified intronic polymorphic site in PTEN, IVS8+32G/T, was analysed in a single affected member from each CS-like family to investigate hemizygosity at the PTEN locus in mutation negative families. This site is moderately heterozygous, with an earlier report finding 50% of samples to be informative.[28] Potential hemizygosity was assessed by the amplification of exon 8 and flanking intronic sequence and digestion with the restriction endonuclease *Hinc*II under conditions suggested by the manufacturer (New England Biolabs, Beverly, MA).

Results

PTEN MUTATION ANALYSIS

A missense point mutation, c.209T→C (L70P), predicted to affect splicing was identified in a single affected patient (1 of 64, 2%) (fig 1). This mutation was not identified in 100 normal alleles. When this occult germline PTEN mutation was identified, the family history was reassessed (fig 2). The subject analysed for this study, III.1, developed follicular thyroid carcinoma at the age of 31. His mother, II.2, had breast adenocarcinoma diagnosed at the age of 49 and again at 53. She also had endometrial carcinoma diagnosed at 63 years. Careful clinical assessment of these two subjects was unable to identify macrocephaly, skin lesions typical of CS, or scrotal tongue. The maternal grandfather, I.1, was diagnosed with leukaemia at the age of 57. Unfortunately, family members other than III.1 were unavailable for analysis. Fresh tumour from III.1, which would have allowed us to study the putative aberrant splicing effect of this mutation, was also unavailable. No mutations were identified in the other 63 unrelated CS-like families.

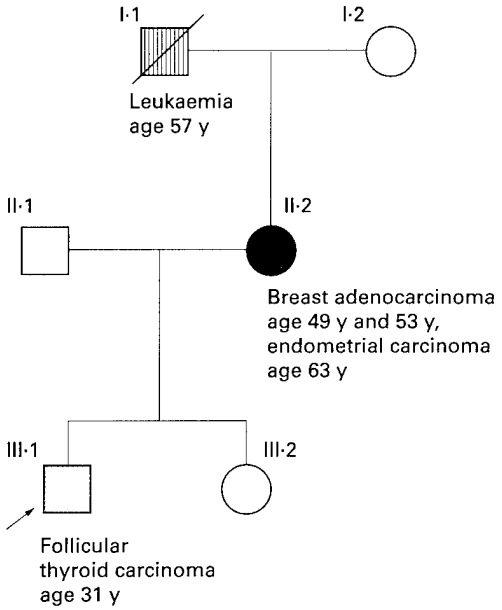

Figure 2 Pedigree of CS-like family with the occult germline PTEN mutation. c.209T→C was identified in DNA extracted from blood leucocytes from patient III.1 who presented with follicular thyroid carcinoma.

PTEN POLYMORPHISM ANALYSIS

Forty-eight percent (30 of 63) of unrelated subjects from PTEN mutation negative CS-like families were found to be heterozygous at the IVS8+32T/G site. This analysis would suggest that, at least in these families, gross germline deletion of PTEN can be excluded.

Discussion

An occult germline PTEN mutation, c.209T→C at the last nucleotide of exon 3 was found in one of 64 (2%) CS-like families. This family's cancers, comprising leukaemia, which may or may not be related, adenocarcinoma of the breast, endometrial carcinoma, and follicular thyroid carcinoma, together do not meet the International Cowden Consortium Criteria used for the diagnosis of CS in this study. However, we cannot exclude the possibility that this family represents a case of low penetrance CS. The family with PTEN mutation in this study contrasts with that in a recent study that reported a PTEN mutation in a family initially classified as having breast and thyroid tumours only but reclassified as CS after mutation analysis led to closer clinical assessment.[36] Closer clinical assessment of the family presented in the current study did not identify additional features of CS.

In the remaining families where no occult germline mutations were identified, it is highly unlikely that these mutations would have gone undetected. Both DGGE and TTGE are highly sensitive mutation detection techniques[37] and both have been shown consistently to detect known PTEN mutations and other sequence polymorphisms (Marsh and Eng, unpublished data, 1998; fig 1). Further, because at least one affected member from nearly half of these mutation negative families was heterozygous at the IVS8+32T/G polymorphism, whole gene deletion is unlikely, at least in these families.

In CS, while missense and truncating mutations are scattered largely along the entirety of PTEN, a mutational "hot spot" exists in exon 5, which contains the PTPase core motif at codons 122-132.[7 16 18] Thus, many mutations in CS are predicted to disrupt the phosphatase function of this protein. Interestingly, the mutation identified in exon 3 falls in the N-terminal half of the PTEN protein that has been shown to have some sequence similarities to the cytoskeletal proteins tensin and auxilin.

Specifically, the leucine residue at codon 70 that is altered by this T to C point mutation (L70P) is conserved in both bovine auxilin and chicken tensin.[14] Thus, it is possible that this mutation may be affecting the phosphatase function of this protein, as one may predict if this putative splice site mutation leads to a truncated protein, and may also function to disrupt normal cellular motility and cell-cell interactions.

Whether germline PTEN mutations are associated with CS and related inherited hamartoma syndromes (Bannayan-Ruvalcaba-Riley syndrome, (BRR, MIM 153480) and juvenile polyposis syndrome (JPS, MIM 174900)), as well as syndromes comprising partial CS phenotypes, is largely unknown. Before the identification of PTEN as the CS gene, it was not inconceivable that the three related hamartoma syndromes and CS-like syndromes were all associated with different mutations in a single gene. We have shown that germline PTEN mutations are associated with the great majority, approximately 80%, of classical CS families.[7 18] Nelen *et al*[17] identified PTEN mutations in 47% of CS cases studied. One other study of 23 CS families identified only 13% of families with germline PTEN mutation.[16] This was perhaps not surprising as limited linkage information in these families suggested the possibility of genetic heterogeneity in CS, even though initial studies of a group of 12 CS families showed no evidence for heterogeneity.[19]

We have also shown that germline PTEN mutations account for at least a proportion of BRR, which is characterised by macrocephaly, lipomatosis, thyroid dysfunction, hamartomatous polyps of the gastrointestinal tract, and pigmented macules of the glans penis, but without a known predisposition to breast and thyroid cancer.[18 38] How mutations in a single gene, at times identical,[18 38] can function to predispose to two overlapping but apparently distinct syndromes, one with malignancy and one without, remains to be elucidated.

Disparate reports concerning the third hamartoma syndrome, JPS, and PTEN mutation or deletion have recently been published.[35 36 39 41] A putative JPS locus, JP1, at 10q22-24 was initially thought to encompass PTEN, although fine structure mapping placed this locus slightly centromeric of PTEN.[42] Subsequently, the 10q22-24 region was excluded as a putative JPS locus by linkage analysis in eight JPS families.[35] Screening of PTEN in 21 classical JPS families and 16 cases of sporadic JPS did not identify any germline mutations.[35 39] In contrast, PTEN mutation has been reported in four patients with "juvenile polyposis",[36 41] although the clinical diagnosis of classic juvenile polyposis in these cases is questionable. Given these genetic data and the phenotypic overlap of these syndromes, we can say with some confidence that if a germline PTEN mutation were detected in a person previously thought to have "juvenile polyposis", then the diagnosis needs to be revised, as that person is likely to have either CS or BRR.

Along the same lines, we have now investigated a cohort of families, each of which contains some of the component tumours of CS but do not meet the Consortium diagnostic criteria for CS. Only one such family was found to have an occult germline PTEN mutation, arguing that such germline alterations play a minor role in families that do not meet the strict CS diagnostic criteria. Nonetheless, this finding is significant for three reasons. Firstly, it suggests that the operational diagnostic criteria for CS established by the International Cowden Consortium are, for the most part, robust and are useful for identifying PTEN mutation positive CS families. Secondly, we must also conclude from our data that other genes are involved which lend susceptibility to a CS-like disease and to site specific breast and non-medullary thyroid cancer. Thirdly, for non-CS subjects identified with occult PTEN mutations, albeit uncommonly, there are important implications for future hamartoma/cancer development that should impact on surveillance.

Unanswered questions remain, however. For example, are CS-like families without germline PTEN mutations at any less risk of cancer than those with mutations? Preliminary genotype-phenotype analyses suggest that classical CS families without germline PTEN mutations are at lower risk of developing malignant breast disease compared to their PTEN mutation positive counterparts.[18] By extrapolation, it would seem that PTEN mutation negative CS-like families should be at decreased risk of developing breast cancer. Unfortunately, this study was unable to confirm this clinically relevant extrapolation. We can conclude, however, that in the majority of cases, germline PTEN mutations lead specifically to a CS or BRR phenotype and that the phenotype of CS-like families is, for the most part, caused by unknown mechanisms.

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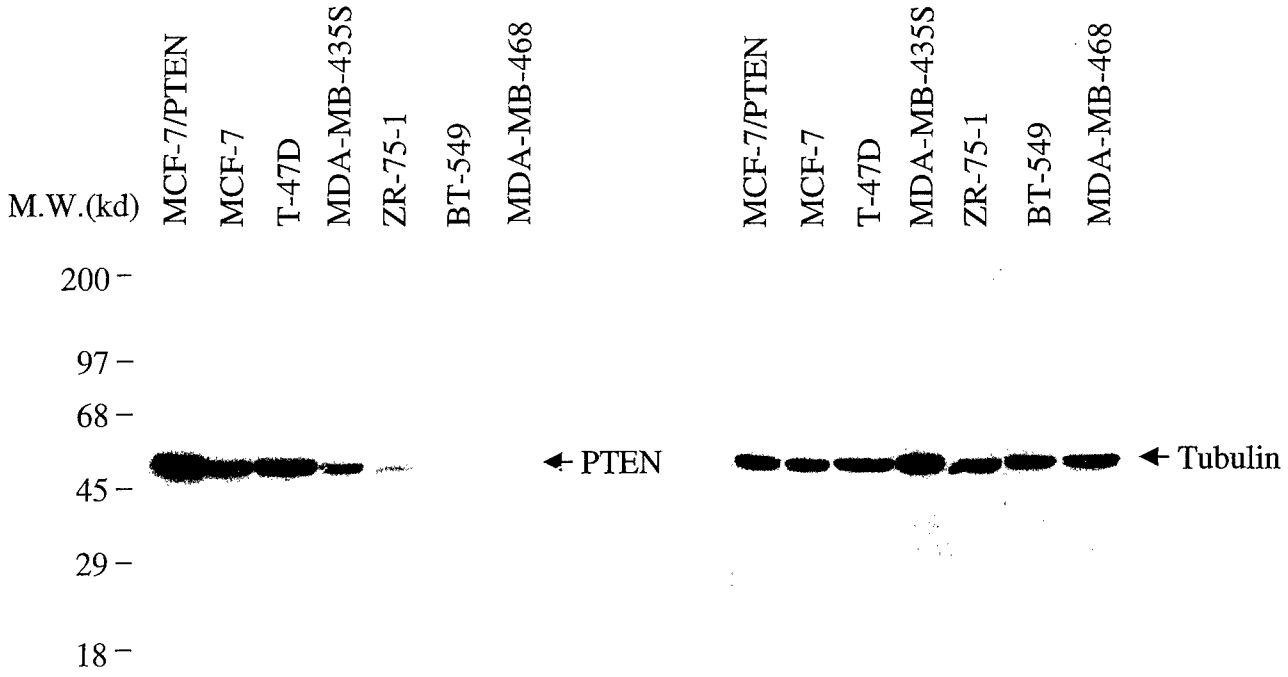

Figure 1. Western blot of 7 breast cancer cell lines using the anti-PTEN monoclonal antibody 6H2.1 (left panel) and using the anti-α-tubulin antibody as a control (right panel). MCF-7, T-47D, and MDA-MB-435S have endogenous PTEN. BT-549 and MDA-MB-468 are PTEN-null. ZR-75-1 has monoallelic PTEN deletion and a missense mutation on the remaining allele. MCF-7/PTEN is the MCF-7 line transfected with a wild-type PTEN construct and a tetracycline-inducible promoter after withdrawal of tetracycline and, hence, induced expression of PTEN.

Results

Specificity of Monoclonal Antibody 6H2.1

Because this study relied on a monoclonal antibody, 6H2.1, specific recognition of PTEN by this antibody is crucial. Western blot analysis using a series of breast cancer lines with known PTEN status and the 6H2.1 anti-PTEN monoclonal antibody demonstrated the specificity of this antibody (Figure 1). Western analysis of three PTEN+/+ lines, MCF-7, T-47D, and MDA-MB-435S, revealed a single band at the molecular weight predicted for PTEN. After induction of MCF-7/PTEN, increased expression of PTEN was evidenced by an increased band intensity (Figure 1). In contrast, ZR-75-1, with a hemizygous deletion of *PTEN* and a missense mutation in the remaining allele, yielded a weak band of the expected size. BT-549 and MDA-MB-468, which are null for PTEN, had no signal. No nonspecific bands were noted. Control blot with anti-α-tubulin antibody revealed signals for all lines.

To test the suitability of the antibody for immunohistochemistry, we used PTEN-transfected U2OS cells as well as a series of cell lines expressing PTEN (Balb C/3T3, Nalm6, DU145) as positive controls. MDA-MB-468, a breast cancer cell line with a hemizygous deletion of PTEN and a truncating mutation of the remaining allele, A172, a glioblastoma cell line with loss of one PTEN allele and a truncating mutation in exon 2 of the remaining allele and PC3, a prostate cell line with homozygous deletion of PTEN, were used as negative controls (data not shown).

PTEN Immunohistochemistry in Primary Breast Carcinomas

Samples from 33 sporadic primary breast carcinomas, which had been examined previously for LOH of markers flanking *PTEN* as well as somatic *PTEN* mutations,[13] were subjected to immunohistochemical analysis using a monoclonal antibody, 6H2.1, raised against the terminal 100 amino acids of human PTEN. Of the 33 total cancers, 29 had accompanying normal tissue; in each of the 29 samples, the normal glandular epithelium showed immunoreactivity to 6H2.1. Interestingly, there was a distinctive staining pattern in the normal tissue. The myoepithelial cells of the normal ducts showed the strongest signal with a nuclear predominance (Figure 2B). In contrast, the amount of staining in the epithelial cell layer was variable. Areas of epithelial ductal hyperplasia with and without atypia stained more strongly than the normal epithelia (Figure 2A). Endothelial cells, especially within neovascular capillaries, and nerves showed strong PTEN expression and were useful as internal positive controls.

Of 33 breast carcinoma samples, 5 (15%) lost all PTEN immunoreactivity and showed negative immunostaining, graded − (Table 1 and Figure 2, E and F). In each of these 5 cases, adjacent non-neoplastic glands (Figure 2F) as well as enclosed non-neoplastic ducts (Figure 2 E) stained positively. Interestingly, the cells within the desmoplastic reaction surrounding each of these 5 carcinomas had high PTEN expression. Six of the 33 (18%) breast cancer specimens stained weakly, graded +, in

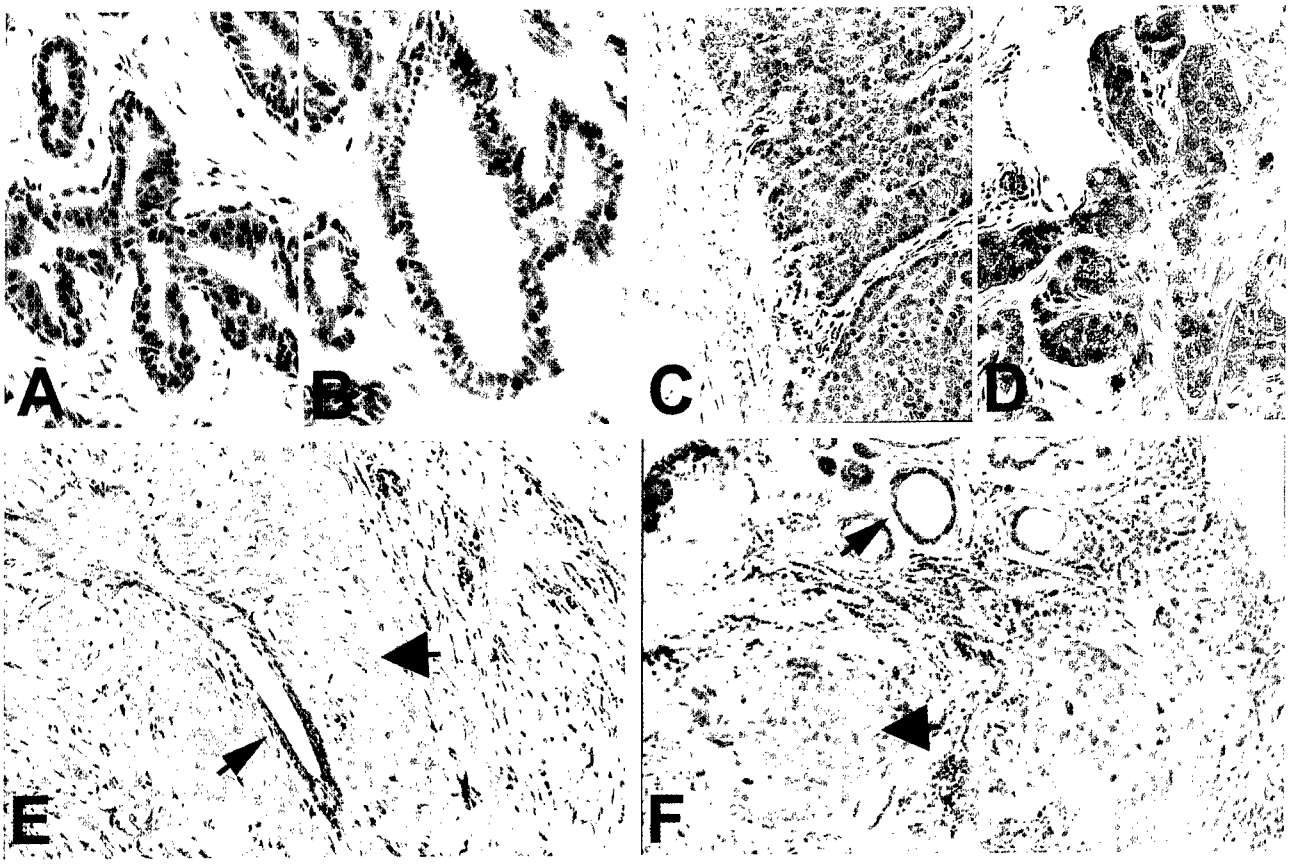

Figure 2. A: Ductal hyperplasia (case 58) with increased staining in the epithelial layer (original magnification, ×60). B: Normal breast glands (case 46) with predominantly nuclear staining in the myoepithelial layer (original magnification, ×60). C (case 48) and D (case 43): Ductal carcinoma with strong PTEN staining (++, original magnification, ×30). E: Ductal PTEN-negative carcinoma (arrowhead, case 58) and surrounding normal duct (arrow). Original magnification, ×30. F: Ductal PTEN-negative carcinoma (arrowhead, case 46) with non-neoplastic normal duct (arrow). Original magnification, ×30.

comparison to the normal tissue (Table 1 and Figure 3). One of these tumors (Sample 40, Table 1) showed positive immunostaining in the intraductal component, whereas the adjacent invasive component lost almost all PTEN protein expression (Figure 3A). The remaining 22 (66%) tumors stained positively, graded ++ (increased staining compared to normal glands). All these tumors showed homogeneous PTEN immunoreactivity throughout the examined section. PTEN immunoreactivity in these 22 tumors as well as their corresponding normal and hyperplastic breast tissue involved the cytoplasmic and nuclear (most likely nuclear membrane) compartment of the cells.

Table 1. Correlation between PTEN Immunostaining and *PTEN* and/or 10q22-23 LOH

	PTEN Immuno ++	PTEN Immuno +	PTEN Immuno −
LOH 5′ Markers	4*	2	4
LOH 3′ Markers	1*	2	5
ROH Flank Markers	18	2*	0
Total Tumors	22	6	5

Correlation between PTEN immunostaining and LOH of 5′ and/or 3′ flanking markers.
Concordance 82%.
LOH, loss of heterozygosity; ROH, retention of heterozygosity.
*Apparent discordance 18%.

Comparison of Immunohistochemical and Structural Mutation Data

Immunohistochemical evidence of PTEN expression was absent or weak in a total of 11 (33%) of 33 breast carcinomas. These breast carcinomas had been previously examined for LOH of markers flanking *PTEN* and also for intragenic *PTEN* mutations;[13] 40% demonstrated LOH but there were no intragenic *PTEN* mutations or biallelic deletion. Whether there is a one-to-one concordance between molecular and immunohistochemical observations is further explored in this report.

LOH analysis for markers in the 10q22–24 interval was previously performed using seven microsatellite markers (centromeric to telomeric): D10S579, D10S215, D10S1765, D10S541, D10S1735, D10S1739, and D10S564.[13] *PTEN* lies between D10S1765 and D10S541, a genetic distance of 1 cM but a physical distance of only several hundred kilobasepairs. For purposes of this study, to compare the immunohistochemical data to the LOH data, *PTEN* was considered to be physically deleted only when one or more immediately flanking (informative) markers centromeric and telomeric of *PTEN* showed LOH. Using this strict and conservative interpretation for monoallelic *PTEN* deletion, 6 of the tumors were shown to have a loss of one allele of the *PTEN* gene, another 7 were shown to have a loss flanking one side of (which may or

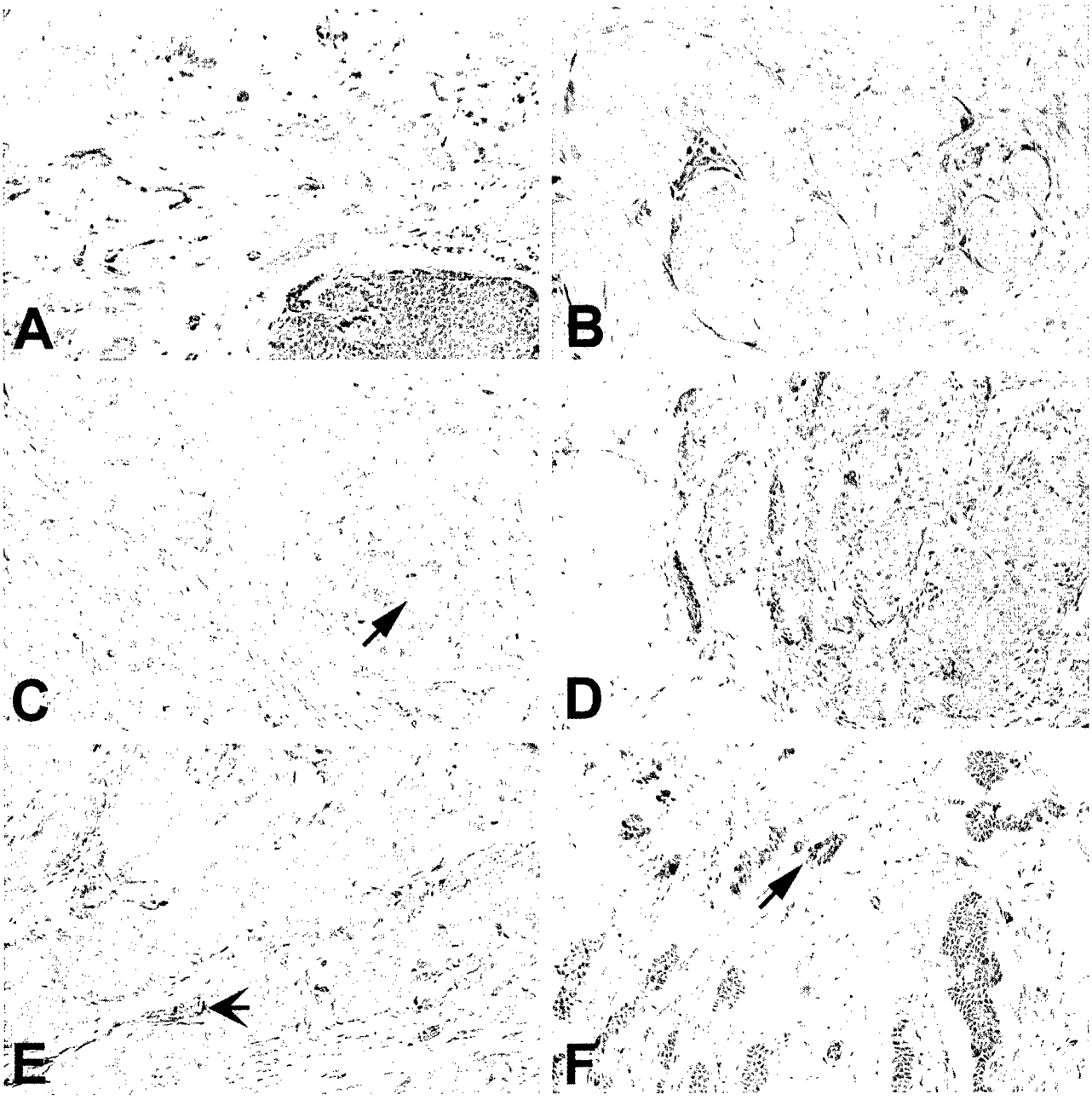

Figure 3. Cases with weak staining (arrows in C and F, non-neoplastic duct; arrow in E, blood vessel). A: Ductal carcinoma (case 40) showing no staining (graded −) in the invasive component (top) adjacent to immunostain-positive intraductal component (bottom). B: Case 66. C: Case 59. D: Case 57. E: Case 45. Original magnification, ×30.

may not include) *PTEN*. For these latter 7 tumors, potential hemizygosity at the *PTEN* locus was further assessed by screening for a T/G polymorphism within *PTEN* intron 8 (IVS8+32T/G), detected by differential digestion with the restriction endonuclease *Hin*cII, and the intragenic polymorphic markers AFM086wg9, D10S2491, and D10S2492. AFM086wg9 lies in intron 2 of *PTEN*. The likely intragenic marker order is centromere – D10S2491 – AFM086wg9 – D10S2492/IVS8+32T/G – telomere (Marsh and Eng, unpublished).

Of the 5 breast carcinomas that exhibited no immunohistochemical evidence of PTEN expression (graded −), 4 showed extensive LOH of markers flanking *PTEN* and hence, *PTEN* itself (Table 1, Column 3 and Table 3). The fifth carcinoma had LOH on the telomeric side (D10S541) of *PTEN*. Further molecular analysis revealed retention of heterozygosity at AFM086wg9 but LOH at the IVS8+32T/G polymorphism, suggesting hemizygous deletion of the 3′ end of *PTEN*. Therefore, all 5 breast carcinomas that had negative PTEN immunostaining also had hemizygous *PTEN* deletion (Table 3). None of these 5 had biallelic deletion of *PTEN* nor did they have a second intragenic *PTEN* hit, ie, mutation of the remaining allele.

Of the 6 carcinomas that had weak PTEN immunostaining, graded +, 4 had been previously shown to have LOH of markers flanking one side or the other of *PTEN* and 2

Table 2. Analysis of Correlation between PTEN Immunostaining and PTEN Intragenic LOH in Cases with Decreased Immunostaining and Apparently Discordant Tumors

		PTEN					
		10q22-23 Markers					
Tumor	Immuno-staining score	S1765	S2491	AFM086	S2492	IVS8	S541
41	++	LOH	NI	LOH	ROH	ROH	NI
52	++	LOH	NI	ROH	ROH	N/A	ROH
53	++	LOH	ROH	ROH	ROH	ROH	LOH
50	++	LOH	ROH	ROH	NI	ROH	NI
40	+	LOH	N/A	NI	N/A	LOH	ROH
59	+	LOH	N/A	LOH	N/A	LOH	ROH
66	+	ROH	NI	LOH	N/A	N/A	LOH
57	+	ROH	LOH	LOH	N/A	N/A	ROH
55	+	ROH	NI	NI	N/A	LOH	LOH
45	+	ROH	NI	ROH	NI	ROH	ROH

Tumor numbers correspond to those of Feilotter et al.[13]
LOH, loss of heterozygosity; ROH, retention of heterozygosity; NI, not informative (germline homozygosity at marker); N/A, not applicable or not done.

showed no LOH of flanking markers (Tables 2 and 3). Further LOH analysis within *PTEN* revealed that the 4 carcinomas with LOH of markers flanking one side of the gene also had LOH of at least one of the intragenic markers (Table 2). Thus, these 4 tumors with decreased immunostaining seemed to have hemizygous deletion of *PTEN* or at least part of it. In the remaining two carcinomas without LOH of markers immediately flanking the gene, further analyses within the gene were uninformative or showed retention of heterozygosity (Tumors 45 and 57, Tables 2 and 3). In all likelihood, *PTEN* might not be altered at the structural level in that particular tumor.

Among the remaining 22 carcinomas that showed immunohistochemical evidence of strong PTEN expression (increased staining compared to normal mammary glands), 18 (82%) showed no LOH and biallelic presence of *PTEN* was demonstrated (Table 1). There were 4 tumors that seemed to be immunostained (grade ++), yet showed LOH flanking *PTEN* (Tables 2 and 3). However, it should be noted that 3 of these 4 tumors had LOH of D10S1765 immediately centromeric of *PTEN* but with either retention of heterozygosity or noninformativeness at D10S541 immediately 3′ of the gene. Further LOH analysis within *PTEN* corroborates the previous observations (Table 2): in tumor 6, 3′ markers within the gene showed retention of heterozygosity and a 5′marker (AFM086wg9) showed LOH; in tumor 5, where D10S1765 showed LOH, markers within the gene (AFM086wg9 and D10S2492) and 3′ of the gene (D10S541) all showed retention of heterozygosity. Similarly, tumor 9, which had LOH at D10S1765, had 3 of 4 intragenic markers with retention of heterozygosity. Tumor 53 was unusual in that both D10S1765 and D10S541 had LOH, although molecular analysis demonstrated all 4 intragenic markers with retained heterozygosity.

Table 3. Summary of PTEN Expression by Immunohistochemistry Compared to Molecular Analysis

PTEN Expression	LOH*	ROH
PTEN−	5	0
PTEN+	5	1
PTEN++	1	21

*LOH of both flanking markers or a minimum of LOH of one intragenic marker.

Table 4. Estrogen/Progesterone Receptor Status of Breast Carcinomas by PTEN Immunostaining Status

PTEN IHC status	ER/PR −	ER/PR +
Negative (−)	3	2
Decreased (+)	0	7
Positive (++)	5	16

ER, estrogen receptor; PR, progesterone receptor.
An equivocal positive receptor status ($n = 5$) was scored as a positive.

Correlation of PTEN Immunohistochemistry and Clinicopathological Parameters

PTEN immunostaining status was compared with such clinicopathological parameters as age at diagnosis, size of primary tumor, tumor grade, lymph node status, and estrogen receptor and progesterone receptor status. Because of the relatively small numbers, especially in the context of subset analyses, no conclusions could be drawn with confidence from our observed correlations. The most interesting association seemed to be that between PTEN expression and hormone receptor status (Table 4). Three of the 5 carcinomas (67%) that had no PTEN protein were estrogen and progesterone receptor-negative compared to 5 of 22 (23%; $P < 0.05$ Fisher's exact test) in the PTEN-immunopositive samples. All 6 carcinomas that had weak PTEN staining were estrogen and progesterone receptor-positive. Other trends are also noteworthy. Although there were only 2 grade I tumors, both had high PTEN expression. All 5 tumors that were 1.5 cm or smaller had high levels of PTEN protein.

Discussion

In this first report of immunohistochemical analyses of PTEN expression in sporadic primary breast carcinomas, we found that 33% of these tumors had either no or decreased expression of PTEN, which generally appeared to correlate with structural monoallelic deletion of the gene. Although it is understandable that tumors with monoallelic loss of *PTEN* have decreased PTEN expression at the protein level, one must explain the 5 samples with no immunoreactivity and structural *PTEN* hemizygosity. None of these samples was found to have intragenic *PTEN* mutations in the remaining allele, either. It is more than plausible, therefore, that an epigenetic phenomenon, such as hypermethylation of the promoter region[28] and decreased protein synthesis or increased protein turnover,[26] might be inactivating the remaining allele. Similarly, for the tumor (case 45) with decreased staining but no structural *PTEN* deletion, similar hypotheses may be raised. Other explanations include point mutations in the putative promoter of the remaining allele or normal tissue contamination of the breast samples, thus giving pseudo-hemizygosity in the face of real homozygous deletion. The latter can be discarded because very careful microdissection of the carcinoma components was performed by a pathologist with extensive experience in microdissection. Further, since the pattern of all positive and negative tumors was homogeneous, regional *PTEN* deletions in tumor subclones are very unlikely. Conversely, the observation of rare immunopositive tumors ($n = 4$) which appear to have LOH of flanking markers can be plausibly explained as well: at least in 3 informative tumors, no deletion of the gene proper or no deletion of most of the 3′ end of the gene has occurred. Hence, the monoclonal antibody, which is raised against the C terminus of PTEN, would still immunostain these samples positively. In this situation, therefore, incomplete 5′ deletion of *PTEN* might still be associated with translation of a truncated immunocompetent PTEN protein. In summary, while structural deletion or mutation of *PTEN* can lead to decreased PTEN protein levels, other mechanisms which lead to complete loss of PTEN expression seem to be prominent as well, at least in the breast carcinoma model.

Whether loss of PTEN expression is an early or late event in breast carcinogenesis is still controversial, although preliminary reports suggest that it is a late event.[11] The observation that loss of PTEN expression is correlated with a negative estrogen and progesterone status and that both grade I tumors had strong PTEN expression also strengthen this hypothesis. There is no doubt that these latter clinicopathological observations need to be investigated further. Nonetheless, these data *in toto* argue that despite the observation that germline *PTEN* mutations cause Cowden syndrome,[4] somatic *PTEN* mutation or functional loss of PTEN expression is associated with tumor progression and not tumor initiation, at least in the breast cancer model. It is also clear from our and other data that breast carcinogenesis does not rely uniformly on the involvement of the PTEN pathway, although how PTEN plays a role in various aspects of normal development and in the pathogenesis of breast carcinoma is not straightforward.

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Commentary

Will the real Cowden syndrome please stand up: revised diagnostic criteria

Charis Eng

Clinical Cancer Genetics and Human Cancer Genetics Programs, Comprehensive Cancer Center, and Division of Human Genetics, Department of Internal Medicine, The Ohio State University, 420 W 12th Avenue (Suite 690 MRF), Columbus, OH 43210, USA; CRC Human Cancer Genetics Research Group, University of Cambridge, Cambridge, UK

Correspondence to:
Professor Eng,
eng-1@medctr.osu.edu

Cowden syndrome (CS, MIM 158350) is an autosomal dominant disorder with age related penetrance characterised by multiple hamartomas and a high risk of breast, thyroid, and perhaps other cancers. These hamartomas can arise in tissues derived from all three embryonic germ cell layers, in accordance with the prominent expression of the susceptibility gene throughout human embryonic and fetal development.[1] The cardinal features of CS include trichilemmomas, which are hamartomas of the infundibulum of the hair follicle, and mucocutaneous papillomatous papules, which occur in the great majority (>90%) of affected subjects.[2 3] Lesions in the breast or thyroid occur in at least two thirds of CS cases. The incidence of CS before gene identification was estimated to be 1 in a million in a population based Dutch clinical epidemiological study.[2 4] However, after gene identification, this figure was revised to 1 in 200 000,[5] which is almost certainly an underestimate. This is because CS has variable expression and often can have nothing but subtle skin signs, and so this condition is difficult to recognise and remains underdiagnosed.

Table 1 International Cowden Consortium operational criteria for the diagnosis of CS, Ver 1995

Pathognomonic criteria
Mucocutaneous lesions
Trichilemmomas, facial
Acral keratoses
Papillomatous lesions
Mucosal lesions
Major criteria
Breast carcinoma
Thyroid carcinoma, especially follicular thyroid carcinoma
Macrocephaly (eg, ≥95th centile)
Lhermitte-Duclos disease (LDD)
Minor criteria
Other thyroid lesions (eg, goitre)
Mental retardation (say, IQ ≤75)
GI hamartomas
Fibrocystic disease of the breast
Lipomas
Fibromas
GU tumours (eg, uterine fibroids) or malformation
Operational diagnosis in a person
(1) Mucocutaneous lesions alone if:
(a) there are 6 or more facial papules, of which 3 or more must be trichilemmoma, or
(b) cutaneous facial papules and oral mucosal papillomatosis, or
(c) oral mucosal papillomatosis and acral keratoses, or
(d) palmoplantar keratoses, 6 or more
(2) 2 major criteria but one must include macrocephaly or LDD
(3) 1 major and 3 minor criteria
(4) 4 minor criteria
Operational diagnosis in a family where one person is diagnostic for Cowden syndrome
(1) The pathognomonic criterion/ia
(2) Any one major criterion with or without minor criteria
(3) Two minor criteria

Before 1996, little was known about the molecular aetiology of the inherited hamartoma syndromes, including CS. For purposes of localising the CS gene, the International Cowden Consortium proposed a set of operational diagnostic criteria to ascertain CS families and to assign affected status within families (table 1).[4 6] These criteria have been adopted by the US based National Comprehensive Cancer Network (NCCN) Genetics/High Risk Cancer Surveillance Panel, whose task is to present evidence based or expert consensus practice guidelines.[7]

The susceptibility gene for CS was mapped to 10q22-23 and identified a year later as *PTEN*.[4 8] PTEN is an almost ubiquitously expressed dual specificity phosphatase which acts as a tumour suppressor[9–11] by mediating cell cycle arrest or apoptosis or both, among other as yet unelucidated functions.[12–14] When CS families and cases are ascertained strictly by the Consortium criteria (table 1), the *PTEN* mutation frequency is approximately 80%.[8 15] However, when these criteria are not used, the mutation frequency ranges from 10-50%.[16–18] Bannayan-Riley-Ruvalcaba syndrome (BRR, MIM 153480), an autosomal dominant developmental disorder characterised by macrocephaly, developmental delay, lipomatosis, haemangiomatosis, and speckled penis, is allelic to CS,[19] with a mutation frequency of 50-60%.[20] The highest *PTEN* mutation frequencies (>92%) are consistently obtained in CS-BRR overlap families (Eng and Hampel, 2000, unpublished observations).[20] Recently, a Proteus syndrome-like subject was found to have a germline *PTEN* mutation and a germline mosaic *PTEN* mutation.[21] This Proteus-like patient presented at birth with marked hypertrophy of the right lower extremity in girth and length, pink verrucoid epidermoid naevi in whirls and plaques on the right side of his body, and macrocephaly. The hemihypertrophy progressed such that massive arteriovenous malformations involving the muscles and bones of the entire right lower extremity and pelvis were noted at the age of 6 years. This patient does not meet the diagnostic criteria for Proteus syndrome[22] nor BRR.[23] A de novo germline *PTEN* R335X was found in this case, and non-germline R130X was found in three different non-contiguous affected tissues from the hypertrophied lower extremity.[21] Whether

Table 2 International Cowden Consortium operational criteria for the diagnosis of CS, Ver 2000

Pathognomonic criteria
Mucocutaneous lesions
Trichilemmomas, facial
Acral keratoses
Papillomatous papules
Mucosal lesions
Major criteria
Breast carcinoma
Thyroid carcinoma (non-medullary), especially follicular thyroid carcinoma
Macrocephaly (megalencephaly) (say, ≥95th centile)
Lhermitte-Duclos disease (LDD)
Endometrial carcinoma
Minor criteria
Other thyroid lesions (eg, adenoma or multinodular goitre)
Mental retardation (say, IQ ≤75)
GI hamartomas
Fibrocystic disease of the breast
Lipomas
Fibromas
GU tumours (eg, **renal cell carcinoma**, uterine fibroids) or malformation
Operational diagnosis in a person
(1) Mucocutaneous lesions alone if:
(a) there are 6 or more facial papules, of which 3 or more must be trichilemmoma, or
(b) cutaneous facial papules and oral mucosal papillomatosis, or
(c) oral mucosal papillomatosis and acral keratoses, or
(d) palmoplantar keratoses, 6 or more
(2) 2 major criteria but one must include macrocephaly or LDD
(3) 1 major and 3 minor criteria
(4) 4 minor criteria
Operational diagnosis in a family where one person is diagnostic for Cowden syndrome
(1) The pathognomonic criterion/ia
(2) Any one major criterion with or without minor criteria
(3) Two minor criteria

Operational diagnostic criteria are reviewed and revised on a continuous basis as new clinical and genetic information becomes available.

other Proteus-like cases will have *PTEN* mutations is unknown and is the subject of continuing research. It has been proposed that these syndromes that are defined by germline *PTEN* mutations be collectively termed *PTEN* Hamartoma Tumour Syndrome or PHTS.[20]

In an effort to determine the full clinical spectrum involved in *PTEN* mutation and to confirm the robustness of the Consortium criteria, a study was performed to examine germline *PTEN* mutations in families and subjects ascertained by the minimal presence of breast cancer and any anatomical thyroid disorder in a single person or in a minimum of two first degree relatives in a family but who did not meet the Consortium criteria for the diagnosis of CS.[24] Of 64 CS-like cases ascertained, one was found to have a germline *PTEN* mutation. This family had bilateral breast cancer, follicular thyroid carcinoma, and endometrial adenocarcinoma. There were only four other families with endometrial cancer. These observations suggest that the Consortium criteria are robust and that the small but finite *PTEN* mutation frequency is important in clinical cancer genetic management. Further, it suggests that the presence of endometrial cancer may increase the likelihood of finding germline *PTEN* mutation, even in CS-like families. In another recent study, a nested cohort comprising 103 eligible women with multiple primary cancers within the 32 826 member Nurses' Health Study were examined for the occult presence of germline *PTEN* mutations.[25] Among 103 cases, five (5%) were found to have germline missense mutations, all of which have been shown to cause some loss of function. Of these five, two cases themselves had endometrial cancer. This study, therefore, suggests that occult germline mutations of *PTEN*, and by extrapolation CS, occur with a higher frequency than previously believed. Further, these data confirm the previous observations[24] that endometrial carcinoma might be an important component cancer of CS and, indeed, its presence in a case or family that is reminiscent of CS but does not meet Consortium criteria might actually help increase the prior probability of finding *PTEN* mutation. Taken together, these molecular based observations, together with previous clinical epidemiological studies,[2] were felt sufficient to revise the Consortium criteria for the diagnosis of CS to include endometrial carcinoma (table 2). These revised criteria will most likely be adopted for the next revision of the NCCN document. Although further long term and formal investigation of whether endometrial carcinoma and other tumours are true components of CS, for purposes of research ascertainment and for clinical practice, exponents of CS and the NCCN panel felt that it would be more conservative, and in the interest of the patient, to acknowledge endometrial carcinoma as a component cancer.

Anecdotal evidence suggests that renal cell carcinoma and malignant melanoma may be minor component neoplasias of CS, although the latter association is difficult to prove because melanoma is common in the general population as well. Nonetheless, they should be kept in mind, especially when considering surveillance in PHTS.

Surveillance recommendations are governed by the component tumours of CS, namely, breast carcinoma, non-medullary thyroid carcinoma, adenocarcinoma of the endometrium, renal cell carcinoma, and possibly melanoma. For males and females, annual comprehensive physical examinations paying particular attention to skin changes and the neck (thyroid) region should be instituted at the age of 18 years or five years younger than the youngest diagnosis of a component cancer in the family.[7] For females, annual clinical breast examination and training in breast self examination should begin around the age of 25 years; annual mammography should begin at 30 or five years younger than the earliest age of breast cancer diagnosis in the family.[7] For the next NCCN revised guidelines, the panel would probably also recommend annual surveillance of the endometrium, blind repel (suction) biopsies of the endometrium in the premenopausal years, perhaps beginning at the age of 35 or five years younger than the youngest age of endometrial cancer diagnosis in the family, as well as annual urine analysis for the presence of blood which may be performed together during the annual physical examination. Further, clinicians who look after such families should be mindful to note any other seemingly non-component neoplasia which might be over-represented in a given family.

Who should undergo CS surveillance? Any person known to have a germline *PTEN* mutation (that is, PHTS) should undergo surveillance. Among classical CS and BRR probands, preliminary data suggest that the presence of a *PTEN* mutation is associated with the

development of breast cancer in any given family.[15 20] Until further data become available, any subject who carries the clinical diagnosis of CS should also undergo surveillance. What is less clear is whether *PTEN* mutation negative BRR should undergo cancer surveillance.

I am deeply grateful to all the patients and families with CS, BRR, and CS-like from around the world who have participated in our studies. I would also like to thank members of my laboratory, numerous collaborators and colleagues, especially Mark Greene and Monica Peacocke, and all the genetic counsellors, especially Heather Hampel and Kathy Schneider, who have contributed in one way or another towards the formulation of these revised criteria. My research activities are funded by the National Institutes of Health, Bethesda, MD, USA, the American Cancer Society, the US Army Breast Cancer Research Program, the Susan G Komen Breast Cancer Research Foundation, and the Mary Kay Ash Charitable Foundation.

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Changes in Endometrial *PTEN* Expression throughout the Human Menstrual Cycle*

GEORGE L. MUTTER, MING-CHIEH LIN, JEFFREY T. FITZGERALD, JENNIFER B. KUM, AND CHARIS ENG

Department of Pathology, Brigham and Women's Hospital (G.L.M., M.-C.L., J.T.F.), Boston, Massachusetts 02115; Department of Adult Oncology, Dana Farber Cancer Institute (J.B.K.), Boston, Massachusetts 02115; Clinical Cancer Genetics and Human Cancer Genetics Programs, Ohio State University Comprehensive Cancer Center (J.B.K., C.E.), Columbus, Ohio 43210

ABSTRACT

Frequent mutation of the *PTEN* tumor suppressor gene in endometrial adenocarcinoma has led to the prediction that its product, a phosphatase that regulates the cell cycle, apoptosis, and possibly cell adhesion, is functionally active within normal endometrial tissues. We examined *PTEN* expression in normal human endometrium during response to changing physiological levels of steroid hormones. *PTEN* ribonucleic acid levels, assessed by RT-PCR, increase severalfold in secretory compared to proliferative endometrium. This suggested that progesterone, a known antineoplastic factor for endometrial adenocarcinoma, increases *PTEN* levels. Immunohistochemistry with an anti-*PTEN* monoclonal antibody displayed a complex pattern of coordinate stromal and epithelial expression. Early in the menstrual cycle under the dominant influence of estrogens, the proliferative endometrium shows ubiquitous cytoplasmic and nuclear *PTEN* expression. After 3–4 days of progesterone exposure, glandular epithelium of early secretory endometrium maintains cytoplasmic *PTEN* protein in an apical distribution offset by expanding *PTEN*-free basal secretory vacuoles. By the midsecretory phase, epithelial *PTEN* is exhausted, but increases dramatically in the cytoplasm of stromal cells undergoing decidual change. We conclude that stromal and epithelial compartments contribute to the hormone-driven changes in endometrial *PTEN* expression and infer that abnormal hormonal conditions may, in turn, disrupt normal patterns of *PTEN* expression in this tissue. (*J Clin Endocrinol Metab* **85:** 2334–2338, 2000)

THE *PTEN* TUMOR suppressor gene is mutated in 34–80% of endometrioid endometrial adenocarcinomas (1–3) and in up to half of premalignant endometrial lesions, atypical endometrial hyperplasias (3–6). Its role in tumor suppression is confirmed by frequent endometrial abnormalities that develop in *PTEN*-deficient mice (7) and the high incidence of breast, thyroid, and endometrial cancers in humans with constitutive mutation of one *PTEN* allele, Cowden's syndrome (8–10). Mutations in the *PTEN* gene have emerged as a primary cause of this most frequent of all gynecological cancers, endometrial adenocarcinoma.

An intriguing feature common to many organs prone to develop somatic *PTEN* mutant tumors is steroid hormone responsiveness. In the case of sporadic endometrial adenocarcinomas, nonphysiological aberrations of sex hormone levels have been repeatedly defined by epidemiological studies as the major risk factor for this disease (11). Is there a relationship between *PTEN* expression and steroid hormone levels that might link the observed high *PTEN* mutational rate and hormonal endometrial risk factors? To date, there is no direct link between steroid hormone response and *PTEN* function. As a primary target organ for sex hormones, the endometrium is an exquisite barometer by which the hormonal environment can be measured. The morphological appearance of endometrium during the latter half of the cycle is sufficiently stereotypical that a trained pathologist can predict the actual menstrual date (±48 h) of a blinded histological specimen. It is thus possible to classify endometrial tissues by histological appearance and infer with a high level of confidence their menstrual age and ambient hormonal conditions.

We have selected normal endometrial tissues from throughout the normal cycle for *PTEN* expression analysis and interpreted our findings in light of the distinctive hormonal profiles that distinguish its phases. Immunohistochemistry permitted further resolution of which cell types contribute to the overall *PTEN* expression within this complex and dynamic tissue.

Materials and Methods

Tissue samples

Snap-frozen endometrial samples were obtained as discarded materials from hysterectomies of women undergoing surgery for benign, nonendometrial, uterine disease (usually uterine prolapse or fibroids). Endometrial histology was evaluated by review of hematoxylin- and eosin-stained paraffin histological sections obtained at the time of tissue allocation. Endometria from four premenopausal (no exogenous hormone administration, age <50 yr) cycling women included two proliferative and two secretory endometria. An additional hysterectomy specimen from a postmenopausal patient with an atrophic endometrium was included along with myometrium as a control.

Paraffin blocks of histologically normal endometria were retrieved by diagnosis from the pathology files of Brigham and Women's Hospital.

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Address all correspondence and requests for reprints to: George L. Mutter, M.D., Department of Pathology, Brigham and Women's Hospital, 75 Francis Street, Boston, Massachusetts 02115. E-mail: gmutter@rics.bwh.harvard.edu.

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All patients were less than 50 yr old, clinically premenopausal, and without intrinsic endometrial disease or recent history of hormone administration. Histological sections were reviewed by a gynecological pathologist (G.L.M.) for assignment of menstrual date according to a standardized 28-day cycle (12). Day assignments of 40 accessioned normal endometria correspond to sequential hormonal and histological events beginning with the first day of menses as follows: menses, days 1–4 (n = 4); proliferative phase, days 5–15 (n = 8); early secretory endometrium, days 16–18 (n = 7); midsecretory endometrium, days 19–24 (n = 7); and late secretory endometrium, days 25–28 (n = 15).

RT-PCR

RNA was isolated by lysis in guanidine isothiocyanate and selective precipitation with lithium chloride (13). RT of 10 μg total RNA with random hexamers and SuperScript reverse transcriptase (Life Technologies, Inc.>zcomx>-BRL, Gaithersburg, MD) was performed according to the manufacturer's instructions. Identical RNA aliquots underwent parallel manipulation, except for the addition of reverse transcriptase. A constant quantity of resultant complementary DNAs or RNAs without RT was amplified by PCR for 27 PCR cycles at an annealing temperature of 50 C with one of three different *PTEN* primer sets and a control β-actin primer (Research Genetics, Inc., Huntsville, AL; catalogue no. M502) (14). The number of PCR cycles was bracketed between 22–32 to identify a linear range of amplification for the PCR conditions used; 27 cycles was the midlinear range for the primers used. PCR reactions were performed in a 50-μL reaction mix [10 mmol/L Tris (pH 8.4), 50 mmol/L KCl, 20 μg/ml gelatin, 1.5 mmol/L MgCl, oligonucleotide primers 0.1 μmol/L of each, 0.2 mmol/L deoxy (d)-ATP, 0.2 mmol/L dGTP, 0.2 mmol/L dCTP, 0.05 mmol/L TTP, and 50–100 nmol/L [^{32}P]TTP; model PTC-100 thermal cycler, MJ Research, Inc., Cambridge, MA). Oligonucleotide primers for the *PTEN* gene spanned exons 5–7 (PT5-a/b), 6–7 (PT6-a/b), and 8–9 (PT8-a/b). PCR primers are as follows: PT5a, TTTCTATGGGGAAGTAAGGA; PT5b, ACGGCTGAGGGAACTC; PT6a, GTCAGAGGCGCTATGTGTAT; PT6b, GTCTTCCCGTCGTGTG; PT8a, AATGTTTCACTTTTGGGTAA; and PT8b, CGGCTCCTCTACTGTTTTT. PCR products were electrophoresed in 0.4-mm thick polyacrylamide gels under nondenaturing conditions (200–500 V in 8% polyacrylamide gel made in 45 mmol/L Tris-borate and 1 mmol/L ethylenediamine tetraacetate). Gels were dried, and autoradiography was performed using preflashed Kodak XAR film (Eastman Kodak Co., Rochester, NY) at −60 C. Autoradiogram optical density was measured with an EC model 910 optical densitometer (EC Apparatus Corp., St. Petersburg, FL), and the resultant plot was integrated using the GS365W Electrophoresis Data System, version 2.0 (Hoeffer Scientific, San Francisco, CA).

Immunocytochemistry

The monoclonal antibody 6H2.1 (3, 15, 16) raised against the last 100 C-terminal amino acids of *PTEN*, developed and supplied by Jacqueline Lees (Massachusetts Institute of Technology, Cambridge, MA), was used in all immunocytochemical analyses. The specificity of this antibody for *PTEN* has been documented previously (15).

Tissue samples were fixed by immersion in buffered formalin and embedded in paraffin following standard histological practices. Four- to 5-mm sections were cut and mounted on SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA). Immunostaining was performed essentially as previously described (15). In summary, the sections were deparaffinized and rehydrated. Hydrated tissue underwent antigen retrieval for 20 min at 98 C in 0.01 mol/L sodium citrate buffer, pH 6.4, in a microwave oven. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide for 30 min. After blocking for 30 min in 0.75% normal serum, the sections were incubated with 6H2.1 (dilution, 1:100) for 1 h at room temperature. Negative control slides received buffer only at this step. The sections were washed in phosphate-buffered saline and then incubated with biotinylated horse antimouse IgG followed by avidin peroxidase using the Vectastain ABC elite kit (Vector Laboratories, Inc., Burlingame, CA). The chromogenic reaction was carried out with 3,3′-diaminobenzidine using copper sulfate amplification, which gives a brown reaction product. After counterstaining with methyl green, the slides were evaluated under a light microscope. The intensity of staining was classified separately for the nucleus/nuclear membrane and the cytoplasm and was graded by two independent observers as strong (+++), moderate (++), weak (+), or absent (−).

Results

PTEN RNA abundance (Table 1) increased by a factor of 5 or more in the transition from proliferative to secretory endometrium, as assessed by three independent PCR assays (Fig. 1, assays PT5-a/b, PT6-a/b, and PT8-a/b).

PTEN immunohistochemistry resolved the tissue-specific (*e.g.* endometrial epithelium, stroma, *etc.*) and subcellular localization of *PTEN* protein in cycling complex endometrial tissues. The distribution of *PTEN* immunohistochemical signal was confined primarily to the endometrium functionalis, the superficial or luminal portion of the endometrial thickness that undergoes dramatic morphological change in response to the changing hormonal conditions that define the menstrual cycle. The deeper endometrial basalis tended to have very faint *PTEN* staining regardless of cycle stage (not shown).

The endometrium functionalis expresses *PTEN* protein in both stromal and glandular epithelial cells, with systematic changes in intensity and subcellular localization during the menstrual cycle (Table 2). Beginning with menstrual endometrium, shed tissue aggregates have nuclear signal in stromal cells, but none in epithelium (Fig. 2A). As the functionalis regenerates during the proliferative phase *PTEN* signal becomes widespread in epithelial and stromal compartments (Fig. 2B). In the early secretory phase, newly formed basal secretory vacuoles exclude *PTEN* protein, which is present only in the apical aspect of glandular epithelial cells (Fig. 2C). At this time, the stromal cells maintain *PTEN* expression in a pattern similar to that of the earlier proliferative phase. In the mid- and late secretory phases, glands are essentially depleted of *PTEN* protein (Fig. 2D). A progesterone-induced change in midsecretory stromal cells, decidualization, corresponds to expansion of the cytoplasmic volume and continues through the later secretory interval

TABLE 1. Increase in *PTEN* RNA in progesterone-exposed endometrium

Assay	Proliferative mean (SD), n = 2	Secretory mean (SD), n = 2	SE/PE ratio	*P* (by *t* test)
PT5-a/b	9,636 (3,797)	53,733 (13,506)	5.6	0.047
PT6-a/b	5,868 (165)	28,023 (4,024)	4.8	0.016
PT8-a/b	2,785 (451)	39,672 (1,179)	14.2	0.001
β-Actin	11,370 (7,196)	14,909 (6,278)	1.3	0.653

Densitometry of RT-PCR autoradiographic signal from Fig. 1 proliferative estrogen-exposed (lanes 3 and 4; n = 2) and secretory progesterone-exposed (lanes 5 and 6; n = 2) endometrium was performed, and averaged results for each tissue type were used to calculate the relative increase in expression within secretory compared to proliferative endometrium (SE/PE ratio). Two-tailed *t* test probability is shown for each primer set. Expression of *PTEN* transcripts as indicated by three PT primer sets increases in secretory relative to proliferative endometrium.

(12). Nuclear signal in decidualized stromal cells at this stage becomes increasingly intense (Fig. 2D), and the cytoplasmic staining becomes somewhat variable relative to that in earlier secretory endometrium.

Discussion

Endometrial expression of *PTEN* is not constant throughout the menstrual cycle, but changes in response to the hormonal environment. Our initial assessment of expression using whole tissue as a RNA source for *PTEN* RT-PCR suggested that the postovulatory secretory phase had increased *PTEN* expression relative to the estrogenic proliferative phase. Immunohistochemical localization of *PTEN* protein within endometrial tissues, however, showed a highly complex distribution in multiple cell types and subcellular locations that cannot be simply summarized by a change in total tissue abundance. The endometrial response to progestins is cell type specific and inverse between epithelial and stromal cells. Estrogen-driven, mitotically active, glandular and stromal cells have a high level of ubiquitous *PTEN* expression in both nuclear and cytoplasmic compartments. With the addition of progesterone, epithelial *PTEN* expression declines to a point where after 3–5 days (cycle days 18–19) this protein is completely extinguished to a level below the threshold of detection. The diminution of epithelial *PTEN* expression begins with a polarized loss of *PTEN* protein from the basal aspect of epithelial cells. At this same time, adjacent stromal cells undergoing cytoplasmic expansion as part of a decidualization process collect abundant nuclear and cytoplasmic *PTEN* protein.

Changes in *PTEN* expression correspond to those endometrial zones that respond to hormonal fluctuation by changes in specialized cellular functions. Areas of endometrium sheltered from cyclical hormone-driven changes have low or absent *PTEN* levels, which remain stable throughout the cycle. This is evident in the endometrial basalis, which does not undergo stromal decidualization or secretory change as seen in more superficial regions (12).

The observation that epithelial *PTEN* expression levels decline in secretory endometrium is unexpected, especially because increasing levels of progesterone are widely known to have antineoplastic effects in this tissue. If *PTEN* had a direct effect on the antitumorigenic properties of progestins, the opposite would be predicted. Two alternate models are worth considering, but will require additional experimentation to evaluate. One is that the *PTEN* effect on endometrial glands is mediated by the adjacent stromal cells. Alternatively, the functional requirement for *PTEN*-mediated tumor suppressor activity might be specific to a highly mitotic estrogenic environment and negated under progestin-dominated conditions that reduce cell division. If this were the case, *PTEN* mutation under unopposed estrogen conditions would result in a high risk of developing carcinoma. This is exactly the combination of circumstances that is known to increase cancer risk: protracted unopposed estrogen exposure (11, 17) and development of a premalignant lesion, many of which we now recognize as having *PTEN* mutations (3). In another study we have shown that endometria stimulated for abnormally long intervals with estrogens begin to display clonal outgrowth of *PTEN*-depleted epithelium, which eventually assumes a physical configuration diagnostic of a precancerous state (3). Correspondingly, pharmacological administration of progestins to patients with endometrial precancers is often effective in causing their ablation, and in primates may increase the expression of tumor suppressor genes such as DMBT1 (18).

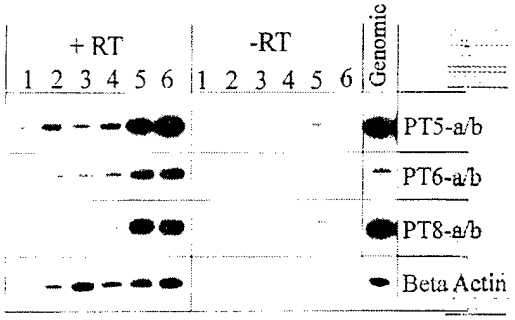

FIG. 1. Endometrial expression of *PTEN* RNA in a changing hormonal environment. *PTEN* expression throughout the normal menstrual cycle was studied by RT-PCR. Equal quantities of normal human endometrial RNA isolated from atrophic (lane 2), estrogen-primed proliferative (2 patients, lanes 3 and 4), and progesterone-exposed secretory (2 patients, lanes 5 and 6) endometria were reverse transcribed (+RT) with random hexamers, and the resultant complementary DNA was used as a PCR template. Myometrium from the postmenopausal patient with atrophic endometrium (lane 2) is included as lane 1. Three different *PTEN* primer sets were used in 27 PCR cycles, spanning exons 5–7 (PT5-a/b), 6–7 (PT6-a/b), and 8–9 (PT8-a/b). All show that *PTEN* RNA levels increased severalfold (see Table I) under progesterone influence (lanes 5 and 6) relative to the estrogenic proliferative phase (lanes 3 and 4) or in the hormonally depleted atrophic state (lane 1). Controls shown include identical RNAs without RT (−RT), genomic DNA, and the constitutively expressed gene β-actin (26). Signal in the +RT lanes can be ascribed to a RNA source, as there is minimal contaminating genomic DNA background (−RT). Each row of data is from a single autoradiogram, with exposure intervals ranging from 4–12 h.

TABLE 2. *PTEN* immunohistochemistry in cycling endometrium functionalis

Phase	Day	Epithelium		Stroma		Notes
		Nucleus	Cytoplasm	Nucleus	Cytoplasm	
Menstrual	0–4	−	−	++	−	
Proliferative	5–15	+	+	++	+	
Early secretory	16–18	+/−	+	++	+	Luminal *PTEN*, excluding basal vacuoles
Midsecretory	19–24	−	−	++	++	
Late secretory	25–28	−	−	+++	varies + to +++	Stromal staining of cytoplasm decreases on predecidualization, nuclear strong throughout

PTEN protein signal was evaluated in the superficial endometrium functionalis, and intensity was recorded for each cell type and subcellular compartment on a scale from no expression (−) to intense expression (+++).

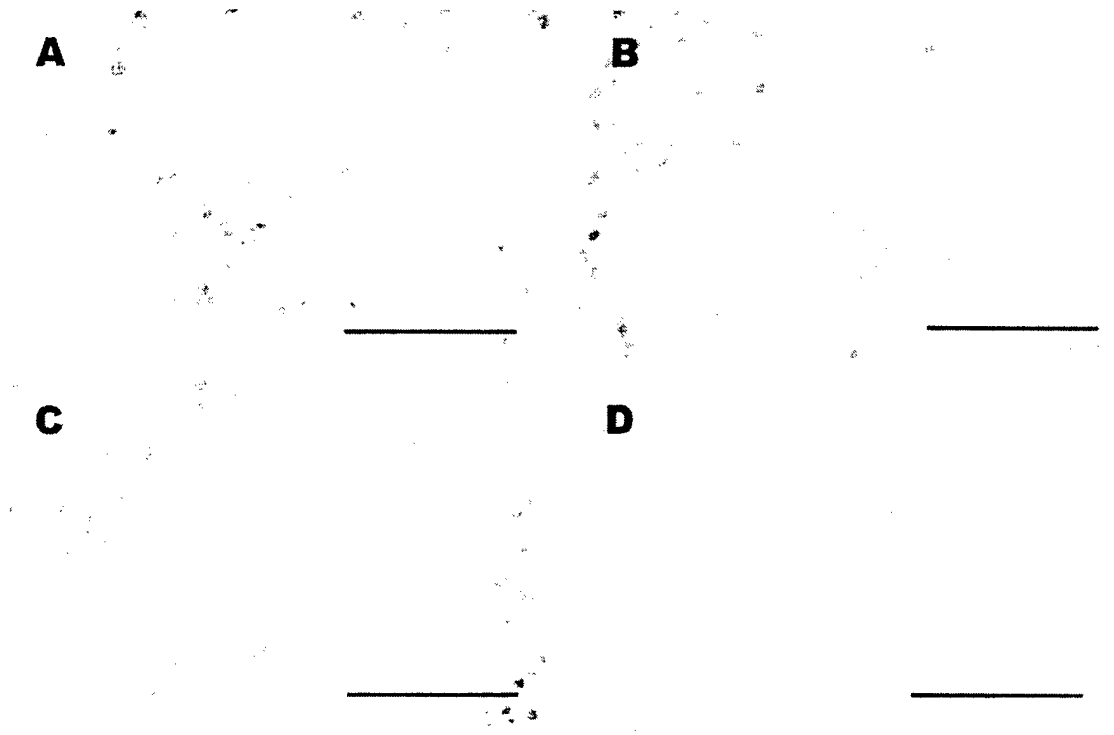

FIG. 2. *PTEN* immunohistochemistry using antibody 6H2.1 displays signal as a *brown* product in sections of menstrual (A), proliferative (B), early secretory (day 17; C), and midsecretory (day 24; D) endometria. *Scale bar*, 100 μm.

A physiological function of *PTEN* exclusive of its postulated role in tumorigenesis is expected. It is essential for complete development, as complete inactivation in knockout mice produces embryonic lethality (7). *PTEN* expression in normal mice is widespread before organogenesis, becoming more restricted thereafter (19), when high levels are seen in skin, breast, thyroid, and brain. These are the very tissues prone to development of neoplasia in adults with acquired or inherited *PTEN* mutations.

Changes in endometrial *PTEN* subcellular localization coincide to shifts in mitotic activity. Mitotically active epithelial and stromal cells have *PTEN* protein in both cytoplasm and nucleus. A relative increase in nuclear localization is seen in nondividing decidualized late stromal cells and apoptotic menstrual stromal cells. To date, *PTEN* has been shown to play some role in cell cycle arrest at the G_1 phase via unknown mediators, apoptosis probably through the PI3K-Akt pathway and cell adhesion via the focal adhesion kinase pathway (20–24). Each of these processes may require *PTEN* to be in specific subcellular localizations. For example, *PTEN* might better regulate cell adhesion and migration through dephosphorylation of focal adhesion kinases in the cytoplasmic compartment (25). If *PTEN* indeed serves to check uncontrolled mitotic division and initiate apoptosis, the fact that these functions are not effective throughout the menstrual cycle requires that *PTEN* expression be coordinated carefully throughout.

In conclusion, *PTEN* expression in normal endometrium is ubiquitous in the estrogenic proliferative phase, but undergoes cell type-specific changes in response to progesterone. Epithelial cells lose *PTEN* protein in the secretory phase, whereas stromal cells increase *PTEN* expression, especially in the cytoplasmic compartment. Epithelial *PTEN* function is probably restricted to the mitotically active glandular epithelium, where its loss by mutation under protracted estrogenic conditions may initiate genesis of a precancerous lesion.

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

George L. Mutter, Ming-Chieh Lin, Jeffrey T. Fitzgerald, Jennifer B. Kum, Jan P. A. Baak, Jacqueline A. Lees, Liang-Ping Weng, Charis Eng

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.

Affiliations of authors: G. L. Mutter, J. T. Fitzgerald, Department of Pathology, Brigham and Women's Hospital, Boston, MA; M.-C. Lin, Department of Pathology, Brigham and Women's Hospital, and Department of Pathology, Taiwan National University Hospital, Taipei; J. B. Kum, Clinical Cancer Genetics and Human Cancer Genetics Programs, Ohio State University Comprehensive Cancer Center, Columbus, and Charles A. Dana Human Cancer Genetics Unit, Dana-Farber Cancer Institute, Boston; J. P. A. Baak, Department of Pathology, Medisch Centrum Alkmaar, and Amsterdam Free University Hospital, The Netherlands; J. A. Lees, Massachusetts Institute of Technology, Cambridge; L.-P. Weng, Clinical Cancer Genetics and Human Cancer Genetics Programs, Ohio State University Comprehensive Cancer Center; C. Eng, Clinical Cancer Genetics and Human Cancer Genetics Programs, Ohio State University Comprehensive Cancer Center, and Cancer Research Campaign Human Cancer Genetics Research Group, University of Cambridge, U.K.

Correspondence to: George L. Mutter, M.D., Department of Pathology, Brigham and Women's Hospital, 75 Francis St., Boston, MA 02115 (e-mail: gmutter@rics.bwh.harvard.edu).

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 (Vectastin 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.

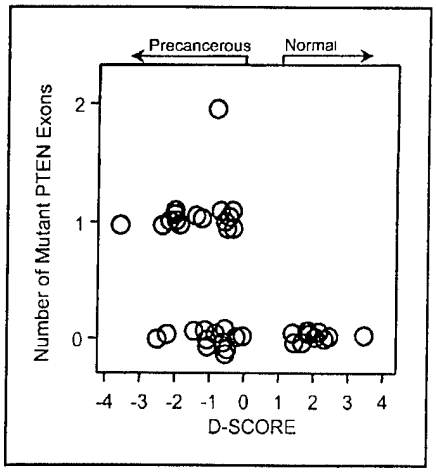

Fig. 1. PTEN mutation and lesion classification by computerized morphometry. Thirty-nine nonmalignant tissues were classified as precancerous or benign on the basis of computerized morphometric D scores, as defined in the text *(12,23)*. Each **circle** shows the D score and the number of mutant PTEN 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 ($0 \leq D \leq 1$) 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.

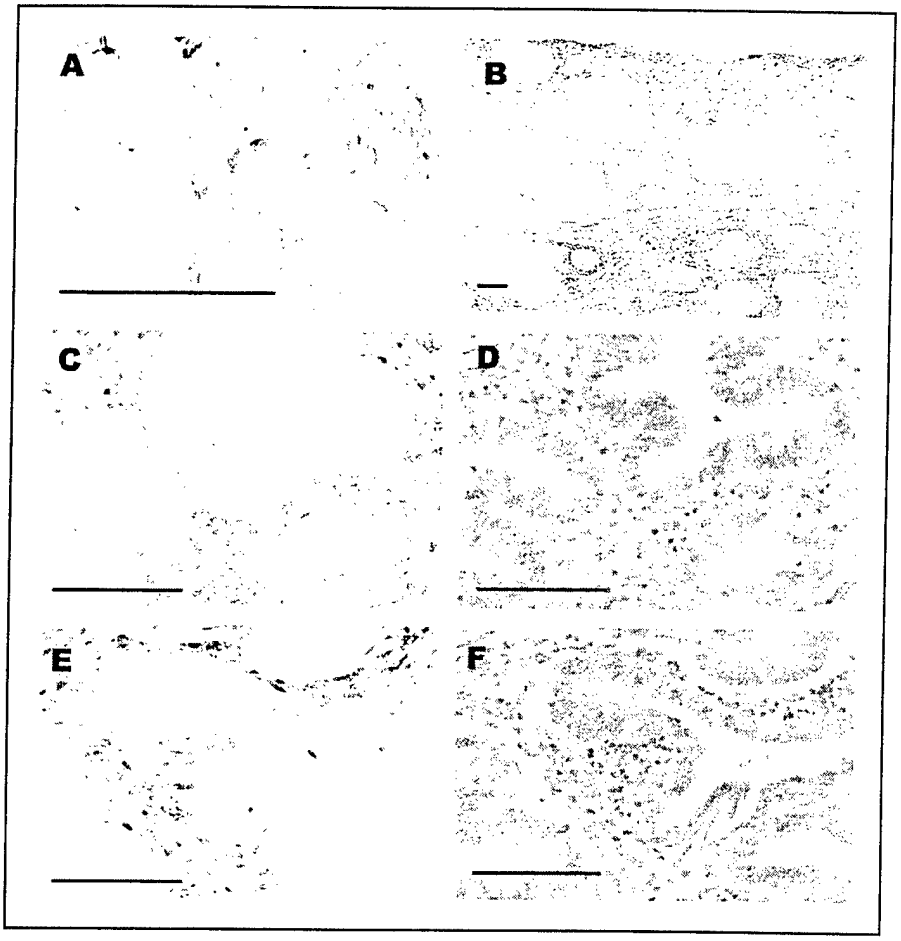

Fig. 2. PTEN protein in endometrial cancer and precancerous endometrial intraepithelial neoplasia. Immunohistochemical staining (**brown**) of PTEN protein with antibody 6H2.1 in **A**) endometrial adenocarcinoma; **B**) a geographic zone of precancerous glands (**upper half and left**) contrasting with 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

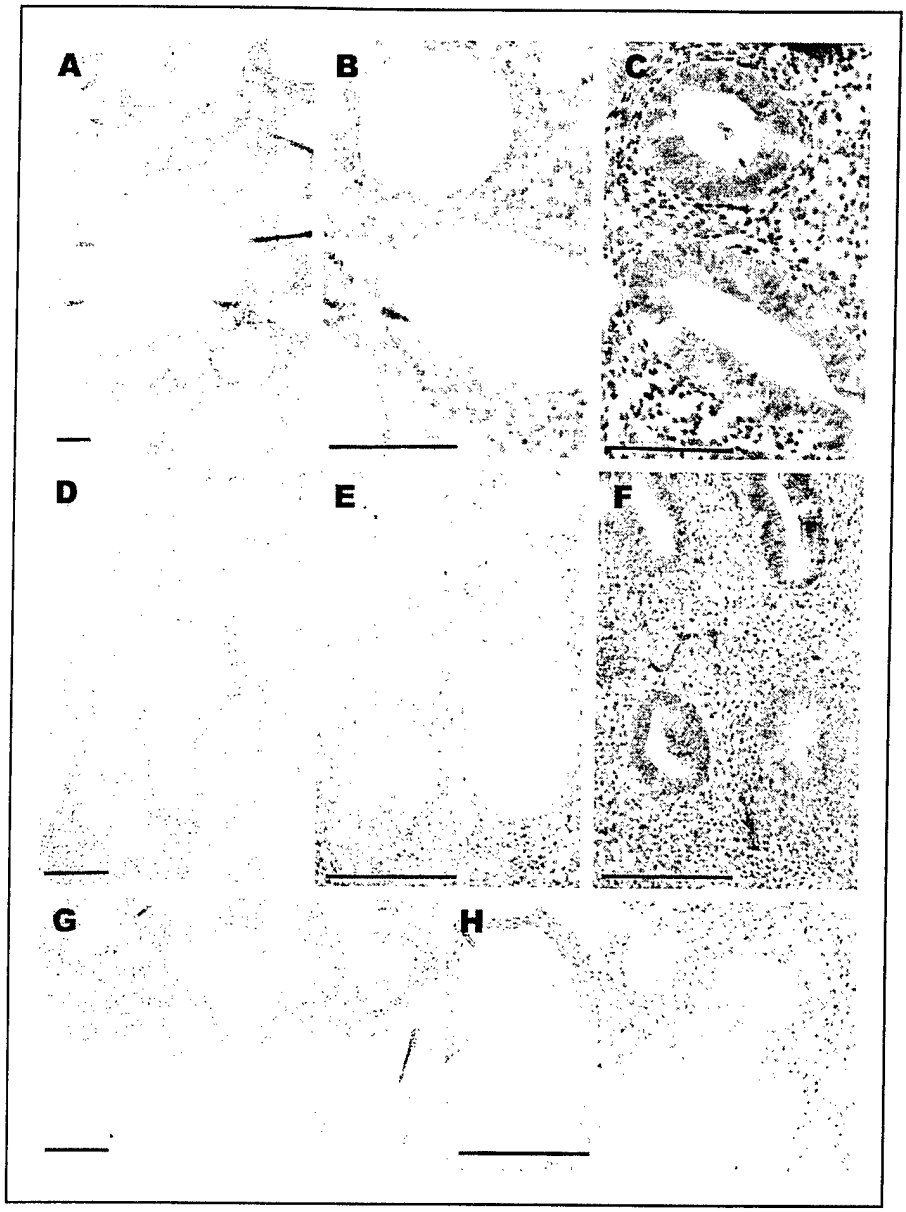

Fig. 3. PTEN protein heterogeneity in precancers and endometria with unopposed estrogen effect. Immunohistochemical staining (**brown**) of PTEN protein with antibody 6H2.1 of **A**) closely packed glands characteristic of precancers defined by computerized morphometry (isolated glands are PTEN negative); **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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Biallelic Inactivating Mutations and an Occult Germline Mutation of *PTEN* in Primary Cervical Carcinomas

Keisuke Kurose,[1] Xiao-Ping Zhou,[1] Tsutomu Araki,[2] and Charis Eng[1,3]*

[1]Clinical Cancer Genetics and Human Cancer Genetics Programs, Comprehensive Cancer Center and Division of Human Genetics, Department of Internal Medicine, Ohio State University, Columbus, Ohio
[2]Department of Obstetrics and Gynecology, Nippon Medical School, Tokyo, Japan
[3]Cancer Research Campaign Human Cancer Genetics Research Group, University of Cambridge, Cambridge, United Kingdom

A tumor suppressor gene on chromosome sub-band 10q23.3, *PTEN*, is frequently mutated or deleted in a variety of human cancers. Germline mutations in *PTEN*, that encodes a dual-specificity phosphatase, have been implicated in two hamartoma-tumor syndromes that exhibit some clinical overlap, Cowden syndrome and Bannayan-Zonana syndrome. Although cervical cancer is not a known component of these two syndromes, loss of heterozygosity (LOH) of markers on chromosome arm 10q is frequently observed in cervical cancers. To determine the potential role that *PTEN* mutation may play in cervical tumorigenesis, we screened 20 primary cervical cancers for LOH of polymorphic markers within and flanking the *PTEN* gene, and for intragenic mutations in the entire coding region and exon-intron boundaries of the *PTEN* gene. LOH was observed in 7 of 19 (36.8%) cases. Further, one sample may have homozygous deletion. Three (15%) intragenic mutations were found: two were somatic missense mutations in exon 5, that encodes the phosphatase motif, and an occult germline intronic sequence variant in intron 7, that we show to be associated with aberrant splicing. All three samples with the mutations also had LOH of the wild-type allele. These data indicate that disruption of *PTEN* by allelic loss or mutation may contribute to tumorigenesis in cervical cancers. In cervical cancer, unlike some other human primary carcinomas, e.g., those of the breast and thyroid, biallelic structural *PTEN* defects seem necessary for carcinogenesis. Further, one in 20 unselected cervical carcinomas was found to have a germline *PTEN* mutation; it is unclear whether the patient with this mutation had Cowden disease or a related syndrome.

INTRODUCTION

Cervical cancer is a common gynecologic malignancy that affects women worldwide, especially in developing countries. Yet, the molecular events underlying tumor development or progression of this type of tumor have not been well characterized. Like many other solid tumors, carcinomas of the cervix are now believed to develop through a multistep process involving activation of oncogenes and inactivation of tumor suppressors (Fearon and Vogelstein, 1990). Cervical cancer is strongly associated with human papillomavirus (HPV), particularly the high-risk HPV16 and HPV18 subtypes. Post-translational interactions between oncogenic HPV viral proteins E6 and E7 and tumor suppressor proteins p53 and pRB result in functional inactivation of these cell cycle regulatory proteins (Munger et al., 1992; von Knebel Doeberitz et al., 1994). Infection with HPV is an important initiating event in the multistep cervical pathogenic process (Alani and Munger, 1998); however, progression to cancer occurs in only a fraction of infected women. This observation suggests that additional somatic genetic changes are required for the completion of the malignant transformation process. Recent research focusing on genetic changes occurring during cervical cancer development has shown clonal abnormalities in many chromosomal regions. One region that has shown a high frequency of allelic loss is chromosome arm 10q: the reported incidence was 28%, although only two markers, on 10q21 and 10q26, were used (Mitra et al., 1994).

The tumor suppressor gene *PTEN/MMAC1/TEP1*, encoding a dual-specificity phosphatase, was isolated from chromosome sub-band 10q23.3 (Li and Sun, 1997; Li et al., 1997; Steck et al., 1997). Germline mutations of *PTEN* have been found in the dominantly inherited Cowden and Bannayan-Zonana syndromes, that are characterized by mul-

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*Correspondence to: Dr. Charis Eng, Human Cancer Genetics Program, Ohio State University, 420 West 12th Avenue, Room 690C MRF, Columbus, OH 43210. E-mail: eng-1@medctr.osu.edu.

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tiple hamartomas and by an increased risk of malignant and benign breast and thyroid tumors (Liaw et al., 1997; Marsh et al., 1997a; Eng, 1998; Marsh et al., 1998a, 1999). Because germline *PTEN* mutations have been found in both these syndromes, they are now referred to as the *PTEN* hamartoma tumor syndromes (PHTS) (Eng and Ji, 1998; Marsh et al., 1999). Further, the spectrum of PHTS has been extended to include a Proteus-like syndrome as well (Zhou et al., 2000).

Somatic mutation or deletion of *PTEN* occurs to a greater or lesser extent in diverse human cancers that show loss of heterozygosity (LOH) in this region, including glioblastoma, endometrial cancer, prostate cancer, and breast cancer (Kong et al., 1997; Li et al., 1997; Steck et al., 1997; Duerr et al., 1998; Feilotter et al., 1998). Genetic, functional and animal modeling studies have substantiated the tumor suppressor function of *PTEN*. *PTEN* is a lipid phosphatase whose major substrate is phosphatidylinositol 3,4,5-triphosphate (PtdIns(3,4,5)P_3), downstream of which lies the Akt (PKB) pathway (Furnari et al., 1998; Li et al., 1998; Machama and Dixon, 1998; Stambolic et al., 1998; Dahia et al., 1999). The serine-threonine kinase Akt, when phosphorylated, protects cells from apoptosis (Dudek et al., 1997). Ectopic expression of *PTEN* results in cell cycle arrest at G1 and/or apoptosis, at least in glioma and breast cancer cell line models (Furnari et al., 1998; Li and Sun, 1998; Weng et al., 1999). Given the prominent role of proteins that mediate apoptosis and cell cycle arrest and 10q LOH in cervical carcinomas, and *PTEN*'s role in the cell cycle and cell death as well as the gene's localization to 10q, *PTEN* is a candidate to play a promoting role in cervical carcinogenesis. Therefore, we examined this gene for allelic loss and intragenic mutation in 20 primary cervical carcinomas.

MATERIALS AND METHODS

Tumor Samples and DNA Extraction

Tumor and corresponding noncancerous tissues were obtained from 20 patients with sporadic cervical carcinomas, who underwent surgery at Nippon Medical School Hospital, Tokyo, Japan. The specimens were immediately frozen and stored at −70°C until nucleic acid extraction. None of these patients had undergone previous radiotherapy or chemotherapy. The tumors consisted of squamous cell carcinoma (n = 13), adenocarcinoma (n = 5), and adenosquamous carcinoma (n = 2) of the cervical cancers. Genomic DNA was extracted with a QIAamp DNA Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions.

LOH Analysis

Five polymorphic markers flanking and within the *PTEN* gene were used for LOH analysis. The markers are ordered from centromere to telomere: D10S579-AFMa086wg9-IVS4+109ins'TCTTA-IVS8+32T/G-D10S541. AFMa086wg9 (*PTEN* intron 2), IVS4+109ins'TCTTA, and IVS8+32T/G lie within *PTEN* (Dahia et al., 1997; Carroll et al., 1999; Marsh et al., 1999). The IVS4+109ins'TCTTA and IVS8+32T/G polymorphisms were screened for by differential digestion with *Aff*II and *Hin*cII, respectively, according to the manufacturer's guidelines (New England Biolabs, Beverly, MA) as described previously (Dahia et al., 1997; Marsh et al., 1998a). PCR conditions for these markers are described elsewhere (Marsh et al., 1997b, 1998b).

PTEN Mutation Analysis

All samples were scanned for mutations by denaturing gradient gel electrophoresis (DGGE). The entire *PTEN* coding region, exon-intron boundaries, and flanking intronic sequences were PCR-amplified and fractionated through denaturing gradient gels according to the conditions described previously (Marsh et al., 1997b). Samples showing DGGE variation were reamplified with another set of primers, specifically for sequence analysis, gel- and column-purified, and subjected to semi-automated sequence analysis as previously published (Dahia et al., 1997).

RNA Extraction and RT-PCR

From approximately 100 mg of cervical cancer tissue, total RNA was extracted by the guanidine thiocyanate method (Chomczynski and Sacchi, 1987), using TRIZOL reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer's guidelines. Total RNA was treated with DNase I (Boehringer Mannheim, Germany) to remove any contaminating genomic DNA before reverse transcription. This RNA was reverse-transcribed with a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Boehringer Mannheim) according to the manufacturer's instructions. RT-PCR using the *PTEN* exonic primers (5.2F, 5′-TCATGTTG-CAGCAATTCAA-3′ (*PTEN* exon 5), and 8/9R, 5′-TGAAGTACAGCTTCACCTTAAA-3′ (*PTEN* exon 8 and 9)) was performed to determine whether splicing abnormalities would result from intronic mutation detected in the sequencing analysis.

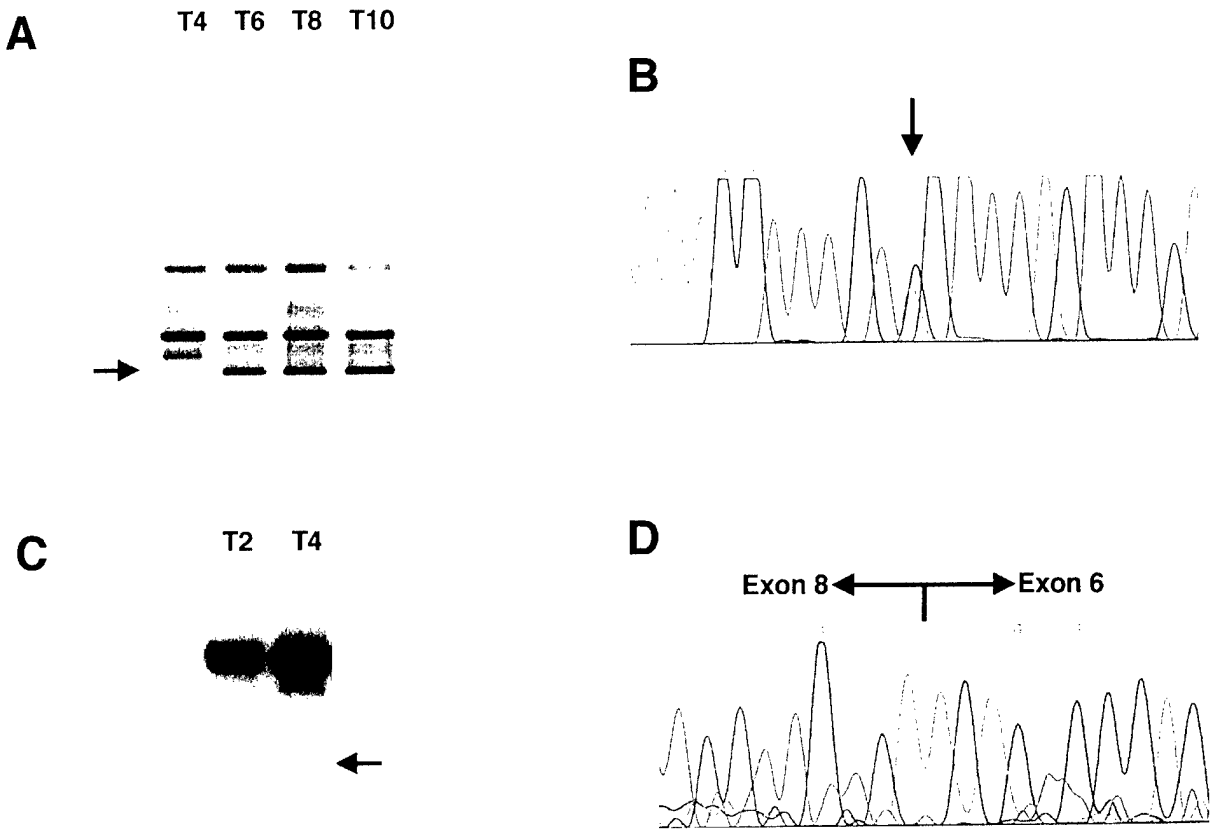

Figure 1. *PTEN* mutation analysis of DNA extracted from Tumor 4, a squamous cell cervical carcinoma. (**A**) Multiplex DGGE analysis of Tumors 4, 6, 8, and 10 revealed a DGGE variant of exon 7 in Tumor 4 (arrow). (**B**) Sequence analysis of genomic DNA from Tumor 4 demonstrated an intronic sequence variant in intron 7 (IVS7+7A>G). (**C**) RT-PCR was performed with exonic sequence primers (see Materials and Methods). cDNA from Tumor 2 generates only the expected-size PCR product. In addition to a fragment of the expected size, Tumor 4 shows an abnormally sized PCR product (arrow; see D below and Results). Another fragment that was slightly shorter than the predicted size was also detected, that is suggested to be non-pathogenic (see Results). (**D**) Sequence analysis of the PCR amplicon representing the aberrant band on RT-PCR for Tumor 4 revealed a frameshifted and truncated product of 194 amino acids that resulted from splicing out of exon 7, the 3′ end of exon 6, and the 5′ end of exon 8 (see text).

TABLE 1. Results of *PTEN* Mutation Analysism

Tumor	LOH	Exon/intron	Mutation	Codon altered	Abnormal splicing
12	+	Exon 5	c.276 C>A	D92E	
32	+	Exon 5	c.476G>C	R159T	
4	+	IVS7	IVS7+7A>G		+

RESULTS

To determine whether *PTEN* is genetically altered during cervical carcinogenesis, we screened 20 primary cervical cancers for mutations in the *PTEN* gene by DGGE analysis. Samples that showed variant bands on DGGE analysis were used as a template for direct sequence analysis. Of the 20 samples, DGGE analysis revealed abnormalities in 3 samples (15%), and sequence analysis of these samples confirmed the presence of mutations. All of the tumors that showed mutations in the *PTEN* gene were squamous cell carcinomas. The identified mutations included two missense mutations and one splice variant (Table 1, Fig. 1). The two missense mutations in Tumors 12 and 32 were somatic, and the corresponding germline was wild-type. Tumor 4 and the corresponding germline showed the same intronic variant. Tumor 12 showed a C-to-A transition at the third nucleotide of codon 92 (exon 5), that would result in a substitution of Glu for Asp (D92E). Tumor 32 exhibited a G-to-C transition at the second nucleotide of codon 159, that would result in the substitution of Thr for Arg (R159T). Germline and somatic DNA

Sample no.	Histology	Stage	*PTEN* mutation	D10S579	AFMa086wg9	IVS4+109ins TCTTA	IVS8+32T/G	D10S541
2	SCC	II b	-	●	-	-	-	●
4	SCC	I b1	+	○	-	●	○	○
6	SCC	I b1	-	○	-	○	-	-
8	SCC	II b	-	○	-	○	○	○
10	ASC	II b	-	○	-	○	●	○
12	SCC	III b	+	-	-	●	○	-
14	SCC	II a	-	-	○	○	○	●
16	AC	I b1	-	-	-	-	-	-
18	SCC	II a	-	○	○	○	○	○
20	SCC	II b	-	○	○	-	-	-
22	SCC	II a	-	-	○	○	○	-
24	SCC	II b	-	-	-	○	○	-
26	AC	II b	-	○	-	○	○	-
28	SCC	I b	-	○	-	-	-	○
30	ASC	I b	-	○	-	○	○	○
32	SCC	I b	+	NA	-	●	●	-
34	AC	II b	-	-	-	●	-	-
36	AC	II a	-	○	-	○	○	-
38	AC	I b	-	○	○	-	-	-
40	SCC	IV c	-	○	-	-	-	○

Figure 2. Results of LOH analysis in cervical cancer. White circles, retention of heterozygosity; black circles, LOH; −, non-informative; NA, not available; SCC, squamous cell carcinoma; ASC, adenosquamous carcinoma; AC, adenocarcinoma.

from Tumor 4 showed an A-to-G transition in intron 7, 7 bases from the exon-intron boundary (IVS7+7A>G; Fig. 1A and B).

RT-PCR was performed to determine whether this intronic variant affected splicing. In addition to a fragment of the expected size, a fragment that was 352 bp shorter than the predicted size was noted (Fig. 1C). Sequence analysis of this aberrant fragment revealed that the last 54 bp within exon 6 and the first 131 bp within exon 8 were spliced out along with exon 7 (167 bp), and the remaining upstream bases of exon 6 were joined to the remaining downstream bases of exon 8, resulting in a frameshift and a truncated product of 194 amino acids (Fig. 1D). Another fragment that was slightly shorter than the predicted size was also detected. Sequence analysis revealed that it lacked exon 6. This fragment was found frequently in a fraction of other cancerous and noncancerous tissues, and probably does not represent a pathogenic transcript (data not shown).

Paired samples of germline and tumor-specific genomic DNA from all 20 cases of cervical carcinoma were analyzed for LOH at five polymorphic markers flanking and within *PTEN*. Among the 20 cervical cancers examined, one was excluded from final analysis because it showed homozygosity in all markers. Of the 19 informative samples, 7 (36.8%; Fig. 2) had LOH at one or more loci. Interestingly, LOH at 10q23 was found in all three tumors with intragenic *PTEN* mutations (Table 1 and Fig. 2).

DISCUSSION

Using 20 sporadic cervical carcinomas originating from Japan, we found that 15%, all of squamous

histology, harbored somatic intragenic *PTEN* mutations, accompanied by loss of the remaining wild-type allele. An additional 3 tumors had hemizygous LOH at *PTEN* and one might have had a homozygous deletion of *PTEN*, resulting in loss of transcript. Sample 14 (Fig. 2) had LOH at D10S541, just 3′ of *PTEN*, and a noninformative marker (D10S579) immediately 5′ of *PTEN*, both of which flank three markers all showing retention of heterozygosity. It has been demonstrated, when closely flanking markers suggest LOH but markers "within" show retention, that this is a manifestation of homozygous deletion and the observation is explained by small amounts of contaminating normal tissue (Cairns et al., 1995). It is, therefore, possible that sample 14 harbors a biallelic deletion of *PTEN*. RT-PCR using Tumor 14 somatic DNA as template and *PTEN* primers resulted in no product, whereas primers for a housekeeping gene, *GUSB*, yielded a product of the expected size (data not shown). The missense mutations within exon 5 almost certainly disrupt PTEN phosphatase function, because normal function requires an intact catalytic domain as well as the flanking alpha-helices, all of which are encoded by exon 5. These two mutations are within the Cowden syndrome mutation cluster in exon 5 (Marsh et al., 1998a). The third mutation, within intron 7 close to the exon-intron boundary, has been shown to cause aberrant splicing and almost certainly also results in a transcript with decreased stability (see Fig. 1C). This type of truncated protein causes loss of the C-terminal C2 domain, that is important for phospholipid membrane binding (Georgescu et al., 1999). This mutation was also identified in the corresponding germline of a 46-year-old woman who had atypical genital bleeding. There was inadequate clinical history recorded for this patient. Therefore it does not note any family history or history to date that suggests the possibility of Cowden syndrome or Bannayan-Zonana syndrome. Cowden syndrome, however, is under-recognized. Thus, it is vital that this patient be followed closely for development of Cowden-related cancers, especially those of the breast, thyroid, and endometrium, because the presence of a germline *PTEN* mutation is a sensitive molecular diagnostic sign for Cowden syndrome (Eng and Ji, 1998; Kurose et al., 1999).

Almost half of all cervical carcinomas have a structural abnormality of *PTEN*; 20% were found to have structural biallelic inactivation of *PTEN*, and an additional 15% had hemizygous *PTEN* deletion. The first few studies on *PTEN* mutations in various sporadic cancers centered around cell lines (Li et al., 1997; Steck et al., 1997). These studies suggested a relatively high frequency of biallelic *PTEN* structural inactivation, especially in glioblastoma multiforme and breast carcinomas. Further studies examining primary human carcinomas have demonstrated that in some tumor types, e.g., glioblastoma multiforme and endometrial carcinomas, structural biallelic inactivation is common (Kong et al., 1997; Duerr et al., 1998; Zhou et al., 1999); in other tumor types, most notably primary breast and prostate carcinomas, only hemizygous deletions have been found with any frequency, whereas intragenic *PTEN* mutations are exceedingly rare (Feilotter et al., 1998, 1999; Singh et al., 1998). Hemizygous *pten* knock-out mouse models have demonstrated that haploinsufficiency is sufficient for tumorigenesis (Di Cristofano et al., 1998, 1999; Stambolic et al., 1998; Podsypanina et al., 1999). Our study of cervical carcinomas suggests that this cancer type, at least of Japanese origin, might be the same category of glioblastoma multiforme and endometrial carcinomas in that up to a quarter of such tumors harbor biallelic structural *PTEN* defects.

Two other groups have examined *PTEN* for structural defects in cervical carcinomas. Tashiro and colleagues found no mutations among 10 squamous cell cervical carcinomas (Tashiro et al., 1997). In a recent study, Su et al. (2000) found one of 50 squamous cell cervical cancers with somatic *PTEN* mutation, and no LOH was detected in any tumor. This is in contrast to the present study, and several explanations are possible. Tashiro et al. (1997) examined the American population, and Su et al. (2000) used Taiwanese Chinese as subjects. Our study focused on cervical carcinomas originating from Japanese women. Although HPV infection is well established as a significant initiating event, it is possible that subsequent somatic genetic pathogenic events differ in different populations. *PTEN* mutation and deletion might be prominent events in the initiation or progression of cervical cancers in the Japanese, whereas other genetic events not directly involving *PTEN* might come into play in other populations, e.g., other molecules upstream or downstream of *PTEN*, *RB1* or *TP53*. Arguments about lack of power in the other two studies can be discarded because the Taiwanese study used 50 tumors of squamous histology. The Tashiro study used only 10 tumors, but our study examined 13 squamous cell carcinomas and found that 3 carried intragenic *PTEN* mutations. Finally, it is likely that technical reasons could account for the different

results among the three studies. If there is a high proportion of normal tissue contamination in the carcinoma samples in the prior two studies, then false negatives might be the result.

In summary, our observations strongly suggest that *PTEN* is involved in the pathogenesis of cervical carcinomas, at least in the Japanese population. It might be interesting to examine events involving the *PTEN*, *RB*, and *TP53* pathways in cervical carcinogenesis in different populations around the world. Our observation of an occult germline *PTEN* mutation in a series of unselected cervical carcinoma cases could hint that this tumor might be a rare component of PHTS, and thus require further formal investigation.

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to the integrin receptor family in MM (Giuffrida et al., 1999). Whether this may have any effect on SV40 binding and internalization in certain types of MM cells with respect to others needs to be further investigated. A confirmation of the role of SV40 as a prognostic cofactor in MM could help correctly enroll patients who can benefit from desperately needed new clinical trials. Our data would support new therapeutic approaches, such as gene therapy employing antisense transcripts that would revert SV40-Tag carcinogenic effects (Waheed et al., 1999), ameliorating disease progression in SV40-positive MM patients.

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Advances in Brief

Molecular Identification of Latent Precancers in Histologically Normal Endometrium[1]

George L. Mutter,[2] Tan A. Ince, Jan P. A. Baak, Gregory A. Kust, Xiao-Ping Zhou, and Charis Eng

Department of Pathology, Brigham and Women's Hospital, Boston, Massachusetts 02115 [G. L. M., T. A. I., J. P. A. B., G. A. K.]; Department of Pathology, Medisch Centrum Alkmaar, Alkmaar, 1815JD the Netherlands [J. P. A. B.]; Department of Pathology, Free University, Amsterdam, 1081 HV the Netherlands [J. P. A. B.]; Clinical Cancer Genetics and Human Cancer Genetics Programs, Ohio State University Comprehensive Cancer Center, Columbus, Ohio 43210 [X-P. Z., C. E.]; and the Cancer Research Campaign Human Cancer Genetics Research Group, University of Cambridge, Cambridge, CB2 2QQ United Kingdom [C. E.]

Abstract

Discovery of somatically mutated cells in human tissues has been less frequent than would be predicted by *in vitro* mutational rates. We analyzed the *PTEN* tumor suppressor gene as an early marker for endometrial carcinogenesis, and we show that 43% of histologically normal premenopausal endometria contain rare glands that fail to express PTEN protein because of mutation and/or deletion. These persist between menstrual cycles. Histopathology of PTEN-null glands is initially unremarkable, but with progression, they form distinctive high-density clusters. These data are consistent with a progression model in which initial mutation is not rate limiting.

Introduction

The endometrium is a highly proliferative and cyclically regenerative tissue in which loss of *PTEN* tumor suppressor gene function heralds the beginning of multistep carcinogenesis. Loss of *PTEN* function occurs in approximately 50% (1) of all endometrial carcinomas, increasing to 83% (2) for tumors with adjacent premalignant lesions. An association between *PTEN* mutation and endometrial cancer risk is further supported by animal studies. Heterozygote *pten* mutant mice uniformly (100%) develop endometrial "hyperplasia," and 21% of these progress to carcinoma (3). EIN[3] lesions of humans, which are monoclonal (4) and frequently *PTEN* mutation-bearing (2), are thought to be precursors of endometrioid carcinoma but cannot be diagnosed in routine histological sections until localized clusters of neoplastic glands have reached a critical dimension (generally over 1 mm) characterized by a crowded architecture and altered cytology (5). *PTEN* mutation appears to be coincident with initiation of these clones, in the manner of a gatekeeper for endometrial carcinogenesis (6).

We previously showed acquisition of PTEN-null endometrial glands in 29% of a very small series of endometria exposed to unopposed estrogens, a known epidemiological risk factor for endometrial adenocarcinoma (2). It is unlikely that these glands acquired mutations within the narrow interval of unopposed estrogen exposure typical of a single abnormal menstrual cycle. More likely, PTEN-null clones are generated over a lifetime of menstrual cycles and retained for a long interval. This was posed as a testable hypothesis: sporadic somatic endometrial mutation of the *PTEN* gene is frequent in the histologically unremarkable endometrium of women of reproductive age, and, once generated, PTEN-null clones are retained or regenerated between menstrual cycles.

From our previous work, we know that immunohistochemically PTEN-null glands are likely to harbor PTEN mutations and/or deletions (2). Therefore, we used highly sensitive PTEN immunohistochemistry to screen histologically normal proliferative endometrium of endogenously cycling (not on replacement hormones) young premenopausal women (<40 years) for PTEN-null glands, and then analyzed these glands for *PTEN* mutation and deletion. Continued presence of PTEN-null glands was evaluated in another series of patients who had paired proliferative endometrial samples separated by at least one menstrual cycle. The prevalence and morphology of PTEN-null glands in persistent proliferative (endometria exposed to a protracted nonphysiological estrogen interval) and EIN endometria, was also determined by immunohistochemistry. EIN (7) is used to describe readily diagnosable lesions (5) that have been shown by computerized morphometric analysis (4) to have diagnostic features that increase risk for concurrent (8) or future (9) endometrial adenocarcinoma.

Materials and Methods

Case Selection. One hundred thirty-eight paraffin-embedded endometrial biopsies and curettings obtained in 1998–2000 (Department of Pathology, Brigham and Women's Hospital) were allocated to proliferative, persistent proliferative, or EIN diagnostic classes based on slide review consensus of two gynecological pathologists (G. L. M. and T. A. I.). "Normal" proliferative endometria all came from premenopausal women <40 years of age (average age, 34.0 ± 4.5) who were not taking supplemental hormones. Persistent proliferative endometria (mean age, 45.2 ± 9.3) had mitotically active but cytologically uniform glands with occasional cystically dilated glands, and were ascribed either to endogenous (anovulation) or exogenous (pharmacological) estrogen sources based on clinical history. Endometrial polyps disqualified a case from the proliferative and persistent proliferative categories. EIN diagnosis (mean age 54.1 ± 7.8) was made visually, according to published criteria (5). We have not used the WHO endometrial hyperplasia classification system in our studies because of its poor reproducibility, and discordance with discrete biological groups defined by genetic analysis (4).

Ninety repeat biopsies were retrieved by diagnostic review from 45 individual women with proliferative endometrium on more than one occasion. Most repeat biopsies were symptomatically indicated (usually bleeding), but some were incidental to unrelated findings such as uterine fibroids or polyps.

Immunohistochemistry. Dewaxed rehydrated 4-μm paraffin sections underwent microwave antigen retrieval before adding primary anti-PTEN antibody 6H2.1 (Cascade Biosciences, Winchester, MA) at 1:300 dilution. Anti-estrogen receptor antibody ER-ID5 (Dako), and anti-progesterone receptor antibody 1A6 (Dako) were used at 1:300 and 1:100 dilutions, respectively. Primary antibody was incubated overnight at 4°C, washed, incubated with appropriate secondary biotinylated immunoglobulin (Vectastain ABC kit; Vector Laboratories, Inc., Burlingame, CA) and signal detected by sequential addition of avidin peroxidase and 3,3′-diaminobenzidine. Epithelial staining was scored by two pathologists (G. L. M., T. A. I.) using endometrial stroma and/or normal endometrial epithelium as an internal positive control and negative run controls without addition of primary antibody. All of the tissue fragments were

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[2] To whom requests for reprints should be addressed, at Department of Pathology, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115. Phone: (617) 732-6096; FAX: (617) 738-6996; E-mail: gmutter@rics.bwh.harvard.edu.

[3] The abbreviations used are: EIN, endometrial intraepithelial neoplasia; DGGE, denaturing gradient gel electrophoresis.

Table 1 *Clinicopathological features of endometria by PTEN immunohistochemistry and slide diagnosis*

	% PTEN-null by diagnosis (fraction)			
	Proliferative	Persistent proliferative	EIN	Total
Sample				
Curettage	42.9 (6/14)	54.5 (6/11)	75.0 (6/8)	54.5 (18/33)
Biopsy	42.9 (18/42)	56.7 (17/30)	59.3 (16/27)	51.5 (51/99)
Clinical indication				
Bleeding	42.9 (12/28)	56.3 (18/32)	55.0 (11/20)	51.3 (41/80)
Infertility/recurrent abortion	41.7 (5/12)	0.0 (0/0)	100.0 (1/1)	46.2 (6/13)
Prior hyperplasia	0.0 (0/1)	0.0 (0/0)	71.4 (5/7)	75.0 (6/8)
Anatomic (fibroids, septum, polyp, thick stripe)	50.0 (3/6)	100.0 (1/1)	100.0 (1/1)	62.5 (5/8)
Other (pain, endometriosis)	20 (1/5)	33.3 (1/3)	0.0 (0/2)	20.0 (2/10)
Unspecified	75.0 (3/4)	60.0 (3/5)	100 (4/4)	76.9 (10/13)
Hormones				
Endogenous	42.9 (24/56)	55.9 (19/34)	64.3 (18/28)	51.7 (61/118)
Exogenous	0.0 (0/0)	57.1 (4/7)	57.1 (4/7)	57.1 (8/14)
Menopause				
Pre	42.9 (24/56)	57.1 (20/35)	66.7 (14/21)	51.8 (58/112)
Post	0.00 (0/0)	50.0 (3/6)	57.1 (8/14)	55.0 (11/20)
Total	42.9 (24/56)	56.1 (23/41)	62.9 (22/35)	52.3 (69/132)

examined, and individual glands were scored as PTEN null when signal was absent in the nuclear and cytoplasmic compartments of most cells in that gland. Hormone receptors were scored by signal intensity in the nuclear compartment.

Genomic Analysis. Matched PTEN expressing and nonexpressing proliferating endometrial epithelial cells were sampled using laser capture microdissection directed by PTEN immunohistochemistry of flanking serial sections. Approximately 10–50 ng of DNA per sample was PCR amplified using primers which define the coding region and flanking introns of all 9 *PTEN* gene exons. PCR products were subjected to DGGE, which in our hands is virtually 100% sensitive and specific in detecting sequence-confirmed PTEN mutations (10). DNA samples that show DGGE variants are resubjected to PCR and semi-automated direct sequencing (ABI377a or PE3600).

For each patient, DNA from PTEN expressing and nonexpressing epithelial cells was subjected to PTEN deletional analysis by PCR using 5′-tagged fluorophor primers, which amplify microsatellites flanking and within the *PTEN* gene, D10S579, D10S2491, and D10S541, and then electrophoresed through an ABI377a gel and analyzed with GeneScan software (11). Marker heterozygosity manifests as two peaks on a GeneScan gel, representing two different alleles present at that marker. Matched sets of DNA samples from PTEN expressing and nonexpressing glands are compared at each marker, and if one peak is reduced by at least one-third, loss of heterozygosity has occurred, which represents deletion of one of the alleles and, usually, that chromosomal region.

Morphometry. A 1-mm circular window (surface area, 0.785 mm^2) containing 100 randomly distributed points was superimposed on digitized photomicrographs of PTEN immunohistochemically stained endometria, and points over the fragment of interest (*PTS100*) tallied by composition of underlying tissue [stroma (*STROMA100*); PTEN-expressing or "positive" glands (*POS100*); PTEN-null glands (*NULL100*). Excluded were seven fragmented or small (<1/2 sample window) samples; four cases diagnosed on H&E slides as EIN, in which the targeted PTEN-null glands did not involve the EIN focus; and one PTEN-null EIN focus, which was distorted by tangential sectioning on recut. Surface area assigned to glands included combined epithelial and luminal compartments. Geometric centroids of each gland profile were marked, and the number of PTEN-null gland centroids (*NULLCT*) and PTEN-expressing gland centroids (*POSCT*) that were within the window were counted. Variables were calculated as follows: (*a*) volume percentage stroma (*VPS*) = 100 × (*STROMA100/PTS100*); (b) volume percentage PTEN-null gland (*VPNULL*) = 100 × (*NULL100/PTS100*); (*c*) volume percentage PTEN-positive gland (*VPPOS*) = 100 × (*POS100/PTS100*); (*d*) density of PTEN-null glands (*DENNULL*) = (*NULLCT/PTS100*) × (100 points in window/0.785 mm^2 window size); (*e*) density of PTEN-positive glands (*DENPOS*) = (*POSCT/PTS100*) × (100 points in window/0.785 mm^2 window size); (*f*) size of PTEN-null glands (*SZNULL*) = (*NULL100/NULLCT*) × (0.785 mm^2)/100 points in window); and (*g*) size of PTEN-expressing glands (*SZPOS*) = (*POS100/POSCT*) × (0.785 mm^2)/100 points in window).

Results

PTEN-null endometrial rates were 43, 56, and 63% in proliferative, persistent proliferative, and EIN diagnostic categories, respectively (Table 1; Fig. 1; all of the histological images are available online).[4] There was a linear trend by decade of age for increasing PTEN-null rates in older women (Coachman's test of linear trend, $P = 0.014$). Average age of women with and without PTEN-null glands was 43.8 ± 9.7 and 40.2 ± 11.6 years, respectively. PTEN-null glands in the three diagnostic groups are present in women biopsied for a variety of reasons; therefore, these results are applicable to a broad range of women seeking routine medical care (Table 1).

The occurrence of PTEN-null glands in 43% (24 of 56) of histologically normal proliferative endometrium (confirmed by staining two sections in each case) was unexpectedly high. In general, only a few histologically unaltered glands were PTEN-null among hundreds of proliferating glands in these otherwise unremarkable endometria. Because PTEN expression responds to the hormonal environment (12), estrogen and progesterone receptor immunohistochemistry were performed on flanking serial tissue sections and showed in all cases that the PTEN-null and -expressing glands in proliferative endometria retained comparable receptor quantities. Nineteen of 24 proliferative endometria with PTEN-null glands had sufficient material for microdissection. Matched DNA from PTEN-expressing and -nonexpressing glands from the same patient were coprocessed for direct comparison of PTEN mutation and deletion.[5] All of the PTEN-expressing matched control glands had a wild-type (normal) genotype, whereas 84% (16 of 19) of nonexpressing glands had a mutation ($n = 8$) and/or loss of at least one 10q23 heterozygous marker ($n = 13$) in the region of the *PTEN* locus.

The appearance of rare histologically normal glands harboring *PTEN* mutations would be inconsequential if they are completely shed with normal menstruation. We, thus, performed PTEN immunohistochemistry on 34 premenopausal women (no hormonal therapy; average age, 42.3 ± 6.2 years) with unremarkable proliferative endometrium on two separate occasions (interval averaged 400 days; range, 26–1167 days). Twelve of 34 women had PTEN-null glands initially (Table 2), scattered throughout varying depths of the endometrial thickness, and 83% (10 of 12) of these continued to be present on follow-up. PTEN status of paired biopsies in Table 2 is highly associated with initial phenotype (Fisher's exact test, $P = 0.01$; odds ratio, 10.71). A woman with PTEN-null glands in her endometrium is five times more likely to have PTEN-null glands on repeat biopsy than not. A separate series of 11 postmenopausal women (mean age, 58.1 ± 2.6 years) with two proliferative endometria separated by an

[4] Internet address: www.endometrium.org.

[5] Detailed genotype table, and additional supplemental data, are available at Cancer Research Online [http://cancerres.aacrjournals.org].

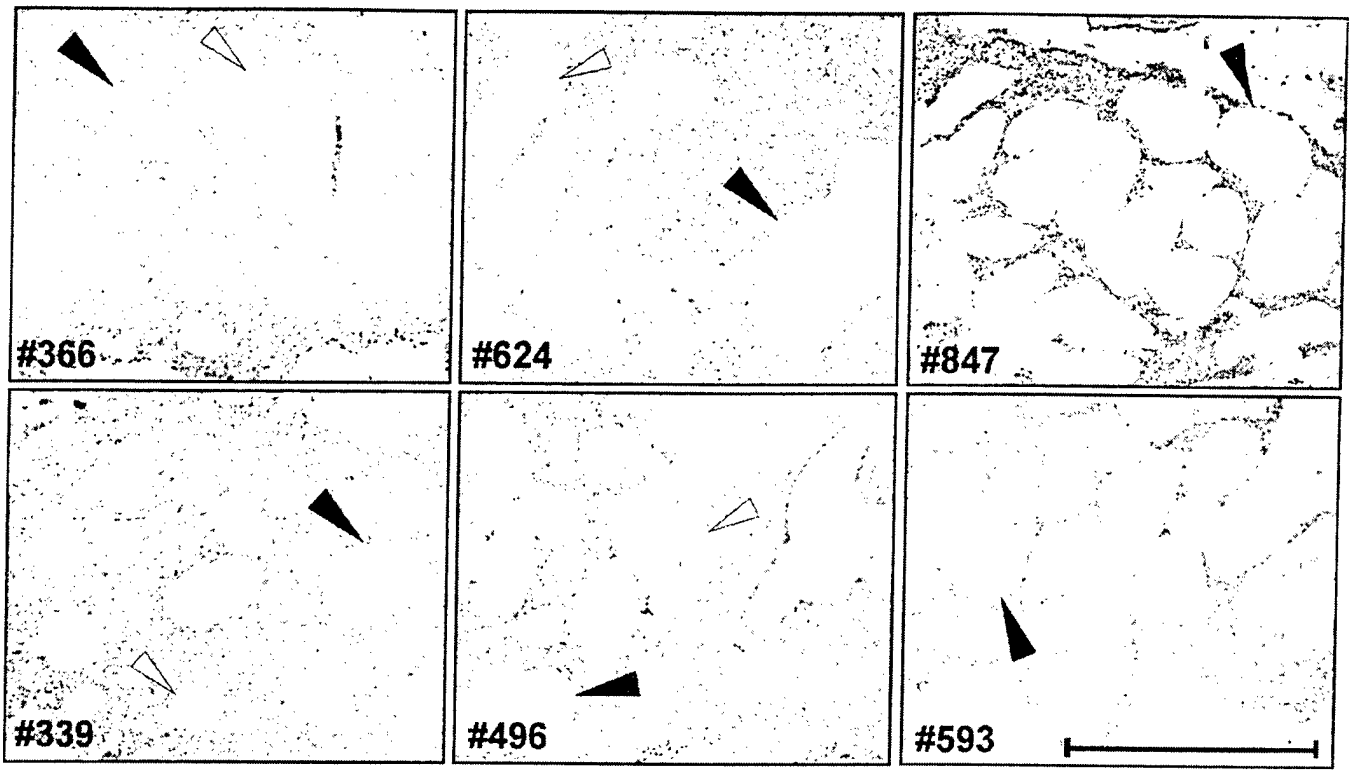

Fig. 1. PTEN immunohistochemistry in endometrium. PTEN-null glands (*black arrowheads*) appear pale green against dark brown staining of surrounding stroma in proliferative (*left, cases 339, 366*), persistent proliferative (*center, cases 624, 496*), and EIN (*right, cases 847, 593*) endometria from six different patients. Brown PTEN-positive glands (*white arrowhead*) are present in proliferative and persistent proliferative examples for comparison. *Scale bar*, 500 μm.

average of 494 days (range, 142–985 days) and sampled during the estrogenic phase of sequential estrogen/progestin replacement therapy were used for PTEN immunohistochemistry. Two had PTEN-null glands initially, retained by both on follow-up. 11% (1 of 9) with PTEN-expressing first biopsies developed PTEN-null glands in the second biopsy. These 11 patients are not shown in Table 2. Combined statistical analysis of all repeat biopsies studied (34 premenopausal, 11 post-menopausal) also shows a high association of PTEN status between first and second biopsy (Fisher's exact test, $P < 0.001$; odds ratio, 17.3).

Changes in the histological structure of PTEN-null clones were documented by morphometric analysis of PTEN-immunostained normal proliferative, persistent proliferative, and EIN endometria (Fig. 2).

Discussion

Insights into the earliest stages of human carcinogenesis are limited by our ability to identify precursor lesions *in vivo*. Estimates of the rate of sporadic mutagenesis in human cells, on the order of 10^{-7} mutations per gene per cell division (13), suggest that the number of cells with "first hits" of a multistep carcinogenesis (14) pathway may number in the hundreds for every gram (10^9 cells/gram) of proliferative tissue. Although it has been possible *in vitro* to directly observe such events using sensitive model systems, complex primary tissues present many confounding factors including sampling errors, insensitive methods of ascertainment, and dynamic fluxuations in the selection of altered clones.

Table 2 *PTEN status in repeat biopsies of premenopausal women with endogenously cycling proliferative endometrium*[a]

	Second sample PTEN-positive	Second sample PTEN-null	Total
First sample PTEN-positive	15	7	22
First sample PTEN-null	2	10	12
Total	17	17	34

[a] Initial (first sample) and repeat (second sample) endometrial samples scored as PTEN-nonexpressing (null) or having only PTEN-expressing glands (positive).

Initiation of *PTEN*-mutant clones is common in endometria of premenopausal women and, once acquired, is stably maintained by a cell population that is incompletely shed during menses. These findings were possible because of the unique suitability of endometrium for discovery and surveillance of acquired somatic mutations. Easily sampled superficial endometrium is regenerated cyclically from a deeper basalis layer; therefore, the resultant biopsy genotype reflects that of the functional stem cells in this tissue and can be nondestructively monitored on multiple occasions. Loss of PTEN protein in proliferative glands cannot be attributed to local estrogen unresponsiveness, because they retain estrogen and progesterone receptors at normal levels. Rather, mutation and/or deletion of the *PTEN* gene in 84% of PTEN-null glands microdissected from histologically normal proliferative endometrium confirms that this is a primary event capable of being perpetuated in the mutant clones. In fact, PTEN-null cells that we observed had undergone some clonal expansion, forming entire glands devoid of PTEN protein that contrast clearly with surrounding stroma and unaffected glands. Mutations in these "normal-appearing" tissues are found only after immuno-directed microdissection. In bulk tissue, they would be easily overlooked because of dilution, and this may be the reason for underestimation of mutations in normal-appearing tissue in earlier studies (2). Our data show that mutational events that are rare for a single cell may achieve a high prevalence if the tissue contains a large number of dividing cells (the average woman grows about 1 kg of endometrial tissue in her reproductive years) with strong positive selection and/or retention factors.

PTEN immunohistochemistry is able to bring out what is perhaps the earliest stage of nonfamilial carcinogenesis yet identified in any human tissue, before histological change is manifest and in patients

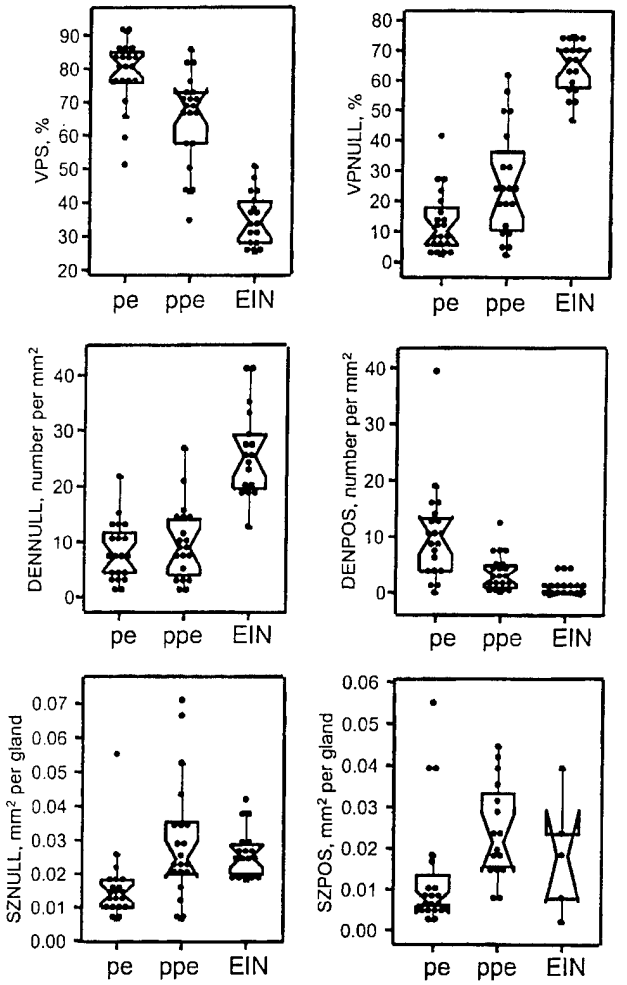

Fig. 2. Morphometric progression of PTEN-null glands. Box-plot of PTEN-null gland morphometric variables (*Y* axis), *X* axis arranged from left to right in order if a putative progression sequence of proliferative (*PE*, $n = 20$), persistent proliferative (*PPE*, $n = 20$), and EIN ($n = 17$) endometria stained for PTEN protein. Volume percentage stroma (*VPS*) declines steadily because of increasing PTEN-null gland volume (*VPNULL*). Two elements sequentially contribute to rising *VPNULL*. The first, in the proliferative-to-persistent-proliferative transition, is enlargement of individual PTEN-null glands (*SZNULL*). The density of PTEN-null glands (*DENNULL*) then increases between persistent proliferative and EIN endometria. PTEN-expressing glands increase in size (*SZPOS*) in the proliferative-to-persistent-proliferative transition but rapidly decline in density.

without concurrent carcinoma. Almost all of the well-known examples of mutation-bearing premalignant disease, including EIN, colonic polyps, and *in situ* carcinomas, have morphological and cytological changes recognizable by light microscopy, which indicates a significant change in phenotype. Precedents in the colon (15) and breast (16) in which histologically normal epithelium may bear genetic changes as seen in adjacent concurrent adenocarcinomas have not yet been demonstrated in patients without cancer. The fact that >80% of PTEN-null glands in proliferative endometrium have *PTEN* mutations seen in endometrial cancer suggests that it is this subpopulation of glands that preferentially will give rise to EIN.

The fate of PTEN-null endometrial clones throughout the life cycle and under varying hormonal conditions will provide new insights into endometrial carcinogenesis. Although ~40% of premenopausal women acquire PTEN-null glands, only 2.4% (17) of them will ever get endometrial cancer, most following menopause. This suggests that acquiring an initial mutation is not the rate-limiting step in endometrial carcinogenesis. Once initiated, ~15% (4) of premalignant endometrial lesions have microsatellite instability, expected to confer a mutator phenotype that may accelerate subsequent progression to carcinoma. Expansion or contraction of mutant clones would also be expected to modify the likelihood that additional genetic mutations will occur in a cell already having a first mutation (18). Hormonal factors are attractive as putative modulators of this process, because mutant clones lose their normal ability to increase PTEN expression (12) in response to estrogens. Unopposed estrogens increase endometrial cancer risk for several years after exposure (19), and this may be reduced or erased by subsequent administration of oral contraceptives (20). We present limited, repeat biopsy data from 11 postmenopausal women on combinatorial hormonal replacement therapy, which shows a lower prevalence (18%; 2 of 11) and acquisition rate (11%; 1 of 9) of PTEN-null glands compared with endogenously cycling premenopausal women (35% or 12 of 34 and 32% or 7 of 22, respectively). This may indicate either an age-related phenomenon or a suppression of PTEN-null clones by pharmacological means. Distinguishing between these possibilities is of great interest in defining a mechanism whereby endogenous and exogenous hormonal factors modify endometrial cancer risk.

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Frequent Loss of PTEN Expression Is Linked to Elevated Phosphorylated Akt Levels, but Not Associated with p27 and Cyclin D1 Expression, in Primary Epithelial Ovarian Carcinomas

Keisuke Kurose,* Xiao-Ping Zhou,* Tsutomu Araki,† Stephen A. Cannistra,‡ Eamonn R. Maher,§ and Charis Eng*¶

From the Clinical Cancer Genetics and Human Cancer Genetics Programs, Comprehensive Cancer Center, and the Division of Human Genetics, Department of Internal Medicine, The Ohio State University, Columbus, Ohio; the Department of Obstetrics and Gynecology,† Nippon Medical School, Tokyo, Japan; the Division of Hematology/Oncology,‡ Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts; the Department of Paediatrics and Child Health,§ Section of Medical and Molecular Genetics, University of Birmingham Medical School, Birmingham, United Kingdom; and the Cancer Research Campaign Human Cancer Genetics Research Group,¶ University of Cambridge, Cambridge, United Kingdom*

PTEN (*MMAC1/TEP1*), a tumor suppressor gene on chromosome subband 10q23.3, is variably mutated and/or deleted in a variety of human cancers. Germline mutations in *PTEN*, which encode a dual-specificity phosphatase, have been implicated in at least two hamartoma tumor syndromes that exhibit some clinical overlap, Cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome. Among several series of ovarian cancers, the frequency of loss of heterozygosity (LOH) of markers flanking and within *PTEN*, is ~30 to 50%, and the somatic intragenic *PTEN* mutation frequency is <10%. In this study, we screened primary adenocarcinomas of the ovary for LOH of polymorphic markers within and flanking the *PTEN* gene and for intragenic mutations of the *PTEN* gene and compared them to PTEN expression using immunohistochemistry. Furthermore, we sought to detect the expression of the presumed downstream targets of PTEN, such as P-Akt, p27, and cyclin D1 by immunohistochemistry. LOH at 10q23 was observed in 29 of 64 (45%) cases. Of the 117 samples, 6 somatic intragenic *PTEN* mutations, 1 germline mutation, and 1 novel polymorphism were found in 7 (6%) patients. Immunostaining of 49 ovarian cancer samples revealed that 13 (27%) were PTEN immunostain-negative, 25 (51%) had reduced staining, and the rest (22%) were PTEN expression-positive. Among the 44 informative tumors assessed for 10q23 LOH and PTEN immunostaining, there was an association between 10q23 LOH and decreased or absent staining (P = 0.0317). Of note, there were five (11%) tumors with neither mutation nor deletion that exhibited no PTEN expression and 10 (25%) others without mutation or deletion but had decreased PTEN expression. Among the 49 tumors available for immunohistochemistry, 28 (57%) showed P-Akt-positive staining, 24 (49%) had decreased p27 staining, and cyclin D1 was overexpressed in 35 (79%) cases. In general, P-Akt expression was inversely correlated with PTEN expression (P = 0.0083). These data suggest that disruption of *PTEN* by several mechanisms, allelic loss, intragenic mutation, or epigenetic silencing, all contribute to epithelial ovarian carcinogenesis, and that epigenetic silencing is a significant mechanism. The Akt pathway is prominently involved, but clearly not in all cases. Surprisingly, despite *in vitro* demonstration that p27 and cyclin D1 lies downstream of PTEN and Akt, there was no correlation between p27 and cyclin D1 expression and PTEN or P-Akt status. Thus, *in vivo*, although PTEN and Akt play a prominent role in ovarian carcinogenesis, p27 and cyclin D1 might not be the primary downstream targets. Alternatively, these observations could also suggest that pathways involving other than Akt, p27 and cyclin D1 that lie downstream of PTEN play roles in ovarian carcinogenesis. *(Am J Pathol 2001, 158:2097–2106)*

Ovarian cancer is the most common cancer in women to be diagnosed at an advanced stage and is the fifth leading cause of cancer deaths among women in the United States.[1] Various genetic alterations have been associated with ovarian cancer, such as somatic ampli-

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Address reprint requests to Dr. Charis Eng, Human Cancer Genetics Program, The Ohio State University Comprehensive Cancer Center, 420 West 12th Ave., Room 690C TMRF, Columbus, OH 43210. E-mail: eng-1@medctr.osu.edu.

fication of the oncogenes HER-2/*neu*, *KRAS*, and *C-MYC*, somatic mutation of the *TP53* tumor suppressor gene and germline mutations of *BRCA1* and *BRCA2*.[2-4] Because carcinogenesis is a multistep process,[5] it is likely that several as yet unknown genes play a role in the development and/or progression of ovarian cancer. Previous studies focusing on genetic changes occurring during ovarian cancer development have revealed clonal abnormalities in many chromosomal regions. Earlier allelotyping studies of ovarian cancer found loss of heterozygosity (LOH) at 10q in 11 to 21% of ovarian cancers, although only three markers, not in the 10q23 region, were used.[6,7] Recent reports using microsatellite markers that were specifically chosen for 10q23 identified a LOH frequency of 31 to 52%.[8-10]

The tumor suppressor gene *PTEN/MMAC1/TEP1*, encoding a dual-specificity phosphatase, has been cloned and mapped to chromosome subband 10q23.3.[11-13] Germline *PTEN* mutations have been found in the inherited autosomal-dominant Cowden and Bannayan-Riley-Ruvalcaba syndromes, which are characterized by multiple hamartomas and by an increased risk of malignant and benign breast and thyroid tumors.[14-19] Recently, a *Proteus*-like syndrome was found to result from germline and germline mosaic *PTEN* mutations.[20] Ovarian cancer, in general, is not considered part of these syndromes. Somatic mutation and/or deletion of *PTEN* occurs to a greater or lesser extent in a wide variety of human cancers that show LOH in this region, including glioblastoma, endometrial cancer, prostate cancer, and breast cancer.[12,13,21-24]

Genetic, functional, and animal modeling studies have substantiated the tumor suppressor function of PTEN. PTEN is a lipid phosphatase whose major substrate is phosphatidylinositol-3,4,5-triphosphate [PtdIns(3,4,5)P_3], downstream of which lies the Akt (PKB) pathway.[25-29] The serine-threonine kinase Akt, when phosphorylated, protects cells from apoptosis.[30,31] PTEN may also be involved in cell migration, spreading, and focal adhesion formation through dephosphorylating focal adhesion kinase, presumably through its protein phosphatase activity.[32,33] Ectopic expression of PTEN results in cell-cycle arrest at G_1 and/or apoptosis at least in the glioma and breast cancer cell line models.[27,28,34] It has been demonstrated that this function of PTEN is Akt-dependent, and can be rescued by expression of phosphorylated Akt.[27,35] One of the targets of PTEN in its ability to block cell-cycle progression at the G_1 phase has been suggested to be the cyclin-dependent kinase inhibitor p27.[28,36-38] It has been shown that up-regulation of p27 by PTEN has occurred in different cell lines.[36,37] However, it remains to be established whether the increase of p27 expression is truly a direct effect of PTEN's action *in vivo* and which intermediate steps are involved in synthesis of this cell-cycle inhibition signal. Another potential mechanism of cell-cycle control by *PTEN* may be through inhibition of cyclin D1 accumulation. AKT phosphorylates GSK3 (glycogen synthase kinase 3), leading to its inactivation.[39] Active GSK3 phosphorylates cyclin D1, targeting it for degradation.[40] Therefore, Akt seems to promote cyclin D1 accumulation.[41,42]

Although only rare mutations of the *PTEN* gene were reported in ovarian cancer,[8-10,43-46] because of PTEN's role in the cell cycle and cell death as well as the gene's localization to 10q23, *PTEN* is an excellent candidate to play an important role in ovarian carcinogenesis. Therefore, we sought to determine whether structural alterations in *PTEN* occurred with any frequency in ovarian cancer, if loss of PTEN expression, detected by immunohistochemistry, is a major mechanism of loss of function, and if there is a correlation between structural alterations of *PTEN* gene and PTEN protein expression. Further, we looked for any alteration in the expression of the presumed downstream targets of PTEN, such as P-Akt, p27, and cyclin D1 by immunohistochemistry, and investigated the correlation among all these variables.

Materials and Methods

Tumor Samples and DNA Extraction

One hundred seventeen epithelial ovarian tumors were obtained from patients undergoing surgery for primary epithelial ovarian cancer. Thirty-eight tumors were obtained from The Ohio State University, Columbus, OH (OSU), 25 were from the Beth Israel-Deaconess Medical Center, Boston, MA (BOS), 31 were from the University of Birmingham, UK (UK), and 23 were from Nippon Medical School, Tokyo, Japan (NMS). Apart from the 31 ovarian carcinomas from the UK that are of unknown histological sub-type, 32 were serous tumors, 28 were endometrioid cancers, 7 were clear cell cancers, 6 were mucinous tumors, 6 were mixed epithelial tumors, 6 were undifferentiated tumors, and 1 was a squamous cell carcinoma. Corresponding noncancerous tissues were available only from OSU, BOS, and NMS samples. Corresponding paraffin blocks were available only from OSU and BOS samples. Genomic DNA was extracted from tumor and matched normal tissue with a QIAamp DNA Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions, or by microdissection of normal and tumor areas of paraffin-embedded archival tissue using standard protocols.[47]

LOH Analysis

Sixty-eight ovarian cancers (35 from OSU, 11 from BOS, and 22 from NMS) in which both tumor and corresponding normal DNA were available were analyzed for LOH at five polymorphic markers flanking and within the *PTEN* gene. The markers are ordered from centromere to telomere: D10S579-D10S1765-IVS4 + 109ins/delTCTTA-IVS8 + 32T/G-D10S541. *PTEN* lies between D10S1765 and D10S541, a genetic distance of 1 cM but a physical distance of only several hundred kilobase pairs. D10S1765 is within 500 kb upstream of the transcriptional start site of *PTEN* and D10S541 is within 300 kb of the translational stop site. IVS4 + 109ins/delTCTTA and IVS8 + 32T/G lie within *PTEN*.[19,48,49] The IVS4 + 109ins/delTCTTA and IVS8 + 32T/G polymorphisms were screened for by differential digestion with *Afl*II and *Hin*cII, respectively, according to

the manufacturer's guidelines (New England Biolabs, Beverly, MA) as described previously.[16,48] D10S579, D10S1765, and D10S541 were screened as documented previously.[50] Polymerase chain reaction (PCR) conditions for these markers are described elsewhere.[51,52]

PTEN Mutation Analysis

All samples were scanned for mutations by denaturing gradient gel electrophoresis (DGGE) as previously described.[24] The entire *PTEN*-coding region, exon-intron boundaries, and flanking intronic sequences were PCR amplified and fractionated through denaturing gradient gels according to the conditions described previously.[24,51] Samples showing DGGE variation were re-amplified with another set of primers, specifically for sequence analysis, gel- and column-purified and subjected to semi-automated sequence analysis as previously published.[48]

RNA Extraction and Reverse Transcriptase (RT)-PCR

From ~100 mg of ovarian cancer tissue, total RNA was extracted by the guanidine thiocyanate method,[53] using Trizol reagent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's guidelines. Total RNA was treated with DNase I (Boehringer Mannheim, Mannheim, Germany) to remove any contaminating genomic DNA before reverse transcription. This RNA was reverse-transcribed with a first strand cDNA synthesis kit for RT-PCR (AMV; Boehringer Mannheim) according to the manufacturer's recommendations. RT-PCR using the *PTEN* exonic primers [1F, 5′-TCAAGAGGATGGATTCGACTT-3′ (*PTEN* exon 1), and 5.1R, 5′-TCATTACACCAGTTCGTCC-3′ (*PTEN* exon 5)] was performed to determine whether splicing abnormalities would result from intronic mutation detected in the sequencing analysis.

Immunohistochemistry

The monoclonal antibody 6H2.1 raised against the last 100 C-terminal amino acids of PTEN[54] was used. Specificity of this antibody for *PTEN* has been documented elsewhere.[50,54] Polyclonal antibody against P-Akt was obtained from Upstate Biotechnology (Lake Placid, NY). Monoclonal antibodies against p27 and cyclin D1 were obtained from Transduction Laboratories (San Diego, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

The tissue samples were fixed by immersion in buffered formalin and embedded in paraffin according to standard procedures.[47] Sections (4 μm) were cut and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Subsequently, the sections were deparaffinized and hydrated by passing through xylene and a graded series of ethanol. Antigen retrieval was performed for 20 minutes at 98°C in 0.01 mol/L sodium citrate buffer, pH 6.4, in a microwave oven. To block endogenous peroxidase activity, the sections were incubated with 0.3% hydrogen peroxide for 30 minutes. After blocking for 30 minutes in 0.75% normal serum, the sections were incubated with each antibody overnight at 4°C. The sections were washed in phosphate-buffered saline and then incubated with biotinylated second antibody followed by avidin peroxidase using the Vectastain ABC elite kit (Vector Laboratories, Burlingame, CA). The chromogenic reaction was performed with 3–3′ diaminobenzidine using nickel cobalt amplification.[55] After counterstaining with Nuclear Fast Red (Rowley Biochemical, Danvers, MA) and mounting, the slides were evaluated under a light microscope. The immunostaining patterns and intensities were independently evaluated by two investigators (KK and X-PZ) and randomly spot evaluated by a third investigator (CE). The multiple independent reading of the slides was performed to ensure quality control and consistency of results. Further, all sections, especially those that are immunostain-negative, were checked to contain internal positive and negative staining controls. Such consistency and accuracy have been previously documented over a broad range of tissue types throughout time [24,50,54,56,57].

Immunohistochemical Analyses

Immunostaining was evaluated without knowledge of the clinical and pathological parameters. For PTEN immunostaining, according to the amount of staining, the tumors were divided into three groups: the group assigned ++ showed increased or equal staining intensity compared to the corresponding normal tissue; the group assigned + had decreased staining intensity; and the group assigned − had no trace of staining. For P-Akt, the samples were divided into two groups according to the amount of staining. The group assigned + showed increased staining intensity compared to the corresponding normal tissue and the group assigned − had no staining. For p27, positive samples were scored according to the frequency of immunopositive cells as <5%, 5 to 50%, >50% cells immunopositive. Samples from patients with <50% p27-positive tumor cells were considered low expressors, whereas those with >50% p27-positive tumor cells were considered high expressors according to the published conventions.[58,59] We also examined cyclin D1 immunostaining. We scored cyclin D1 immunostaining by the percentage of total cells that were positive in both the nucleus and cytoplasm. Slides were graded as: negative (0 to 10% cells stained), + (10 to 50% cells stained), ++ (>50% cells stained). A cutoff value of 10% staining was used to separate normal staining (<10%) from cyclin D1-overexpressing cells (>10%).

The Fisher exact test and Wilcoxon rank sum test were used for statistical analysis of the results. The nonparametric Wilcoxon test was also chosen because immunostaining intensities (especially for cyclin D1 and p27) may be considered a continuous variable. A $P < 0.05$ was considered statistically significant.

Table 1. Results of PTEN Mutation Analysis in Primary Ovarian Cancer

Tumor	10q23 LOH	Exon/intron	Mutation	Codon altered	Germline
NMS2	–	IVS 1	IVS1+41C>G	Polymorphism	Germline
NMS26	+	Exon 3	c.166T>G	F56V	Somatic
UK63	NA	Exon 1	c.70G>T	D24Y	NA
UK33	NA	Exon 5	c.463T>A	Y155N	NA
UK66	NA	Exon 5	469–470insG	Stop at codon 179	NA
UK33	NA	Exon 7	741–742insA	Stop at codon 252	NA
UK18	NA	Exon 8	c.862G>T	E288X	NA
BOS11	+	IVS 3	IVS3-1G>T	Stop at codon 76 (Ex. 4 deletion)	Germline

NA, not available.

Results

LOH Analysis

We analyzed 68 ovarian cancers (35 from OSU, 11 from BOS, and 22 from NMS), for which tumor and corresponding normal DNA were available, for LOH at five polymorphic markers flanking *PTEN* (D10S579, D10S1765, and D10S541) and within *PTEN* (IVS4 + 109ins/delTCTTA and IVS8 + 32G/T). Among the 68 samples examined, 4 were excluded from final analysis because they were not informative at all markers. LOH at 10q23 was scored when one or more of the panel of five polymorphic loci showed LOH, as is standard. Of the 64 informative samples that were informative for at least one marker, 29 (45%) had LOH at one or more loci.

PTEN Mutation Analysis

To determine whether *PTEN* is genetically altered during ovarian carcinogenesis, we screened primary ovarian cancers for mutations in the *PTEN* gene by DGGE analysis. Samples that showed variant bands on DGGE analysis were used as template for direct sequence analysis. Of the 117 total samples, DGGE analysis revealed 8 variants in 7 samples (6%), and sequence analysis of these samples confirmed the presence of sequence variation. The identified variants included three missense, one nonsense, two frameshift mutations, and two intronic variants (Table 1). The UK samples showed the most frequent mutations (4 of 31, 13%), and Japanese and United States samples harbored mutations in 2 of 23 (9%) and 1 of 63 (2%), respectively. There was a statistical significance in mutation frequency between UK and US samples ($P = 0.022$, chi-square test). The one missense mutation in tumor NMS26 was somatic, and the corresponding germline was wild type. Tumors NMS2 and BOS11 had intronic variants in both tumor and corresponding germline DNA. Germline and somatic DNA from tumor NMS2 showed a C-to-G change in intron 1, 41 bases from the exon-intron boundary (IVS1 + 41C>G). Germline and somatic DNA from BOS11 revealed a G-to-T change in intron 3, one base from exon-intron boundary (IVS3–1G>T). Corresponding germline DNA of the remaining samples that showed variants were not available.

RT-PCR was performed to determine whether these intronic variants affected splicing. In tumor NMS2, no abnormal fragments were detected. In tumor BOS11, a fragment that was 44 bp shorter than the predicted size was noted. Sequence analysis of this aberrant fragment revealed skipping of exon 4, resulting in a frameshift and a truncated product of 76 amino acids (data not shown). We concluded that the variant in tumor NMS2 is a novel, naturally occurring, polymorphism and the variant in tumor BOS11 is a germline mutation. Interestingly, LOH at 10q23 was also found in these two tumors that showed intragenic *PTEN* mutations. In addition, UK33 showed two different pathogenic mutations, located in exons 5 and 7 (Table 1).

PTEN Immunohistochemistry in Ovarian Cancer

Samples from 49 sporadic ovarian epithelial carcinomas (38 from OSU and 11 from BOS), which had paraffin-embedded sections available, were examined for PTEN expression using the monoclonal antibody 6H2.1, raised against the terminal 100 amino acids of human PTEN. Vascular endothelial cells showed strong PTEN expression with a nuclear predominance and served as an internal positive control for this study (Figure 1, A and C).[50,54] In contrast, nuclear- and cytoplasmic-staining intensity of fibrocytes was very heterogeneous and varied from strong to weak. Of 49 ovarian cancer samples, 13 (27%) lost all PTEN immunoreactivity and was graded – (Figure 1, A and B). Twenty-five of the 49 (51%) ovarian cancer specimens stained weakly, graded +, in comparison to the normal tissue (Figure 1C). The remaining 11 (22%) tumors stained positively, graded ++ (increased or equal staining intensity compared to the corresponding normal tissue) (Figure 1D). In general, the quality and intensity of PTEN immunostaining in the nucleus and cytoplasm was relatively uniform throughout each specimen.

Comparison of Immunohistochemical and Structural Alteration Data of PTEN Gene

There were a total of 46 samples in which both immunohistochemical data and LOH data are available. Among these 46 samples, LOH data from 44 tumors were infor-

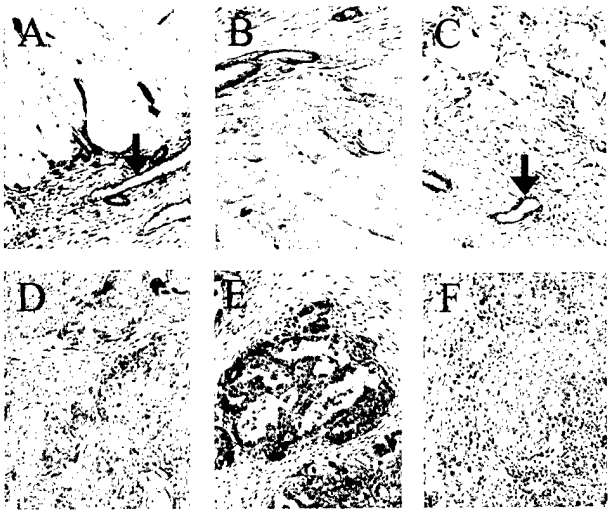

Figure 1. PTEN and P-Akt immunohistochemistry in ovarian cancers. Positive staining (++) of vascular endothelial cells serves as an internal positive control (**arrows**). Original magnification, ×10. **A** and **B:** Ovarian cancer with PTEN-negative staining (−). **C:** Ovarian cancer showing weak staining (+). **D:** Ovarian cancer exhibiting positive staining (++) for PTEN. **E:** Ovarian cancer with positive (+) P-Akt staining. **F:** Ovarian cancer exhibiting negative (−) P-Akt staining.

mative. Ten (23%) of the 44 samples were graded ++ for PTEN expression by immunohistochemistry, and 8 of these 10 did not show evidence of 10q23 LOH (Table 2 and Figure 2). The remaining two samples (OSU50 and BOS19) might be viewed as apparently discordant when LOH data are compared to those obtained from immunohistochemistry (Figure 2). Sample OSU50 showed LOH at D10S1765 (5′ of *PTEN*) and retention of both alleles at the remaining four loci, notably those within *PTEN*. Sample BOS19 showed LOH at D10S579 (5′ of *PTEN*), retention of both alleles at D10S1765, and noninformative at the remaining three loci (Figure 2). Because these samples showed LOH only 5′ of *PTEN*, it is doubtful whether it is deleted within *PTEN*, thus explaining the ++ immunohistochemistry.

Thirteen samples (30%) were immunostain-negative. Among these, eight showed evidence of 10q23/*PTEN* deletion and five had no evidence of *PTEN* allele loss (Table 2 and Figure 2). Four of these five samples (OSU14, OSU28, OSU58, and OSU68) showed retention of both alleles at the flanking markers, and noninformative at the intragenic *PTEN* polymorphic markers. Therefore, there is a possibility that a part or entire *PTEN* might be deleted (Figure 2).

Twenty-one samples (48%) were immunostain-positive. Of these 21, 11 might be classified as having LOH

Table 2. Summary of PTEN Immunostaining and 10q23 LOH

	PTEN staining ++	PTEN staining +	PTEN staining −
Loss of heterozygosity	2 (20%)	11 (52%)	8 (62%)
Retention of heterozygosity	8 (80%)	10 (48%)	5 (38%)
Total	10	21	13

Statistical correlation of PTEN intensity *versus* LOH at 10q23 performed using a Wilcoxon rank sum test yielded results of P = 0.0317, indicating a significant increase in LOH at 10q23 with decreasing PTEN stain intensity.

Sample no	*PTEN* staining	*PTEN* mutation	D10S579	D10S1765	IVS4+109ins/del TCTTA	IVS8+32T/G	D10S541
OSU50	++	-	○	●	○	○	○
BOS19	++	-	●	○	-	-	-
OSU8	+	-	-	○	-	○	-
OSU24	+	-	○	-	-	-	○
OSU30	+	-	○	○	-	-	-
OSU32	+	-	○	○	○	○	○
OSU36	+	-	-	-	-	-	○
OSU38	+	-	○	-	-	-	○
OSU46	+	-	-	○	○	○	○
OSU60	+	-	○	○	-	-	○
BOS2	+	-	○	○	-	-	○
BOS6	+	-	○	○	-	-	○
OSU4	-	-	○	○	-	○	-
OSU14	-	-	○	○	-	-	○
OSU28	-	-	○	○	-	-	○
OSU58	-	-	-	○	-	-	-
OSU68	-	-	-	○	-	-	-

Figure 2. Correlation between PTEN immunostaining and LOH at 10q23 with apparently discordant tumors in ovarian cancer. **Open circles**, retention of heterozygosity; **filled circles**, LOH; −, noninformative or failed PCR.

representing hemizygous *PTEN* allelic loss that could correspond to the diminished immunostaining. The remaining 10 with weak immunoreactivity retained heterozygosity at their respective informative loci (Table 2 and Figure 2).

We investigated potential relationships between the presence of LOH at 10q23 and the intensity of PTEN immunostaining. Among the 44 informative tumors assessed for LOH and PTEN immunostaining, there seemed to be an association between decreased or absent staining and 10q23 LOH. The frequency of tumors that showed LOH at 10q23 steadily increased from tumors with ++ immunostaining (20% LOH), + staining (52% LOH), to negative (−) staining (62% LOH) (P = 0.0317 Wilcoxon rank-sum test) (Table 2). However, it is also clear that one-third (15 of 44) of tumors did not show structural deletion or mutation but showed diminished or no PTEN expression.

P-Akt, p27, Cyclin D1 Immunohistochemistry in Ovarian Cancer

Of 49 ovarian cancer samples, 28 (57%) stained positive, graded +, in comparison to the normal tissue (Figure 1E). Twenty-one of the 49 (43%) ovarian cancer specimens lost all P-Akt immunoreactivity and showed negative immunostaining, graded − (Figure 1F). P-Akt immunostaining showed a nuclear predominance, however, weak cytoplasmic staining was also observed. Among these same 49 ovarian cancers, 4 (8%) showed negative immunostaining with p27 antibody, graded − (Figure 3A). Twenty of the 49 (41%) ovarian cancer specimens stained weakly, graded +, in comparison to the normal tissue. Thus, 24 samples (49%) were classified as low expressors. The remaining 25 (51%) tumors stained positively, graded ++ (Figure 3B) and were classified as high expressors. p27 immunostaining was mostly nuclear; however, weak cytoplasmic staining was also observed. Overexpression of cyclin D1 was detected in

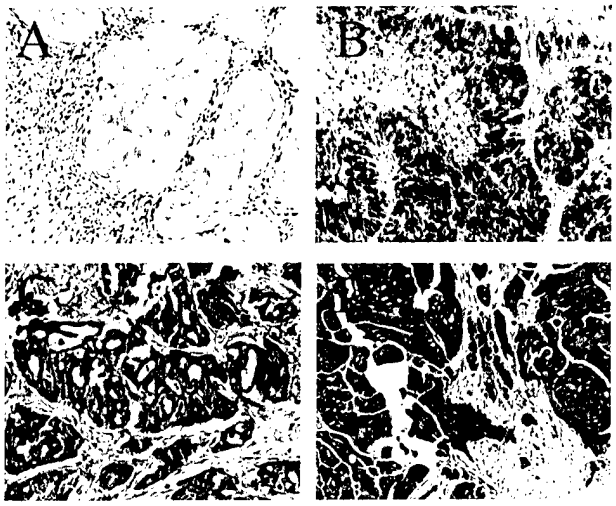

Figure 3. p27 and cyclin D1 immunohistochemistry in ovarian cancers. Original magnification, ×10. Ovarian cancer with p27-negative (−, **A**), and p27-positive (++, **B**) staining. **C:** Ovarian cancer showing cyclin D1 overexpression (++) in simultaneous nucleus and cytoplasm. **D:** Ovarian cancer showing cyclin D1 overexpression (++) in exclusively cytoplasm.

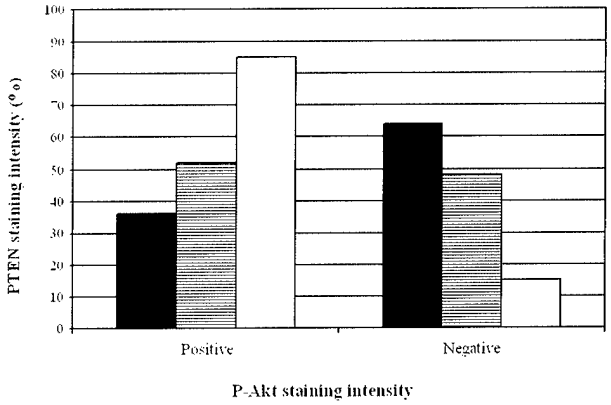

Figure 4. Correlation between PTEN and P-Akt immunostaining intensity. Strong (**filled square**), decreased (**lined box**), and absent (**open square**) PTEN staining. The *y*-axis represents percentage of samples with various intensities of PTEN staining. Statistical correlation was found between PTEN and P-Akt staining ($P = 0.0083$).

35/49 (71%) of the ovarian cancers examined. The level of overexpression was variable between the slides and was graded accordingly. Cyclin D1 was overexpressed predominantly in the nuclei of tumor cells in 6 of 49 (12%) of the samples, and simultaneously in the nucleus and cytoplasm of tumor cells in 10 of 49 (20%) (Figure 3C). Therefore, 16 of 49 (33%) of the tumors examined showed nuclear overexpression of cyclin D1 (Table 3). In 19 of 49 (39%) of the tumors examined, overexpression was detected only in the cytoplasm of the tumor cells (Figure 3D). Therefore, 29 of 49 (59%) of the tumors examined showed cytoplasmic overexpression of cyclin D1 (Table 3). Representative examples of simultaneous nuclear and cytoplasmic, and exclusively cytoplasmic staining are shown (Figure 3, C and D).

We investigated potential relationships of expression of PTEN, P-Akt, p27, and cyclin D1. Among the 49 informative tumors assessed for immunostaining, there seemed to be an association between positive P-Akt staining and decreased or absent staining of PTEN (Figure 4). The proportion of tumors that had positive P-Akt immunostaining steadily increased from tumors with ++ PTEN expression (36%), to those with no PTEN expression (85%) ($P = 0.0083$ Wilcoxon rank sum test; Figure 4). Of the 11 tumors that showed ++ PTEN expression, 7 (64%) of these tumors stained p27 ++, 4 (36%) of these showed p27 + staining, and no tumors showed negative p27 staining. (Figure 4). However, this correlation between PTEN expression and p27 expression did not achieve statistical significance ($P > 0.05$, chi-square test; $P = 0.4$, Fisher two-tailed exact test). No significant association was found between PTEN or P-Akt expression and cyclin D1.

Discussion

Our study of PTEN in primary epithelial ovarian carcinomas has revealed that genetic and epigenetic inactivation of PTEN together play a significant role in tumorigenesis. In this study, we have found that the incidence of LOH at 10q23 was 45% in primary epithelial ovarian cancers, a figure consistent with previous allelotyping.[8–10] Examining 117 sporadic ovarian cancers, we found that 6 cases (5%) harbored intragenic *PTEN* mutations, again consistent with previous reports (0 to 10%).[8–10,43–46] Obata and colleagues[9] identified that intragenic *PTEN* mutations are much more common in endometrioid histology (7 of 34; 21%). However, Saito and colleagues[10] reported intragenic *PTEN* mutations were found not only in endometrioid type (1 of 5; 20%) but also in serous (1 of 10; 10%) and mucinous (1 of 4; 25%) type. Unfortunately, histological information of UK samples was not available. NMS26 and BOS11, two *PTEN* mutation-positive samples in which histological information was available, were classified as mucinous (1 of 6; 17%) and endometrioid (1 of 28; 4%) type, respectively. Of 13 samples that showed negative PTEN immunoreactivity, 6 were endometrioid cancers (6 of 13; 46%), 4 were serous tumors (4 of 21; 19%), 2 were mixed epithelial tumors (2 of 6; 33%), and 1 was an undifferentiated tumor (1 of 4; 25%). Thus, PTEN seems to play a role across several subtypes of ovarian cancer and is not predominant among the endometrioid subtype, as previously believed.

Table 3. Cyclin D1 Immunostaining and Localization in Ovarian Cancer

	Cyclin D1 (cytoplasm only)	Cyclin D1 (nuclear only)	Cyclin D1 (both)	Negative
Overexpression*	19 (39%)	6 (12%)	10 (20%)	14 (29%)

*, >10% cells stained.

Among the samples that had intragenic *PTEN* mutations, only two samples had both tumor and normal DNA available for investigation. Interestingly, both these samples (NMS26 and BOS11) showed biallelic structural inactivation by intragenic *PTEN* mutation plus loss of the remaining wild-type allele. Although normal DNA was not available, the exon 5 and exon 7 double-intragenic mutations in UK33 almost certainly represents mutations involving both alleles (Table 1). Previous studies have shown that biallelic structural inactivation occurs in ovarian cancer, either by somatic intragenic *PTEN* mutation plus loss of the remaining wild-type allele, by double-somatic intragenic *PTEN* mutations, or by homozygous deletion at 10q23.[9,10] Our study and these data suggest that a subset of ovarian cancer, albeit small, might be in the same category as glioblastoma multiforme, a subset of endometrial cancers and cervical cancers, where biallelic structural *PTEN* inactivation is also an important mechanism.[21,23,60,61]

In case BOS11, mutation within intron 3, one base from the exon-intron boundary (IVS3–1G>T), has been shown to cause aberrant splicing and almost certainly also results in a transcript with decreased stability. This mutation was also identified in the corresponding germline DNA; thus, among 107 apparently sporadic ovarian cancer cases, we detected at least one (1%) carrying an occult germline *PTEN* mutation. It is, therefore, vital that this patient be followed closely for development of Cowden-related cancers, especially those of the breast, thyroid, and endometrium, because the presence of a germline *PTEN* mutation is a sensitive molecular diagnostic marker for Cowden syndrome.[18,62] If our observations can be replicated, clinicians might wish to inquire about other features of Cowden syndrome and to take a good family history when faced with apparently sporadic ovarian cancer cases. Hereditary ovarian cancer occurs as a part of three clinically distinct syndromes, site-specific ovarian cancer and breast-ovarian cancer, both of which are because of germline mutations in *BRCA1* and *BRCA2* in >90% of such cases,[63] and hereditary nonpolyposis colorectal cancer. It is possible that Cowden syndrome might be included as a minor differential diagnosis of genetic ovarian cancer cases.

Among the samples that were available for both PTEN immunohistochemistry data and LOH analysis, we found that 77% (34 of 44) of these tumors had either partial or complete expressional loss of PTEN at the protein level. Of these 34 samples, only 3% (1 of 34) had structural biallelic inactivation, 53% (18 of 34) had structural monoallelic deletion of *PTEN*, and the remaining 15 samples (44%) had no evidence of *PTEN* structural anomalies and thus, lost of PTEN expression might be because of epigenetic silencing (Table 2). Especially worthy of note are the 13 tumors with no PTEN expression (−): only 1 had structural biallelic inactivation by intragenic *PTEN* mutation and loss of the remaining wild-type allele; 7 had evidence of loss of one allele only and so, silencing of the remaining wild-type allele might be by epigenetic means; and 5 had complete silencing of both *PTEN* alleles via mechanisms beyond structural alteration. Similarly, among the 21 tumors with weak (+) PTEN immunostaining, 11 had monoallelic deletion that would account for the decreased PTEN expression. However, the remaining 10 had neither *PTEN* mutation nor deletion. These 10 tumors might have monoallelic silencing via epigenetic mechanisms (Table 2 and Figure 2). In contrast, all tumors with strong PTEN expression had both intact *PTEN* alleles. The two seemingly discordant samples with strong expression but LOH only had LOH 5′ of PTEN. Recent accumulating knowledge has suggested that PTEN may be inactivated by several mechanisms other than mutations and/or deletions in a tissue-specific manner [24,29,50,54,56,57,64]. In general, the data to date would suggest that more than one mechanism of PTEN inactivation can occur in a given tumor type. It would seem, however, that certain mechanisms of *PTEN* inactivation predominate in certain tissues. For example, in primary cervical carcinomas and glioblastoma multiforme, the predominant mechanism of PTEN silencing is biallelic structural alteration (mutation; deletion).[61] In metastatic malignant melanoma, biallelic epigenetic mechanisms of PTEN silencing predominate.[57] In nonmedullary thyroid cancers and endocrine pancreatic tumors, PTEN inactivation seems to be mediated by differential subcellular compartmentalization.[50,56] In the case of primary epithelial ovarian cancers (this study), biallelic structural alterations are rare whereas a mixed genetic/epigenetic and biallelic epigenetic silencing are evident in the great majority of tumors with decreased or absent PTEN expression. In contrast to thyroid tumors and endocrine pancreatic tumors, there is no evidence of subcellular compartmentalization as a mechanism of PTEN inactivation in ovarian carcinomas. Nonetheless, the precise epigenetic or other mechanisms, such as methylation or degradation, involved in modulating *PTEN* expression are yet to be elucidated. Further, why subcellular compartmentalization involving the nuclear compartment for a molecule such as PTEN lacking a nuclear localization signal is actively being investigated.[32,50,56,65,66]

It has been well established that PTEN signals down the PI3K-Akt pathway and that PTEN inversely correlates with P-Akt.[25–27,29] Of the 49 tumors examined, 28 (57%) showed positive (+) P-Akt staining and 21 (43%) showed negative (−) staining, a general trend showing this inverse correlation. However, it should be noted that the inverse correlation is not observed among all tumors: six (12%) did not show this inverse correlation. These observations might suggest that in the subset of PTEN null-P-Akt null ovarian cancers, at least one other non-PI3K-Akt pathway downstream of PTEN is the major pathway involved. In contrast, in the subset of PTEN and P-Akt co-expressors, PTEN is likely not involved and P-Akt is anti-apoptotic via other upstream mechanisms. These *in vivo* observations in noncultured tumors corroborate our *in vitro* functional analyses that demonstrate that PTEN can induce cell-cycle arrest and apoptosis via PI3K-Akt-dependent and -independent pathways.[67]

Because of several reports of finding a relationship between PTEN and p27 and between PTEN and cyclin D1, albeit *in vitro*, we sought to examine differential expression levels among these proteins. Several reports have suggested that PTEN induced p27 expression

downstream of Akt[36, 38] and that this was a mechanism of cell-cycle arrest mediated by PTEN. If this were true, we would have observed a direct correlation between PTEN expression and p27 expression. Although we saw an associative trend, this was not statistically significant nor was it convincing. Similarly, the literature has suggested that PTEN down-regulates cyclin D1.[68] If this were true, then we should have observed a trend of increased cyclin D1 expression with decreased PTEN expression. In our series, cyclin D1 expression levels and subcellular location were random irrespective of PTEN or P-Akt status. Our observations might suggest that *in vivo*, at least in primary epithelial ovarian carcinomas, p27 and cyclin D1 are not prominent or direct targets of PTEN action.

In summary, despite the low frequency of intragenic somatic *PTEN* mutations in primary ovarian cancer, we have found a high frequency of decreased or absent PTEN protein expression, associated with increased P-Akt expression. Our data argues for the prominent role of PTEN inactivation in ovarian carcinomas via multiple mechanisms, ranging from biallelic genetic alteration to biallelic epigenetic silencing. Further, although the Akt pathway is involved in PTEN's role in a proportion of ovarian tumorigenesis, p27 and cyclin D1, previously described to be downstream of PTEN, do not seem to have this relationship with PTEN, at least not in epithelial ovarian carcinogenesis. Therefore, it would seem that not only are mechanisms of PTEN inactivation tissue-specific but downstream pathways of PTEN as well.

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Impact of gender and parent-of-origin on phenotypic expression of hereditary non-polyposis colon cancer (HNPCC) in 12 Newfoundland families with a founder MSH2 mutation. *J. Green[1], M. O'Driscoll[1], A. Barnes[1], E. Maher[2], K. Shields[3], P. Bridge[3], P. Parfrey[1].* 1) Disciplines of Medical Genetics and Medicine, Memorial Univ, St.Johns, NF; 2) Section of Med/Molecular Genetics, Univ of Birmingham, UK; 3) Molecular Diagnostic Laboratory, Alberta Children's Hospital, Calgary, AB.

A group of 12 families with a founder MSH2 mutation (nt943+3 A to T) has been identified from a genetic isolate in Newfoundland. Medical records were reviewed to identify types and age at onset of cancer, and age at death. Genetic testing was offered to those at 50% risk. Ascertainment bias was limited by analyzing only sibships where >50% had known genetic status (N=302). 147 were mutation positive (87 clinically affected), 94 mutation negative, and 61 of unknown mutation status. **METHODS:** Kaplan-Meier life table analysis was used to calculate cumulative age to death, and age to onset of any cancer, of CRC, and of endometrial or ovarian cancer. Relative risk was estimated by calculating hazard ratios using a Cox regression model. **RESULTS:** The age-related risks of any cancer, of CRC, or of death from cancer were significantly higher in males than in females (RR=1.7, p=0.02; RR=2.5, p=0.001; RR=2.0, p=0.03 respectively). 24% of women at risk developed endometrial cancer by age 50, and 70% by age 70; whereas 24% developed ovarian cancer by age 50, and 37% by age 70. The mutation was transmitted by the mother (N=91) more frequently than by the father (N=47), associated with the decreased longevity and decreased fertility in affected males. Females who inherited the mutation from their father had a 3.5 RR (p=0.001) of developing any cancer, and a 4.1 RR (p=0.003) of dying from cancer compared to those who inherited the mutation from their mother. **DISCUSSION:** Investigation of large kindreds from the same geographic area and sharing the same mutation may provide new insights into the determinants of phenotype. Search for modifiers of the expression of HNPCC mutations should take account of the difference in risk of cancer, and risk of death from cancer, depending on the sex of the mutation-carrier, and of the parent transmitting the mutation.

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Germline hMLH3 mutations in patients with suspected HNPCC. *Y. Wu[1], M.J.W Berends[2], R.G.J Mensink[1], E. Verlind[1], R.H. Sijmons[1], A.G.J. van der Zee[4], H. Hollema[3], J.H. Kleibeuker[2], C.H.C.M. Buys[1], R.M.W. Hofstra[1].* 1) Dept Medical Genetics, Univ Groningen, Groningen, Netherlands; 2) Dept Gastroenterology, Univ Groningen, Groningen, Netherlands; 3) Dept Pathology, Univ Groningen, Groningen, Netherlands; 4) Dept Gynaecology, Univ Groningen, Groningen, Netherlands.

Hereditary nonpolyposis colorectal cancer (HNPCC), as defined by the so-called Amsterdam criteria, is an autosomal dominant disorder. So far, in five different mismatch repair genes germline mutations have been found associated with HNPCC. These genes account for 50-75% of all HNPCC families. A role of the MutL homologue hMLH3 in HNPCC has not yet been reported. We scanned hMLH3 for mutations in all 11 exons by use of DGGE followed by sequencing of aberrant DNA fragments in patients from 17 unrelated HNPCC families meeting the classic Amsterdam criteria and 208 index patients with suspected HNPCC. We detected seven germline mutations of hMLH3, all in patients suspected for HNPCC. None of the mutations were found in over 200 control individuals. The mutations included one frameshift mutation (2578delA) and six missense mutations (Gln24Glu, Glu624Gln, Arg647Cys, Ser817Gly, Gly981Ser and Ala1370Thr) that all result in substitutions of amino acids belonging to different polarity groups. We could obtain tumor materials for MSI analysis from five of the seven patients with germline hMLH3 mutations. We found that the colorectal carcinoma from the patient with the frameshift mutation displayed an MSI-low phenotype. Tumors from the other patients with the missense mutations in two cases proved MSI-high, in two other cases MSI-low. Our results show involvement of hMLH3 in a proportion of patients with suspected HNPCC.

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Altered AXIN2 in Colorectal Cancer with Defective Mismatch Repair. *W. Liu, X. Dong, M. Mai, R.S. Seelan, K. Taniguchi, K.K. Krishnadath, K.C. Halling, J.M. Cunningham, C. Qian, E. Christensen, P.C. Roche, D.I. Smith, S.N. Thibodeau.* Dept Lab Med & Pathology, Mayo Clinic/Mayo Medical School, Rochester, MN.

Colorectal cancer (CRC) with defective DNA mismatch repair (MMR) is associated with alterations in one of several DNA MMR genes. However, the downstream functional consequences of such defects in the development of CRC are poorly understood. We previously cloned the human homolog of mouse conductin (AXIN2). AXIN2 interacts with APC, GSK3b and b-catenin and makes it a potential mutational target for colorectal cancer. We have determined its genomic structure and screened 105 CRC tumors (45 had defective MMR and the remaining 60 were MMR proficient) for mutations by DHPLC. Eleven frameshift mutations were identified and confirmed by direct sequence analysis. Significantly, mutations were identified only in CRC with defective MMR (11/45). None were detected in MMR proficient tumors (0/60; P < 10-5, Fishers exact test). b-catenin was shown to be accumulated in cell nuclei in 10/11 tumors with AXIN2 mutations and no APC or b-catenin mutations were detected in these tumors, demonstrating the pathologic importance of such alterations. The functional importance of these mutations was shown by the accumulation of b-catenin in the nuclei when mutant AXIN2 was transfected into normal fibroblast cells and by the strong Tcf dependent transcription upon co-transfection with mutant AXIN2 but not with wild-type AXIN2 in a Tcf reporter assay. Our data indicate that AXIN2 mutation plays an important role in CRC development by linking defective MMR to the APC pathway.

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Hemochromatosis gene *(HFE)* mutations are associated with an increased risk of colon cancer. *L.M. Silverman, N.J. Shaheen, R.S. Sandler, T.O. Keku, L.B. Lawrence, C.F. Martin, R. Maynard, E.M. Rohlfs.* University of North Carolina, Chapel Hill, NC.

In order to assess the association of elevated iron stores with an increased risk of colorectal neoplasia, we performed a population-based, case-control study of hemochromatosis gene *(HFE)* mutations in patients with colon cancer. Cases were 415 (41.5% African-American) patients with colon cancer from a 33 county area identified through the North Carolina Central Cancer Registry. Age-, sex, and race-matched controls (n=776, 35.7% African-American) were obtained from the same area by using Medicare records and the driver's license registry. All cases and controls completed extensive questionnaires to assess multiple environmental exposures, including dietary iron intake. Venous blood samples were obtained and assays for *(HFE)* mutations were performed on extracted DNA by polymerase chain reaction, restriction enzyme digestion, and agarose gel electrophoresis to identify both the C282Y and H63D mutations. Data were analyzed by Fisher's exact test and logistic regression. When controlled for age, sex, race, and dietary iron intake, subjects with colon cancer were significantly more likely to possess either the C282Y or H63D mutations than were controls (adjustedOR=1.40, 95% CI: 1.05-1.86). Although African-Americans are less likely than Caucasians to possess the C282Y mutation, it was a strong risk factor for colon cancer (aOR=2.31, 95% CI: 1.24-4.29). Additionally, women with any *(HFE)* mutation were also at increased risk (aOR=1.79, 95% CI: 1.18-2.72). Thus, it appears that *(HFE)* mutations are associated with an increased risk of colon cancer, with women and African-Americans having the highest risk. This increased risk may be iron-mediated or *(HFE)* may be associated with other genes that predispose to colon cancer. The work was funded by an American College of Gastroenterology Junior Faculty Development Award and R01 CA66635.

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Predicting the risk of gastric cancer using *H. pylori* gastritis patterns associated with family history of gastric cancer. *A.R. Sepulveda[1], L.E. Peterson[1], J. Shelton[1], D.Y. Graham[1], O. Gutierrez[2].* 1) Department of Medicine/Gastroenterology, Baylor College of Medicine, Houston, Texas; 2) Universidad National Bogota, Colombia.

Progression of H. pylori-associated gastritis is the main pathway for gastric cancer (GC) development. Host factors may represent genetic susceptibility traits that aggregate in families with GC, and might influence the outcome of H. pylori infection. The role of family history of GC in the spectrum of H. pylori-associated histological changes of the gastric mucosa, and identification of specific patterns to predict an increased risk of GC development were investigated. Histopathological changes associated with H. pylori infection were assessed in 111 individuals with family history of GC and in 77 without. Gastric biopsies using a 12-site (5 antrum (A1-5), 6 corpus (B1-6), 1 cardia) protocol were obtained. Genta stain was used to assess H. pylori, PMN and mononuclear infiltration, lymphoid follicle (LF) load, intestinal metaplasia and atrophy using the Houston-scoring system. Individuals with positive family history of GC develop pangastritis with significantly higher (p<0.05) H. pylori bacterial scores in the gastric corpus while those without family history initially develop antral predominant gastritis with progressive antralization of the corpus with age. A significantly higher load of LF (p<0.05) was seen in the corpus of younger individuals (age<36) and in the antrum of older individuals (age 48+) with a positive family history of GC. The odds ratio for positive family history of GC based on A5LF was 4.18 (95% CI, 1.66-10.5), for B5LF was 2.82 (95% CI, 1.38-5.77), and for both was 11.81 (95% CI, 3.53-39.54) accounting for covariance. Individuals with family history of GC develop a unique histological pattern of gastritis in response to H. pylori infection. Using family history as a surrogate measure of gastric cancer risk, histological examination of lymphoid follicle load at two biopsy sites in the antrum (A5) and corpus (B5) permits the attribution of increased odds of GC risk. This information may be applicable to gastric cancer screening and surveillance in at-risk populations.

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Different mechanisms of PTEN inactivation in sporadic cancers are tissue-specific. *C. Eng[1], O. Gimm[1], A. Perren[1], X.P. Zhou[1], G. Mutter[2].* 1) Human Cancer Genetics, Ohio State Univ, Columbus; 2) Brigham & Womens Hospital, Boston.

Germline PTEN mutations cause Cowden syndrome, a hamartoma syndrome with a risk of breast (BrCa), thyroid and endometrial cancers (EC). To determine if PTEN silencing can occur without genetic alteration, we performed genetic analysis and expression analysis by immunohistochemistry (mAb 6H2.1) in sporadic non-cultured Br Ca, thyroid tumors, EC, malignant melanoma (MM) and endocrine pancreatic tumors (EPT). There seemed to be 3 patterns of PTEN inactivation. One involves a first DNA mutation/deletion hit followed by epigenetic silencing (Br Ca, EC). No intragenic mutation was found among 33 sporadic invasive BrCa; 11 had hemizygous deletions, 50% of which showed no PTEN expression. In 33 EC, only 33% had deletions or mutations involving both PTEN alleles, yet 61% expressed no protein. In matched precancers, 55% had intragenic mutation while 75% had no expression. Hence, PTEN mutation is an early event initiating endometrial precancers and epigenetic PTEN silencing can precede genetic alteration in the earliest precancers. The second pattern involves both hits being epigenetic. Of 34 MM, 20% had hemizygous deletion and no intragenic mutation, yet 65% had no or decreased PTEN expression. Of the 5 with no PTEN expression, 4 had no structural abnormalities. Subcellular partitioning as a possible mechanism of PTEN inactivation is the third pattern. In 139 thyroid tumors, hemizygous deletion (25-60%) and decreased PTEN expression were associated (p<0.01). Decreasing PTEN expression was observed with declining degree of differentiation. Decreasing nuclear PTEN expression seemed to precede that in the cytoplasm. Among 33 EPT, only 1 had an intragenic frameshift mutation and deletion of the other allele, resulting in no PTEN expression. 8/16 informative cases had hemizygous deletion that was associated with malignant status (P<0.05) but not with PTEN expression. The remaining 32 EPT which expressed PTEN had a predominantly cytoplasmic expression, in contrast to normal islet cells, in which PTEN expression was nuclear. Overall, these observations suggest that specific mechanisms of inactivation predominate in a tissue-specific manner.

from paraffin embedded tissue. Total 129 pieces from colorectal specimens from 5 patients with UCAN who underwent colorectal resection were examined. Fluorescent PCR with 18 microsatellite markers were performed and the loss of heterozygosity (LOH) as well as microsatellite instability (MSI) was assessed. All 5 UC showed MSI in low frequency (MSI-L) from non-dysplastic inflammatory mucosa (4.3% (33/769)), dysplastic epithelium (4.1% (30/733)), to cancer (3.8% (23/603)). LOH at chromosomal 18q was detected high frequency in cancer (56.9% (87/153)) as well as dyspastic epithelium (42.2% (57/135)), while the 18q loss in non-dysplastic inflammatory mucosa was only 10.9% (18/165). These result suggest that LOH at 18q may occur in low frequency among non-dysplastic epithelium which may have a potential for malignant transformation, but frequency of 18q loss significantly elevates from premalignant dysplastic epithelium to UCAN. Frequency of MSI-L did not increased in accordance with each stages, which may suggest that MSI occurs early in UC and may not be involved in the development of cancer.

#1050 Frequent Loss of PTEN Expression is Linked to Elevated Phosphorylated AKT Levels, but not Associated with p27 and Cyclin D1 Expression, in Primary Epithelial Ovarian Carcinomas. Keisuke Kurose, Xiao-Ping Zhou, Tsutomu Araki, Stephen A. Cannistra, Eamonn R. Maher, and Charis Eng. *Beth Israel Deaconess Medical Center, Boston, MA, Edgbaston Women's Hospital, Birmingham, UK, Nippon Medical School, Tokyo, Japan, and The Ohio State University, Columbus, OH.*

PTEN (MMAC1/TEP1), a tumor suppressor gene on chromosome sub-band 10q23.3, is variably mutated and/or deleted in a variety of human cancers. Germline mutations in PTEN, which encodes a dual specificity phosphatase, have been implicated in at least two hamartoma-tumor syndromes that exhibit some clinical overlap, Cowden syndrome and Bannayan-Zonana syndrome. Among several series of ovarian cancers, the frequency of loss of heterozygosity (LOH) of markers flanking and within PTEN, is approximately 30 to 50%, and the somatic intragenic PTEN mutation frequency is less than 10%. In this study, we screened primary adenocarcinomas of the ovary for LOH of polymorphic markers within and flanking the PTEN gene, for intragenic mutations of the PTEN gene, and for PTEN expression using immunohistochemistry. Furthermore, we sought to detect the expression of the presumed downstream targets of PTEN, such as P-Akt, p27, and cyclin D1 by immunohistochemistry. LOH at 10q23 was observed in 29 of 64 (45%) cases. Of the 117 samples, 6 somatic intragenic PTEN mutations, 1 germline mutation, and 1 novel polymorphism were found in 7 (6%) patients. Immunostaining of 49 ovarian cancer samples revealed that 13 (27%) were PTEN immunostain negative, 25 (51%) had reduced staining, and the rest (22%) were PTEN-positive. Among the 44 informative tumors assessed for 10q23 LOH and PTEN immunostaining, there was an association between 10q23 LOH and decreased or absent staining ($P=0.0317$). Of note, there were 5 (11%) tumors with neither mutation nor deletion which exhibited no PTEN expression, and 10 (25%) others without mutation or deletion but had decreased PTEN expression. Among the 49 tumors available for immunohistochemistry, 28 (57%) showed P-Akt positive staining, 24 (49%) had decreased p27 staining, and cyclin D1 was overexpressed in 35 (79%) cases. In general, P-Akt expression was inversely correlated with PTEN expression ($P=0.0083$). These data suggest that disruption of PTEN by several mechanisms, allelic loss, intragenic mutation, or epigenetic silencing, all contribute to epithelial ovarian carcinogenesis, and that epigenetic silencing is a significant mechanism. The Akt pathway is prominently involved, but clearly not in all cases. Surprisingly, despite in vitro demonstration that p27 and cyclin D1 lies downstream of PTEN and Akt, there was no correlation between p27 and cyclin D1 expression and PTEN or P-Akt status. Thus, in vivo, while PTEN and Akt plays a prominent role in ovarian carcinogenesis, p27 and cyclin D1 might not be the primary downstream targets.

#1051 Complex, Risk-Associated Haplotypes of the *CDKN1A* Gene. Louis Geller, Garrett P. Larson, Steven Flanagan, and Theodore G. Krontiris. *Divisions of Molecular Medicine and Neuroscience, Beckman Research Institute of the City of Hope, Duarte, CA.*

CDKN1A (p21, CIP1, WAF1) is a potent negative regulator of the mammalian cell cycle. As a major downstream target of *TP53*, CDKN1Ap inhibits the G1-to-S cell cycle checkpoint. We recently applied a new approach to the analysis of gene-gene interactions to identify four rare pathogenic SNPs within the promoter, coding, and 3' untranslated regions of *CDKN1A*. In addition to risk-associated sequence variants, other SNPs were quite prevalent within the promoter and flanking regions. Using a combination of PCR and cloning methodologies, we identified 12 complex haplotypes from breast cancer patients and unaffected controls. The 4 cancer-associated SNPs were present on 3 different haplotypes. Over 60 SNPs, insertions, and deletions were present within the 28 kb region characterized. The region within and surrounding *CDKN1A* contained evidence for at least three independent crossovers and many gene conversion events. Linkage disequilibrium extended over the entire 28 kb region. The complex haplotype architecture at this locus, as well as the distribution of rare, cancer-associated variants over multiple haplotypes, has important implications for reduced efficiency and sensitivity of genome-wide association tests based on linkage disequilibrium of SNPs.

CLINICAL RESEARCH 5: Genitourinary Cancers

#1052 Utilization of a Urine Based Assay for BLCA-4 in the Detection of Bladder Cancer. Thu-Suong Tran Nguyen, Tracy Davido, Badrinath R. Konety, and Robert H. Getzenberg. *Memorial Sloan-Kettering, New York, NY, and University of Pittsburgh, Pittsburgh, PA.*

There currently is a need to develop novel markers for bladder cancer that can be utilized to supplement or replace cytology to detect the disease with high specificity and sensitivity. We have identified six nuclear matrix proteins (NMPs) with which it is possible to differentiate human bladder tumors from normal bladder, and which are not found in other types of cancers or normal tissues. Currently, we have peptide sequences and antibodies for several of these NMPs. In this application, we will focus on BLCA-4 the first of these proteins to be characterized. Immunoblot analysis utilizing BLCA-4 reveals that it is able to differentiate individuals with bladder cancer from those that do not have the disease and is found throughout the bladder in individuals with bladder cancer. Recently, utilizing an ELISA, we have been successful in detecting BLCA-4 in the urine and determining that the levels are significantly higher in patients with bladder cancer ($p=2.4\times10^{-6}$). All normal individuals (51) had urinary BLCA-4 levels below the prospectively utilized cutoff of 13 O.D. units per mg of protein with an average value of 4.02 ± 4.21, whereas 52 of the 54 individuals with bladder cancer had urinary BLCA-4 levels above this cutoff with an average value of 43.36 ± 49.52. The current results from our studies reveal a specificity of 100% and a sensitivity of 96.4%. Examination of individuals with spinal cord injuries has demonstrated that BLCA-4 is not elevated in individuals with cystitis. Investigations in animal models of bladder cancer indicate that the expression of this protein appears signficantly prior to the observance of grossly visible tumors in the bladder. Analysis of the cDNA sequence of BLCA-4 reveals a high homology with the ETS domain of the ELK3 oncogene for a portion of the protein. This homology suggests that BLCA-4 may serve as a regulator of gene expression in bladder cancer and we are now exploring the functional role of BLCA-4 in bladder cancer. BLCA-4 appears to be the first bladder cancer specific marker to be able to identify patients with bladder cancer from those without the disease with a high specificity and which may play an important role in the regulation of bladder gene expression. The assay that we have developed can provide an opportunity for the early and easy detection of bladder cancer. Supported by NIH CA82522.

#1053 Do Serum Metalloproteinase-2 and -9 (MMP-2, MMP-9) and CYFRA 21-1 Have Any Importance in the Surveillance of Patients with Advanced Bladder Cancer? Charalampos Andreadis, Grammati Galaktidou, Alexandros Kortsaris, Despina Mouratidou, Nikolaos Salem, and Elias Papadopoulos. *Dpt of Urology, University of Alexandroupolis, Alexandroupolis, Greece, and Theagenion Cancer Hospital, Thessaloniki, Greece.*

The contribution of metalloproteinases in the process of tumor invasion and metastasis is well known. On the other hand, the cytokeratin subgroups 7, 8, 18, 19 are expressed on the urethelium. We investigated the serum levels of MMP-2, MMP-9 and cytokeratin 19-fragments (named CYFRA 21-1) in patients with transitional cell bladder cancer. The MMP-2, MMP-9 and CYFRA 21-1 serum levels were measured by immunoassay systems in 40 patients with histologically confirmed transitional cell bladder cancer. Their data were compared with 10 healthy controls. Eighteen patients had local invasive (T2-4N0M0) disease and 22 metastatic (TanyN1-3M0-1) disease. The sera levels of CYFRA 21-1 in pts with local and metastatic disease were $0,895\pm$SD 0,55 and $7,79\pm$SD 2,66 ng/ml respectively (controls: $1,42\pm$SD 1,01 ng/ml). The MMP-2 levels were $721,6\pm$SD 240,7 and $819,4\pm$SD 314,1 ng/ml (controls: 586,9 ng/ml) and the MMP-9 $632,9\pm$SD 284,3 and $813,8\pm$SD 245,7 ng/ml (controls: $358,3\pm$SD 142,1 ng/ml). Using t-test statistical methods we found out that the CYFRA 21-1, MMP-2 and MMP-9 levels of pts with metastatic disease were significant higher than in controls [$p<0.001$, p:0.01 and p:0.01 respectively]. There was not any statistical significance between the groups of pts with local disease and controls and with the two groups of pts with local and metastatic disease. In the most of the cases there was an accordance between disease process and the serum level of the 3 tumor markers. We conclude that the sera MMP-2, MMP-9 and CYFRA 21-1 may have a significant role in the surveillance of pts with invasive bladder cancer. Is required a larger number of pts for cleaner confirmation.

#1054 Quantitative Assay to Detect Telomerase Activity in Bladder Carcinoma and Exfoliated Cells in Urine. Roberta Fedriga, Roberta Gunelli, Francesco Bacci, Dino Amadori, and Daniele Calistri. *Dept. Oncology Morgani-Pierantoni Hospital, Forlì, Italy, Dept. Urology Morgagni-Pierantoni Hospital, Forlì, Italy, Div. Patology Morgani-Pierantoni Hospital, Forlì, Italy, and Istituto Oncologico Romagnolo, Forlì, Italy.*

The standard procedure used for diagnosing of bladder carcinoma is cystoscopy; this approach is not suitable as a screening method due to its invasiveness. On the other hand, urine cytology, which is a simple and non invasive method of analysis, has poor sensitivity and produces a high percentage of false negatives. Therefore, non invasive and sensitive assays for the diagnostic screening of bladder cancer are needed. Activation of the enzyme telomerase is considered essential for the immortalization of cells through the maintenance of a constant telomere length. Telomerase activity is detected in a high percentage of tumors of various origin, including urothelial cancers. In contrast, telomerase activity is