PTEN Mutations and Microsatellite Instability in Complex Atypical Hyperplasia, a Precursor Lesion to Uterine Endometrioid Carcinoma

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Abstract

The two most common types of genetic alterations yet identified in uterine endometrioid carcinoma (UEC) are PTEN mutations and microsatellite instability (MI). Furthermore, MI-positive UECs (defined as tumors with detectable alterations at two or more different microsatellite loci) are significantly more likely to contain PTEN mutations than are MI-negative UECs. To determine whether PTEN inactivation is a relatively early event in endometrial tumorigenesis, we evaluated complex atypical hyperplasia (CAH), the direct precursor to UEC, for the presence of PTEN mutations. Mutations were present in 3 of 11 (27%) CAHs with synchronous UEC and in 4 of 18 (22%) CAHs that were not associated with invasive carcinoma. One case with synchronous CAH and UEC contained a germ-line PTEN mutation. In addition, we evaluated the same series of CAHs for MI. We identified four MI-positive CAHs with synchronous UEC but did not detect the MI phenotype in any CAHs without associated invasive carcinoma. A PTEN-mutant (germ-line mutation) MI-negative CAH was synchronous with a PTEN-mutant MI-positive UEC. These results suggest that mutation of PTEN can be an early event in the pathogenesis of UEC and may precede the development of the MI phenotype in a subset of cases.

Introduction

Endometrial cancer is the most common gynecological malignancy and is the fourth most common cancer of women in the United States (1). Endometrial carcinoma consists of several distinct histological types, of which UEC is the most common. UEC often arises from endometrial epithelium via a histopathological continuum of hyperplastic lesions, culminating in CAH. Based on clinical and histopathological data, CAH is thought to be the direct precursor to at least a subset of UECs. Clinically, women with a diagnosis of CAH frequently have synchronous UEC, and women with CAH without concurrent invasive carcinoma have a 30% risk of developing UEC if untreated (2). In addition, CAH and UEC share histopathological features (e.g., cytological atypia and glandular complexity) but are distinguished by the presence of stromal invasion in UEC (3). Although the association of CAH and UEC has been well established, the molecular genetic changes that underlie the development of CAH and the progression from CAH to UEC have not been characterized.

PTEN is a tumor suppressor gene located within chromosomal region 10q23, and PTEN mutations have been identified in multiple tumor types including glioblastoma multiforme and prostate, breast, and thyroid cancer (4, 5). Previously, we and others identified inactivating PTEN mutations in 35–50% of UECs (6–8), which represents the highest known frequency of PTEN mutations in any primary tumor. Of note, we observed PTEN mutations in low-, moderate-, and high-grade UECs, suggesting that PTEN inactivation may occur relatively early in the pathogenesis of UEC. PTEN mutations have also been identified in patients with Cowden disease, an inherited hamartomatic syndrome that carries an increased risk of thyroid, breast, and possibly endometrial cancer (9). PTEN is homologous to tyrosine kinase and dual-specificity phosphatases and has intrinsic phosphatase activity that is abrogated by specific mutations found in primary tumors (including UEC) and in Cowden disease kindreds (10).

MI was first detected in tumors from patients with hereditary nonpolyposis colorectal carcinoma and in sporadic colorectal carcinomas. Subsequently, the MI phenotype was attributed to defects in DNA mismatch repair genes (11). Endometrial carcinoma is the most common extracolonic tumor in women with hereditary nonpolyposis colorectal carcinoma (12), and approximately 20% of sporadic UECs display the MI phenotype (13, 14). These observations support the hypothesis that DNA mismatch repair deficiency has a pathogenetic role in some cases of UEC, and mutations in DNA mismatch repair genes have been identified in some MI-positive UECs (15). Moreover, recent studies have found a significant association between PTEN mutations and the MI phenotype in UEC (approximately 75–85% of MI-positive UECs and 30–35% of MI-negative UECs contain PTEN mutations), yet the basis of this association is presently unknown (6, 7).

Given the high frequency of PTEN mutations in UEC and their presence in low-, moderate-, and high-grade UECs, we chose to investigate a set of CAHs for the presence of PTEN mutations. In addition, to further characterize the association of PTEN mutations and MI in endometrial tumorigenesis, we analyzed the same set of CAHs for the MI phenotype.

Materials and Methods

Specimen Collection. Twenty-nine cases of CAH were identified in a retrospective review of the surgical pathology files of The Johns Hopkins Hospital from 1990 to 1996. Distinct areas of CAH were identified in hysterectomy specimens from 11 patients with synchronous UEC; in 5 cases, the invasive carcinomas had previously been analyzed for PTEN mutations and for MI (6, 15). The remaining 18 cases had a diagnosis of CAH without invasive carcinoma, and hysterectomy specimens were available for 17 of the 18 cases to exclude the possibility of concurrent UEC. The cases were all reviewed by the same gynecological pathologist (L. H. E.).

Microdissection and DNA Extraction. Multiple 5-μm sections of paraffin-embedded tissue were deparaffinized and stained with hematoxylin. DNA extraction was performed by a short xylene incubation followed by digestion with 200 ng/ml proteinase K in 50 mmol/liter Tris (pH 9.0), as described previously (16). Two gynecological pathologists (L. H. E. and R. J. K.) identified areas of CAH for microdissection. For cases with synchro-
nous CAH and UEC, areas free of contaminating invasive carcinoma were carefully selected. All cases were microdissected to ensure greater than 70% CAH cells. Normal endometrium and/or myometrium were microdissected for extraction of matched normal DNA. **PTEN Mutational Analysis.** Extracted CAH DNA was analyzed for intragenic mutations in the PTEN gene using exon-specific PCR amplification and direct DNA sequencing. Four CAHs had synchronous UECs with previously identified PTEN mutations (6), and for these cases, DNA sequence analysis was directed at the exon(s) containing the mutation(s) in the associated invasive carcinoma. The remaining 25 cases were assayed for intragenic PTEN mutations in exons 3, 5, 7, and 8, because these exons are most frequently mutated in UEC (6). Individual exons were amplified from 2-100 ng of genomic DNA using intron-based primers as described previously (6). PCR fragments were purified either by enzymatic treatment with shrimp alkaline phosphatase/exonuclease (Amersham Life Science, Cleveland, OH) or by phenol-chloroform extraction followed by sodium perchlorate/isopropanol precipitation as described previously (16). The purified PCR products were sequenced using the ThermoSequenase kit with incorporation of 32P-labeled deoxyribonucleotides (Amersham Life Science). The exons were sequenced using one or both of the primers used for amplification, except for exon 8, which was sequenced using a primer specific for sequencing (17). The radio-labeled PCR products were fractionated by electrophoresis on a denaturing 6% polyacrylamide gel with 8 mol/liter urea and visualized by autoradiography. A negative control was included with each PCR reaction and then sequenced to exclude the possibility of contamination. Mutations were reamplified from genomic CAH DNA and resequenced to ensure reproducibility, and normal DNA was amplified and sequenced to determine whether mutations were germ-line or somatic in origin. All insertion and deletion mutations were reamplified and cloned using the TA Cloning Kit (Invitrogen, Carlsbad, CA), and multiple individual clones were sequenced to characterize the exact nature of each mutation.

**MI Analysis.** Paired normal and CAH DNA were analyzed for MI by PCR amplification of eight microsatellite loci. Seven microsatellite markers (2S147, 18S58, 18S69, 2S119, 2S123, 10S197, and 13S175) on four different chromosomes (MAP pair primers; Research Genetics, Huntsville, AL) and an additional microsatellite marker consisting of a (AT) repeat within the gene (chromosome 18q) were amplified with incorporation of [32P]dCTP (10 mCi/ml; Amersham Life Science) as described previously (13). Cases were considered MI positive if they showed alterations in the size of microsatellite sequences for at least two different loci.

**Results**

**PTEN Mutational Analysis.** Four CAHs were synchronous with PTEN-mutant UECs, and in these cases, only the exon(s) with mutations in the corresponding UEC was sequenced. In two (cases C and E) of the four cases, sequence analysis revealed identical PTEN exon 5 mutations in the CAH and synchronous UEC. Normal DNA was sequenced for both cases to determine whether the mutations were germ line or somatic in origin, and in case C, the identical 75-bp deletion was found in the CAH, the UEC, and in multiple types of normal tissue. This deletion includes the entire highly conserved core phosphatase domain of the Pten protein. Sequence analysis of case E revealed a C-to-T transition in the 1518CAH and the UEC, which predicts a proline-to-leucine substitution that was not present in matched normal DNA. The remaining seven CAHs with synchronous UEC were analyzed for mutations in exons 3, 5, 7, and 8 of the PTEN gene. Sequence analysis of case H demonstrated a T-to-C transition within exon 3 of the PTEN gene that is predicted to cause a tyrosine-to-histidine substitution within the portion of the Pten protein that is homologous to tensin. This mutation was detected in CAH DNA from case H but not in matched normal DNA. In sum, 3 mutations were found in 11 cases of CAH with synchronous carcinoma (27.2%; Table 1).

Exons 3, 5, 7, and 8 of the PTEN gene were amplified and directly sequenced from 18 CAHs that were not associated with invasive carcinoma. Intragenic PTEN mutations were found in 4 of 18 cases (22.2%). Of the four mutations, three were frameshift mutations, and one was a missense mutation (Table 2). The missense mutation (case 15) is a G-to-T transversion within the highly conserved phosphatase domain that is expected to result in an arginine-to-leucine substitution at codon 130 (Fig. 1). This missense mutation has been identified in a Cowden disease kindred (18). The remaining insertion and deletion mutations are predicted to lead to truncated proteins. To further characterize the frameshift mutations, the specific exon for each mutation was reamplified, the PCR products were cloned, and multiple clones were sequenced. The specific insertion and deletion mutations are detailed in Table 2 (cases 5, 9, and 14). Sequence analysis of matched normal DNA for each case was negative for mutation.

**MI Analysis.** Ten of 11 CAHs with synchronous UEC were informative at 5 or more microsatellite loci (Table 2). In four cases, the CAHs were synchronous with MI-positive UECs, and in two of these cases, the CAHs were also MI positive (Fig. 2). We also identified the MI phenotype in two of six CAHs synchronous with UECs whose MI status is not known. Five CAHs synchronous with invasive carcinoma showed a shift at a single microsatellite locus (one case was informative at only two loci), and two cases were negative at all loci tested. Fifteen of 18 CAHs that were not associated with UEC were informative for at least 5 microsatellite loci, and not one of these cases met the criteria for MI (Table 2). Two of these cases showed microsatellite alterations at a single locus. The remaining 13 informative cases did not show a shift at any loci tested.

We detected a PTEN mutation and the MI phenotype in both the CAH and synchronous UEC in one case. We did not identify PTEN mutations in the three remaining MI-positive CAHs with synchronous invasive carcinoma. One MI-negative PTEN-mutant (germ-line mutation) CAH was synchronous with a MI-positive PTEN-mutant UEC (Case C). The CAH showed a shift at one of five microsatellite loci, whereas the UEC displayed a shift at all five microsatellite loci tested. Each of the four PTEN-mutant CAHs not associated with invasive carcinoma was informative at a minimum of six microsatellite loci, and none showed a shift at a single locus tested.

<table>
<thead>
<tr>
<th>Case</th>
<th>CAH PTEN gene status</th>
<th>UEC PTEN gene status</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td>No mutation</td>
<td>No mutation</td>
</tr>
<tr>
<td>B</td>
<td>Wild-type X3 and X7</td>
<td>X3 DE A (62 or 63)</td>
</tr>
<tr>
<td>C</td>
<td>X5 DE° 75bp (121-145) in frame</td>
<td>X5 DE 75bp (121-145) in frame</td>
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<tr>
<td>D</td>
<td>Wild-type X4</td>
<td>X4 DE 2bp AA (73 or/and 74) Stop (76)</td>
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<tr>
<td>E</td>
<td>X5 MS CCA-CTA-Pro-Leu (95)</td>
<td>X5 MS CCA-CTA-Pro-Leu (95)</td>
</tr>
<tr>
<td>G</td>
<td>No mutation</td>
<td>NA</td>
</tr>
<tr>
<td>H</td>
<td>X3 MS TAC-CAC-Tyr-His (68)</td>
<td>NA</td>
</tr>
<tr>
<td>I</td>
<td>No mutation</td>
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</tr>
<tr>
<td>J</td>
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</tr>
<tr>
<td>K</td>
<td>No mutation</td>
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° DE, deletion; MS, missense; NS, nonsense; NA, not analyzed.
Table 2. PTEN Mutations and Microsatellite Instability in Complex Atypical Hyperplasias

<table>
<thead>
<tr>
<th>Case</th>
<th>CAH with synchronous UEC</th>
<th>PTEN Mutation*</th>
<th>Microsatellite Loci</th>
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<tr>
<td>A</td>
<td>Yes*</td>
<td>neg</td>
<td>2S147 18S58 AT</td>
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<td>B</td>
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<td>neg</td>
<td>18S69</td>
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<tr>
<td>C</td>
<td>Yes*</td>
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<tr>
<td>E</td>
<td>Yes*</td>
<td>neg</td>
<td>10S197</td>
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<tr>
<td>F</td>
<td>Yes*</td>
<td>neg</td>
<td>13S175</td>
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<td>I</td>
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<tr>
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</table>

* The associated UECs are MI positive.

Discussion

In our previous study, PTEN mutations were identified in low-, moderate-, and high-grade tumors, suggesting that PTEN inactivation may be a relatively early alteration in the molecular pathogenesis of UEC (6). To determine whether PTEN alterations preceed the development of invasive carcinoma, we analyzed CAH, an in situ precursor to UEC. Concordant PTEN mutations were found in two of four CAHs with synchronous UECs, suggesting that PTEN inactivation can occur early in endometrioid tumorigenesis. This prompted a search for PTEN mutations in a larger series of CAHs. We identified intragenic PTEN mutations in 3 of 11 (27.2%) CAHs with synchronous UEC and in 4 of 18 (22.2%) CAHs not associated with invasive carcinoma. Compared with the frequency of PTEN inactivation in UEC (35-50%), the observation that 22-27% of CAHs contain PTEN mutations provides evidence that such alterations can occur early in endometrial tumorigenesis. As many as half of all PTEN mutations may be present in CAHs in the absence of an invasive component, suggesting that PTEN inactivation may influence the development of CAH and/or facilitate the progression from CAH to UEC. This is in contrast to data in glial tumors, where PTEN mutations have been found in anaplastic astrocytomas and in glioblastomas but not in low-grade gliomas, and in prostate cancer, where PTEN inactivation is also thought to be a relatively late step (19, 20).

In this study, we chose to limit our analysis to the four exons of the PTEN gene (exons 3, 5, 7, and 8) with the highest frequency of mutation in UEC. Thus, it is possible that we failed to identify all of the intragenic PTEN mutations present in our series of CAHs, particularly because frameshift mutations have been identified throughout the entire coding region of the gene in several tumor types (21). In addition, we did not attempt to identify homozygous and/or hemizygous deletions of the PTEN gene, which have been observed in other tumors (5, 22) but not in UEC. Consequently, the reported frequency of PTEN inactivation in CAH may be a slight underestimation.

We identified a germ-line PTEN mutation in one patient with CAH and concurrent UEC. This is the first report to our knowledge of a patient with a germ-line PTEN mutation who presented with endometrial carcinoma, although two studies have detected germ-line PTEN mutations in Cowden disease patients with endometrial carcinoma or with both breast and endometrial cancer (23, 24). Germ-line PTEN mutations have been identified in at least two different genetic syndromes, indicating that inherited mutations in PTEN may be pleiotropic. Further investigation will be needed to determine whether there are families with germ-line PTEN mutations who have an increased susceptibility to endometrial carcinoma.

Our analysis of MI in CAH clearly demonstrates that the MI phenotype can be detected in some CAHs associated with concurrent invasive carcinoma. We detected the MI phenotype in two of four CAHs synchronous with MI-positive UECs. In addition, two of six CAHs synchronous with invasive carcinomas whose MI status was not known (these UECs are currently being evaluated) were MI
positive. This observation suggests that the acquisition of the MI phenotype is an early event in the progression of some but not all MI-positive endometrioid carcinomas. Although our analysis identified several MI-positive CAHs that were synchronous with UEC, we did not identify the MI phenotype in any CAHs without associated invasive carcinoma. This is consistent with previous studies, one that identified three MI-positive CAHs synchronous with MI-positive UECs, and a second that did not detect the MI phenotype in two CAHs adjacent to MI-positive carcinomas (25, 26). However, previous studies did not examine cases exclusively with CAH. The MI phenotype is commonly thought to reflect defects in DNA mismatch repair, but its absence may not necessarily imply an intact DNA mismatch repair pathway. DNA mismatch repair defects must conceivably be present for a finite period of time to facilitate the development of the MI phenotype; which is detectable as alterations in the length of at least two distinct microsatellite DNA sequences. In five CAHs synchronous with invasive carcinoma and in two CAHs not associated with UEC, we detected a shift at a single microsatellite locus. This observation may reflect the inherent evolution of microsatellite loci, or it may indicate an early expression of the MI phenotype in lesions that have recently acquired defects in DNA mismatch repair. Alternatively, the absence of the MI phenotype in CAHs without associated carcinoma may reflect an accelerated rate of tumorigenesis in MI-positive lesions, as has been proposed for colorectal cancer (11). The rapid accumulation of cancer-causing gene mutations in MI-positive CAHs would facilitate the progression to invasive carcinoma, making the detection of MI-positive CAHs without associated UEC very unlikely. A more sensitive assay for detecting subtle changes in microsatellite loci or a direct assay for DNA mismatch repair deficiency in primary tumors could determine whether DNA mismatch repair defects are present in CAHs before they progress to invasive carcinoma.

The association of PTEN mutations and the MI phenotype in UECs leads one to speculate that the PTEN gene may be a preferential mutational target in endometrial tumors with defects in DNA mismatch repair. However, the finding that the frequency of PTEN mutations (4 of 18 cases) is greater than the frequency of the MI phenotype (0 of 15 cases) in CAHs not associated with invasive carcinoma suggests that PTEN inactivation may precede the development of MI in some sporadic MI-positive UECs. In addition, we identified one MI-negative PTEN-mutant CAH (the PTEN mutation was germ line) that was synchronous with a MI-positive PTEN-mutant UEC. These possibilities are not mutually exclusive, and the order of the two events in the development of endometrial carcinoma may not be critical.

In conclusion, this study clearly demonstrates that PTEN mutations can occur relatively early in endometrial tumorigenesis and may point to a cellular pathway that is fundamentally important in the development of endometrioid carcinoma.

Acknowledgments

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References

PTEN MUTATIONS AND MI IN CAH


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