Somatic Mutation of PTEN in Vulvar Cancer


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ABSTRACT

PTEN, a candidate tumor suppressor gene located at chromosome 10q23.3, has been shown to be mutated in ~40% of endometrial cancers. Such mutations have also been identified in endometrial hyperplasia, indicating that inactivation of the PTEN tumor suppressor gene is an early event in the genesis of some endometrial cancers. In this study, we have extended the analysis of PTEN in gynecological cancer to include adenocarcinoma of the cervix and vulvar carcinomas. Microdissected tissue (including normal tissues), preneoplastic, and neoplastic lesions were analyzed from 9 patients with cervical cancer and 10 patients with vulvar cancer. Only 1 cervical adenocarcinoma displayed a PTEN mutation. In contrast, five of eight vulvar carcinomas studied harbored PTEN mutations. Alterations were identified in carcinoma in situ as well as squamous cell carcinoma of the vulva. In two patients, PTEN mutations were identified in mucosal regions with mild or focal dysplasia. These results suggest that PTEN is frequently altered in vulvar carcinomas and can be found associated with early dysplastic changes in vulvar mucosa.

INTRODUCTION

PTEN encodes a dual-specific phosphatase, displaying homology to the cytoskeletal proteins tensin and auxilin. Since its discovery (1, 2), mutations in the PTEN gene have been identified in numerous cancers (3) including glioblastomas (4, 5), melanomas (6), prostate carcinomas (7, 8), and endometrial carcinomas (9–12). In most cancers, PTEN alterations have been identified as a late-stage event, consistent with its reported involvement in the regulation of migration and invasion (13–15). In contrast, PTEN mutations have been identified in endometrial hyperplasia (16), a premalignant precursor of invasive endometrial adenocarcinoma. Hence, PTEN mutations are considered to be an early and frequent event in endometrial neoplastic progression.

Since the time of these observations, numerous investigations have extended these studies to include alternative gynecological malignancies representing different histological tumor types of ovarian and cervical carcinomas (17–23). Although PTEN mutations have been identified in such lesions, the frequency of change was considerably lower than that reported for endometrial carcinomas (9–12). In this study, we have evaluated the status of PTEN in vulvar tumors and adenocarcinomas of the cervix, two gynecological lesions not reported previously. We report a low frequency of PTEN mutations in cervical adenocarcinomas, contrasting with vulvar carcinomas, where PTEN mutations were identified in 60% of the lesions studied. In addition, PTEN mutations were identified in hyperplastic and dysplastic lesions of the vulva, implicating this molecular change as an early event in vulvar neoplastic progression.

MATERIALS AND METHODS

Cell Lines

Human breast cancer cell line MDA-MB-468 and prostate cancer cell lines PC-3 and LNCaP were included as controls in PTEN analysis based on previous reports of a deletion in exon 4, a homozygous deletion in exons 3 through 9, and a mutation in exon 1 of PTEN (24), respectively.

Extraction of DNA

Cell Lines. Cultured cells were incubated in lysis buffer [10 mM Tris-HCl (pH 8.3), 100 mM NaCl, 1 mM EDTA, 1% SDS, and 100 μg/ml proteinase K] overnight at 37°C. High molecular weight DNA was isolated after phenol-chloroform extraction and ethanol precipitation.

Tumor Tissue. H&E-stained sections of endometrium, vulva, and cervix were reviewed by a pathologist to identify and characterize malignant lesions within each tissue section. Normal, preneoplastic, and neoplastic regions within a section were marked by the pathologist on a reference slide. This slide was used as a guide to dissect individual tissue areas from one tissue block. Each dissected region of tissue was re-embedded, sectioned, and stained to confirm the pathology. Three to five 8-μm tissue sections were cut from each tissue block for extraction of DNA, and a final section was stained to compare the pathology of tissue with the reference slide. Tissue sections were incubated in 200 μl of lysis buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, 0.45% Tween 20, and 0.5 mg/ml proteinase K] for 2 h at 55°C with regular shaking. The mixture was boiled for 15
min, and 10 μl of this preparation were used as the substrate for DNA amplification. After the addition of 1 mM EDTA, the remainder of the DNA was stored at −20°C.

PCR

Primer sequences for amplification of PTEN are shown in Table 1. Overlapping primers were designed for exons 5 and 8 to decrease the size of the PCR product for SSCP analysis. PCR amplification was carried out in a 25-μl volume containing 100 ng of genomic cell line, normal or tumor DNA, 1× PCR buffer containing 15 mM MgCl2, 200 μM each of dATP, dGTP, and dTTP, and 120 μM dCTP, 1 unit of Taq DNA polymerase (Perkin-Elmer Gene Amp, Foster City, CA), 0.4 μM of forward and reverse primers, and 2.0 μCi of [γ-32P]dCTP (Amersham, Arlington Heights, IL). After an initial denaturation at 95°C for 5 min, 35 cycles at 95°C for 60 s, specific annealing temperature (parentheses in Table 1) for 45 s and 72°C for 15 s were performed. The last cycle was followed by a 10-min extension at 72°C.

SSCP Analysis

Four μl of the PCR product were mixed with 16 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol FF), heat-denatured at 95°C for 5 min, and rapidly loaded onto nondenaturing polyacrylamide gels. All samples were run under two different gel conditions: 6% acrylamide and 10% glycerol run at room temperature with a cooling fan at 30 W; and 8% acrylamide with no glycerol run at 4°C at 30 W. The gels were dried at 80°C and autoradiographed using Kodak BioMax film (Eastman Kodak, Rochester, NY) for 24–96 h. The tumor was scored as having a mutation if an abnormal SSCP pattern was detected in two or more repeat experiments involving separate PCR reactions.

DNA Sequencing

Aberrantly migrating DNA fragments were excised from the gel and extracted after boiling in water for 15 min. DNA was ethanol-precipitated in glycogen and used for PCR as described above. One μl of the PCR product was subcloned and used in the transformation of competent cells provided in the TA Cloning kit (InVitrogen, San Diego, CA) according to the manufacturer’s instructions. Colonies containing PCR product were grown overnight in Luria-Bertani medium (tryptone 1% w/v, yeast extract 0.5% w/v, NaCl 0.5% w/v, and 10 mM NaOH), and the plasmids were extracted. Sequencing was performed according to the dideoxy-chain-termination method using a Sequenase Version 2.0 kit (United States Biochemical Corp., Cleveland, OH) according to the manu-
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RESULTS

Molecular analysis of individual exons of PTEN was performed using PCR-SSCP (Fig. 1). Eleven primer sets were used to amplify the nine exons of PTEN (Table 1). Sections from each tissue type were assessed by the pathologist identifying regions of normal, preneoplastic, and carcinoma tissue for dissection. Multiple lesions within the same section were isolated and analyzed separately. In all, 48 tissue samples have been analyzed for the presence of PTEN mutations.

Endometrial Cancer. We investigated the status of PTEN in endometrial lesions from nine patients. Lesions from eight of the nine patients represented invasive adenocarcinomas, with one patient lesion displaying atypical hyperplasia. Two of the invasive carcinomas had accompanying preneoplastic lesions including hyperplasia and hyperplasia with focal atypia. Normal tissue from one patient was analyzed alongside an atypical hyperplastic lesion taken from the same tissue section. Analysis of SCC tumor tissue taken from the same section. Each of the two additional patients were recorded as CIS (Table 2).

Table 2 Patient number and pathology of lesions evaluated

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Condition</th>
<th>Patient no.</th>
<th>Condition</th>
<th>Patient no.</th>
<th>Condition</th>
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<td>ACIS</td>
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<td>18</td>
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<td>28</td>
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*Bold lettering indicates the presence of a PTEN mutation.

Vulvar Cancer. Vulvar tumors from 10 patients were isolated and analyzed for PTEN alterations. Lesions included 8 SCCs, 1 with normal tissue, 1 with CIS, and 2 others with dysplasia and/or CIS taken from the same section. Tissues from two additional patients were recorded as CIS (Table 2).

PTEN alterations were identified in 6 of 10 patients (60%), 5 with SCC and 1 with CIS. In patient 10, a point mutation in exon 2 (Fig. 3C), changing a phenylalanine to a serine, and a 5-bp deletion in intron 3 was identified in mucosa displaying focal mild dysplasia (VIN I) but not in tumor tissue taken from the same section. Analysis of SCC from a different part of the tumor (Fig. 2C) revealed the presence of the same point mutation and intronic deletion as was recorded in the dysplastic tissue from this patient (Fig. 2D). In patient 11, three different point mutations were identified in dysplasia (Fig. 2E, VIN III), CIS, and tumor tissue microdissected from the same section. Each of the point mutations was recorded in different exons with two point mutations identified in exon 1 of dysplastic tissue. Review of slides prepared during the harvesting of sections for DNA revealed a focus of high-grade dysplasia (Fig. 2E, VIN III) surrounded by normal tissue (Fig. 2F). In the SCC from patient 12, a point mutation in exon seven and a 2-bp deletion in the same exon were identified (Fig. 3D), resulting in a premature stop codon. No PTEN mutations were identified in normal or CIS tissue from this patient. The same deletion event in exon 7 was recorded in the CIS lesion from patient 16. This deletion occurred within a tract of four TG repeats and was not detected in normal epithelium from this patient. A single bp deletion in a (A)n tract in exon 8 of SCC from patient 18 resulted in a premature stop codon. Finally, multiple bp insertions in intron 2 of PTEN were identified in patient 19, predicted to result in a splicing error.

In three patients (nos. 2, 10, and 11), we identified the same silent variants in exon 8. These involved bp 277 (ACA>GAC, Thr>Thr), 290 (GTA>GTT, Val>Val), 295 (CTA>CTT,
Leu>Leu), and 298 (CAA>CAG, Gln>Gln), representing a possible polymorphism.

**Cervical Cancer.** Of nine cervical adenocarcinomas studied, five had accompanying normal tissue from the same section. Four cases represented invasive adenocarcinoma with three accompanied by adenocarcinoma in situ and normal from the same tissue section. Only one carcinoma was identified as harboring a PTEN mutation, patient 28, where two point mutations were identified in exon 9. No PTEN mutations were found in normal tissue from five patients or in five patients with adenocarcinoma in situ.

**Sensitivity of PTEN Mutation Assay.** Although every effort was made to dissect discrete lesions from different tissues, normal tissue components are always present and are contaminants in the analysis. To assess the sensitivity of the assay used for the detection of PTEN mutations in tissue, we performed a mixing experiment of normal and mutant DNA to establish the percentage representation of mutant required for identification in SSCP analysis. Fig. 4 shows these results where mutant PTEN-PCR product is identifiable when present as 5% of the total DNA.

**DISCUSSION**

Mutations in PTEN have been identified in ~40% of endometrial cancers (9–12). Maxwell et al. (16) have identified PTEN mutations in preneoplastic lesions of endometrial tissue, including hyperplasia and hyperplasia with atypia, demonstrating the involvement of PTEN at an early stage of neoplastic progression. Investigation of PTEN alterations in alternative gynecological malignancies, including ovarian and cervical carcinomas, have demonstrated the presence of mutations at a low frequency in a small subset of tumors (23). Epithelial ovarian tumors exist as four major histological types, with PTEN mutations recorded mainly, if not exclusively, in ovarian tumors of endometrioid origin (20). Similar studies in SCCs of the cervix have revealed a low incidence of PTEN mutations in these lesions (9, 21).

In this study, we have analyzed PTEN in a small number of endometrial adenocarcinomas and two hyperplastic lesions with atypia. Mutations at this locus were identified in three of nine patients, consistent with reports in the literature where PTEN mutations were detected in 33–55% of the endometrial...
Two of the three hyperplastic lesions in this study harbored PTEN mutations, one of which displayed mutations in exons 2 and 8 in both the hyperplastic and adenocarcinomatous lesion from the same patient. These results are supportive of PTEN alterations being an early event in endometrial cancer with a primary role in neoplastic progression.

Extension of this study to include adenocarcinoma of the cervix and vulvar tumors, two lesions in which the PTEN status has not been reported, revealed a low frequency of PTEN alterations in cervical adenocarcinomas with only one of nine cases identified with a PTEN mutation. Analysis of cervical adenocarcinoma in situ from eight patients demonstrated no PTEN mutations. The only PTEN change identified was found in an adenocarcinoma, where two missense mutations were recorded in exon 9.

In contrast, 6 of 10 patients with vulvar lesions harbored PTEN mutations. These included SCC, CIS, and tissue originally identified as normal mucosa in reference slides. In the two cases of putative normal tissue harboring PTEN mutations (patients 10 and 11), a review of the slides, prior to and after harvesting sections for DNA extraction, revealed the presence of focal dysplasia that was not present in the reference slide. The intensity of the aberrant band detected in SSCP in both cases suggests a low representation of mutation, approximately 5–10%, consistent with the representation of focal dysplasia in the tissue. Analysis of tissue with focal dysplasia in patient 10 revealed two PTEN changes, one a missense mutation in exon 2 changing a phenylalanine to a serine and a second mutation in intron 3, the significance of which is not known. No PTEN changes were detected in the SCC dissected from the same section. However, analysis of a second region of SCC from a different tissue block of patient 10 revealed the same two mutations as were recorded in the corresponding dysplastic mucosa. Although we do not know the spatial relationship between these two lesions, this result suggests that PTEN mutation may be an early event in neoplastic progression in the vulva. In addition, the finding of two SCCs with different PTEN molecular profiles demonstrates the polyclonal origin of tumor lesions within this tissue. The idea of polyclonality is supported by results from patient 11 where dysplasia, CIS, and SCC from the same section harbored different PTEN mutations. As before, sections of putative normal mucosa from deeper in the tissue block were found to harbor a focus of high-grade dysplasia with sequencing identifying three missense mutations, two in exon 1 and one in exon 8. The CIS and SCC lesions from this patient harbored missense mutations in exons 2 and 4, respectively. The PTEN mutation identified in CIS from this patient was located in codon 44, the site of a rare polymorphism found in this gene (8). The polymorphism in codon 44 is characterized by a C>T at the third base of this codon, resulting in a silent variant. The PTEN mutation identified in the CIS of patient 11 represents a G>A transition in the second base of codon 44, changing a glycine to aspartic acid. Interestingly, all five mutations identified in this patient’s

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5 Unpublished observations.
same deletion was identified in SCC of the vulva (patient 12) and an endometrial lesion displaying atypical hyperplasia (patient 2). This deletion event occurs within a tract of four TG repeats. A second deletion event involving disruption of the (A6) repeat, beginning at nucleotide 963, was identified in patient 18 and has been reported before in numerous different cancers (23). In endometrial tumors, the occurrence of an insertion or deletion of 1 bp within the (A6) repeats, encompassing codons 265–267 or 321–323, is linked to high levels of microsatellite instability. Mononucleotide runs are considered to be sites of slipped misalignment of the template DNA strand, and there is a reported clustering of mutations reported in this region of exon 7 (23). To our knowledge, alterations in mismatch repair genes resulting in microsatellite instability have not been reported in vulvar cancer. Finally, we identified multiple insertion events associated with intron 2 of PTEN in patient 19. These events are predicted to disrupt the acceptor splice site (25) in intron 2.

Table 3  Pathology and PTEN mutations in endometrial, vulvar, and cervical carcinomas

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Pathologya</th>
<th>Exon bp</th>
<th>Codon</th>
<th>Mutation</th>
<th>Predicted effect</th>
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<td>37</td>
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<tr>
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<td>9</td>
<td>1093</td>
<td>365</td>
<td>GTA→ATA</td>
</tr>
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</table>

a NL, normal; DY, dysplasia; AH, atypical hyperplasia; AC, adenocarcinoma; ND, no mutation detected.
b Focal mild dysplasia.
c SCC from the same section as normal.
d SCC from a different tumor region.
e Normal mucosa with a focus of high-grade dysplasia.

Fig. 4  Mixing experiment to determine the lower limit of representation of mutant DNA for detection in SSCP analysis. The percentages shown at the top of the gel represent the level of mutant DNA in the mix.

**tissue represent transition events, possibly involving the same etiological agent.**

Two of the five CIS lesions from vulvar tissue harbored PTEN mutations. Analysis of CIS from patient 16 showed a TG deletion in exon 7, resulting in a premature stop codon. The same deletion was identified in SCC of the vulva (patient 12) and an endometrial lesion displaying atypical hyperplasia (patient 2). This deletion event occurs within a tract of four TG repeats. A second deletion event involving disruption of the (A6) repeat, beginning at nucleotide 963, was identified in patient 18 and has been reported before in numerous different cancers (23). In endometrial tumors, the occurrence of an insertion or deletion of 1 bp within the (A6) repeats, encompassing codons 265–267 or 321–323, is linked to high levels of microsatellite instability. Mononucleotide runs are considered to be sites of slipped misalignment of the template DNA strand, and there is a reported clustering of mutations reported in this region of exon 7 (23). To our knowledge, alterations in mismatch repair genes resulting in microsatellite instability have not been reported in vulvar cancer. Finally, we identified multiple insertion events associated with intron 2 of PTEN in patient 19. These events are predicted to disrupt the acceptor splice site (25) in intron 2.

The identification of molecular events associated with vulvar cancer is limited, including alterations associated with p16, p15 (26), and cyclin D1 (27) involving small numbers of vulvar carcinomas. More studies have focused on the presence of HPV
and p53 mutations in vulvar lesions (28–34). These two oncogenic elements are linked, because the HPV protein E6 binds to and causes rapid degradation of p53. Hence, the presence and expression of HPV or mutated p53 can independently disrupt a common signaling pathway contributing to neoplastic progression. Both HPV and mutation of p53 have been identified as frequent events in vulvar cancer, where inactivation of p53, whether by mutation or interaction with viral proteins, is essential in the pathogenesis of vulvar carcinomas. Within the small sample we have analyzed, the frequency of PTEN mutations is similar to that reported for p53 in vulvar lesions. In addition, we have identified PTEN mutations in dysplastic lesions, suggesting this is a possible early event in vulvar neoplastic progression.

REFERENCES
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