Distinct PTEN mutational spectra in hereditary non-polyposis colon cancer syndrome-related endometrial carcinomas compared to sporadic microsatellite unstable tumors

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Germline PTEN mutations cause Cowden syndrome (CS) and Bannayan–Riley–Ruvalcaba syndrome (BRR), two hamartoma-tumor syndromes with an increased risk of breast, thyroid and endometrial cancers. Somatic genetic and epigenetic inactivation of PTEN is involved in as high as 93% of sporadic endometrial carcinomas (EC), irrespective of microsatellite status, and can occur in the earliest precancers. EC is the most frequent extra-colonic cancer in patients with hereditary non-polyposis colon cancer syndrome (HNPCC), characterized by germline mutations in the mismatch repair (MMR) genes and by microsatellite instability (MSI) in component tumors. To determine whether PTEN is involved in the pathogenesis of EC arising in HNPCC cases, and whether PTEN inactivation precedes MMR deficiency, we obtained 41 ECs from 29 MLH1 or MSH2 mutation positive HNPCC families and subjected them to PTEN expression and mutation analysis. Immunohistochemical analysis revealed 68% (28/41) of the HNPCC-related ECs with absent or weak PTEN expression. The remaining 27% (11/41) of tumors had normal expression and 5% (2/41) with mixed populations showing weak/absent as well as normal expression. Mutation analysis of 20 aberrant PTEN-expressing tumors revealed that 17 (85%) harbored 18 somatic PTEN mutations. All mutations were frameshift, 10 (56%) of which involved the 6(A) tracts in exon 7 or 8. These results suggest that PTEN plays a significant pathogenic role in both HNPCC and sporadic endometrial carcinogenesis, unlike the scenarios for colorectal cancer. Furthermore, we have shown that somatic PTEN mutation, especially frameshift, is a consequence of profound MMR deficiency in HNPCC-related ECs. In contrast, among 60 previously reported MSI+ sporadic ECs with 70 somatic mutations in PTEN, 39 (56%) were frameshift, of which only eight (21%) were affecting the 6(A) tracts in exon 7 or 8 (P =0.01), suggesting that PTEN mutations may precede MMR deficiency.

INTRODUCTION

Endometrial carcinoma (EC) is the most common gynecologic malignancy in the USA, with approximately 38 000 new cases diagnosed each year. Because of recent molecular epidemiologic studies, EC was found to be a likely component tumor of Cowden syndrome (CS), which is an autosomal dominant disorder characterized by multiple hamartomas and a risk of breast and thyroid tumors (1). Germline mutations of PTEN, a tumor suppressor gene on 10q23.3, are associated with 80% of CS as well as seemingly unrelated developmental disorders Bannayan–Riley–Ruvalcaba syndrome (BRR), Proteus syndrome and Proteus-like syndromes (2–6). PTEN is a phosphatase which signals down the phosphoinositol-3-kinase/Akt pathway and mediates cell cycle arrest and apoptosis (7–11).

Initial analyses revealed that somatic mutation and/or deletion of the PTEN tumor suppressor gene was present in 33–55% of low-grade as well as in high-grade endometrial cancers of endometrioid histology, which is the most common subtype (12–14). Studies selecting for the endometrioid subtype and using sensitive detection techniques combined with expression studies have yielded as high as a 93% frequency of somatic genetic and/or epigenetic inactivation of PTEN (15). More importantly, several studies provided strong evidence that loss of function of PTEN is involved in the initial stages of tumor development, even in the earliest precancers, in

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the manner of a gatekeeper for EC, at least of the endometrioid subtype (15–17).

Approximately 20–30% of sporadic ECs have a defect in DNA mismatch repair (MMR) leading to the microsatellite unstable or MSI+ phenotype. MSI has been described as the hallmark of component tumors from individuals with the autosomal dominant hereditary non-polyposis colon cancer (HNPCC) syndrome (18), characterized by germline mutations in the DNA MMR genes (19–23). EC is the most common malignancy in female patients with HNPCC (24). It was initially believed that the frequency of somatic PTEN mutations was higher in sporadic ECs with the MSI+ phenotype compared to those that were MSI− (12,13,25). However, subsequent investigations have revealed similar somatic PTEN mutation frequencies for both MSI+ and MSI− sporadic ECs (15,26,27).

Approximately 15% of sporadic CRCs exhibit the MSI phenotype (28–30); among MSI+ sporadic colon cancers, ∼19% were found to have somatic frameshift mutations in PTEN almost exclusively in one of two 6(A) tracts in exons 7 and 8 (31). In contrast, in MSI unknown or MSI− sporadic colorectal tumors, <5% have been shown to have somatic PTEN mutations, and none has occurred in any poly(N) tracts (P.L.M. Dahia and C.Eng, unpublished data) (32). Existing data in the literature to date suggests that the downstream pathways of HNPCC-related component tumors and those of their sporadic counterparts are quite different (reviewed in 33,34). Therefore, we sought to determine whether PTEN is involved in the pathogenesis of EC arising in HNPCC cases, whether the frequency and spectra of structural alterations in PTEN of ECs arising in HNPCC patients differ from those of sporadic MSI+ ECs using a mutational and expression analysis strategy, and if there is any evidence that the sequence of loss of MMR and PTEN somatic mutation differ between the two as well.

RESULTS

PTEN immunohistochemistry in HNPCC-related ECs

PTEN expression at the protein level in 41 ECs arising in MMR gene mutation positive HNPCC families was evaluated by immunohistochemical analysis. All 41 tumor sections had accompanying stroma and/or normal endometrial epithelium present, which showed strong PTEN immunostaining in the cytoplasm and the nucleus, were graded ++ and served as internal positive controls as described previously (15,35). There were no normal glands that showed decreased or no PTEN expression. If blood vessels were present, their endothelium also expressed PTEN (graded ++), as previously used as internal positive controls as well (36,37). About two-thirds of the ECs, 28 of 41 (68%), had weak (+) or absent (−) cytoplasmic PTEN staining (Fig. 1 and Table 1). Sixteen (39%) ECs lost all PTEN immunoreactivity (−) and 12 (29%) showed weak (+) cytoplasmic PTEN immunostaining.

The remaining 27% (11/41) showed normal (+++) cytoplasmic immunostaining and 5% (2/41) had mixed tumor cell populations showing weak/absent as well as normal immunostaining intensity (Fig. 1 and Table 1).

**PTEN mutations in HNPCC-related ECs**

Twenty of 28 ECs with absent or weak PTEN immunostaining had sufficient material for mutational analysis of the entire PTEN gene. Eighteen somatic PTEN mutations were found in 17 (85%) of 20 tumors (Tables 1 and 2). All 18 mutations were frameshift, 12 (67%) of which occurred in poly(A) tracts (comprising four, five or six A repeats) (Tables 1 and 2). Of note, 10 (56%) tumors had somatic deletions or insertions affecting one of the two 6(A) tracts in exon 7 or 8 (Tables 1 and 2).

Among the 17 mutation positive tumors with PTEN immunohistochemistry data, seven had decreased PTEN expression and harbored a monoallelic PTEN mutation. Nine that were shown not to express PTEN protein had a monoallelic PTEN mutation and one displayed negative immunostaining and carried two different mutations.

**Comparison of somatic PTEN mutational frequency and spectra in HNPCC-related and sporadic ECs**

The somatic PTEN mutations described previously in sporadic ECs with known MSI status are presented in Table 2. PTEN mutation frequencies ranged from 29 to 86% (12,13,26,27,38). Amongst all MSI+ sporadic ECs, including those from our own work (G.Mutter and C.Eng, unpublished data), 60 of 118 (51%) were found to have somatic PTEN mutations (Table 2). Of the 64 truncating mutations detected in 60 ECs, 39 (61%) were frameshift, and only eight of these 39 (21%) occurred in one of the 6(A) tracts in exons 7 and 8. Compared to sporadic MSI+ ECs, the frequency of PTEN mutations in ECs arising in patients with HNPCC was significantly different (P = 0.006).

**Figure 1.** PTEN protein (brown immunostaining) in HNPCC-related ECs. (A) EC with PTEN-positive cytoplasmic staining (++) in all tumor cells. (B) EC showing weak staining (+). (C) EC exhibiting negative (−) PTEN staining in all glands. Magnification, ×10.
Table 1. PTEN expression by immunohistochemistry and somatic PTEN mutations in ECs from germline MMR mutation-positive HNPCC families

<table>
<thead>
<tr>
<th>Case ID</th>
<th>Germline MMR mutation</th>
<th>MLH1 IHC</th>
<th>MSH2 IHC</th>
<th>MSH6 IHC</th>
<th>PTEN IHC</th>
<th>PTEN mutation</th>
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<tr>
<td>F1-47</td>
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<td>–</td>
<td>+/–</td>
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<tr>
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<td>–</td>
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<td>F61-24</td>
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<td>++</td>
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<td>–</td>
<td>+/–</td>
<td>++</td>
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</tr>
<tr>
<td>F93-27</td>
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<td>+</td>
<td>–</td>
<td>–</td>
<td>++</td>
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</tr>
<tr>
<td>F99-13</td>
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<td>–</td>
<td>+</td>
<td>+</td>
<td>++</td>
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<td>F105-9</td>
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<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>c.795-10 (A)/Ex7 del A</td>
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<tr>
<td>F105-24</td>
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<td>+</td>
<td>–</td>
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<td>–/+,++ mix</td>
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<td>–</td>
<td>+</td>
<td>+</td>
<td>c.1040-1(Ex9) del TC</td>
</tr>
</tbody>
</table>

Ex, exon; del, deletion; ins, insertion; blank, not done because PTEN expression is ++ or there was no nucleic acid or tissue left.

*Germline mutations 1–8 represent eight different germline mutations affecting MLH1. Mutation 1, 3.5 kb genomic deletion affecting codons 578–632 of exon 16 of MLH1; mutation 2, IVS5-1g→a; mutation 3, c.1975G→C in exon 17; mutation 4, IVS12+1g→c; mutation 5, c.320T→G in exon 4; mutation 6, IVS11-1g→a; mutation 7, IVS13-1g→t; and mutation 8, c.1975C→T in exon 17. Mutation 9 is a c.1550-1delCA in exon 10 of MSH2 (33). Mutations 1 and 2 are founder mutations in the Finnish population that account for approximately half of all HNPCC mutations found in Finland (44). Protein expression levels of MLH1, MSH2 and MSH6 are scored: +, protein expressed; –, protein not expressed; +/–, protein expressed only in some regions (33).
The proportion of frameshift mutations versus total truncating mutations between these two groups was statistically significantly different ($P = 0.0009$) as well. Of note, frameshift mutations affecting one of the two 6(A) tracts over the total number of truncating mutations (2/19) in sporadic MSI– EC would be significantly different from that in HNPCC-related EC (10/18; $P = 0.0051$).

**DISCUSSION**

In this study, we have found that somatic *PTEN* alterations occur with high frequency in both HNPCC-related ECs and sporadic MSI+ ECs. Interestingly, the actual mutation frequency of sporadic MSI– ECs is no different from sporadic MSI+ ECs. Interestingly, the spectra of somatic frameshift mutations affecting one of the 6(A) tracts over the total number of truncating mutations (2/19) in sporadic MSI– EC was greatly over-represented among HNPCC-related ECs compared to sporadic MSI+ tumors ($P = 0.01$).

Given that *PTEN* plays a major role in both sporadic and hereditary ECs, this study allowed us to determine the temporal sequence of the acquisition of MMR deficiency and somatic *PTEN* mutation, as well as the mutational spectra between HNPCC-related and sporadic MSI+ ECs. These comparisons are valid because all ECs were derived from families with HNPCC with known germline mutations in *MLH1*, *MSH2*, *MSH6* (33). Germline MMR deficiency is thus the etiological factor in the MSI phenotype in these ECs. Additionally, the sporadic counterpart tumors are MSI+, and although mutations in the *MMR* genes are rare causes of MSI in the sporadic setting, methylation of the promoter of *MLH1* has been shown to be associated with the MSI phenotype in the sporadic tumors (39). Comparison of somatic *PTEN* mutational spectra among HNPCC-related ECs and sporadic ones, both MSI+ or MSI– revealed distinct *PTEN* mutational spectra. All somatic *PTEN* mutations found in HNPCC-related ECs were frameshift. Of note, more than half occurred in one of the two 6(A) tracts in exons 7 and 8. It has been demonstrated that the development of EC in HNPCC is selectively associated with the MSH2/MSH6 protein complex deficiency (33). Interestingly, 10 of 17 (59%) of the ECs with *PTEN* frameshift mutations had loss of expression of *MLH1*, *MSH2* and/or *MSH6* detected by immunohistochemistry while only one of three (33%) without any frameshift mutation showed similar loss of expression (Table 1). However, no significant association was found between the presence of *PTEN* frameshift mutation and specific germline *MMR* mutation type (Table 1). Given the causal relationship between defective DNA MMR and slippage errors in microsatellite sequences in HNPCC (40), it is obvious that the unique frameshift mutational spectrum in *PTEN* in this HNPCC-related EC setting, at least those affecting the 6(A) repeats in exon 7 or 8, were the consequence of profound DNA MMR deficiency. This is in contrast to the mutational spectra in sporadic MSI+ ECs, where over half are frameshift mutations with only 20% occurring in one of the two 6(A) tracts. Interestingly, the spectra of somatic *PTEN* mutations in MSI+ and MSI– sporadic tumors are not statistically different in our pooled series (Table 2). Frameshift mutations within mononucleotide repeat tracts are the phenotypic signature of *MMR* deficiency (41,42). Our observations therefore suggest that HNPCC-related ECs arise because of germline mutation in one of the *MMR* genes, followed by somatic inactivation of other *MMR* genes. Because of this profound MMR deficiency, subsequent somatic mutations occur within mononucleotide tracts in putative target genes, such as *PTEN*, which might act to accelerate tumor progression. In sporadic

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**Table 2.** Comparison of somatic *PTEN* mutational spectra and frequency in HNPCC-related ECs and sporadic tumors

<table>
<thead>
<tr>
<th></th>
<th>Mutation frequency&lt;sup&gt;a&lt;/sup&gt;</th>
<th>FS mutation versus truncating mutation&lt;sup&gt;b&lt;/sup&gt;</th>
<th>FS mutation in 6(A) tracts&lt;sup&gt;c&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>HNPCC-related EC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sporadic MSI+ EC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>60/118 (51%)</td>
<td>39/64 (61%)</td>
<td>8/39 (21%)</td>
</tr>
<tr>
<td><em>P</em>-value</td>
<td>0.006</td>
<td>0.0009</td>
<td>0.01</td>
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<tr>
<td>Sporadic MSI– EC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>24/66 (36%)</td>
<td>9/19 (47%)</td>
<td>2/9 (22%)</td>
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<tr>
<td><em>P</em>-value</td>
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<td>0.2929</td>
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<tr>
<td>Sporadic MSI+ EC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>17/20 (85%)</td>
<td>18/18 (100%)</td>
<td>10/18 (56%)</td>
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<tr>
<td><em>P</em>-value</td>
<td>0.0002</td>
<td>0.0004</td>
<td>0.2</td>
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<sup>a</sup> Proportion of tumors with at least one somatic intragenic *PTEN* mutation.

<sup>b</sup> Proportion of total number of any frameshift (FS) mutation over total number of truncating mutations.

<sup>c</sup> Proportion of frameshift mutations affecting one of the two 6(A) tracts in exons 7 and 8.

<sup>d</sup> (12,13,26-27,38; G.Mutter and C.Eng, unpublished data).
MSI+ endometrial carcinogenesis, on the other hand, the first
‘hit’ is not necessarily an alteration of MMR deficiency.
Somatic PTEN mutation may be the first hit in ~50–80%,
estimated as the proportion of tumors with PTEN alterations
that do not show frameshift mutations (Table 2). This is
corroborated by our previous findings that PTEN mutation or
epigeneric PTEN silencing can occur in isolated normal
dometrial glands in up to 40% of premenopausal women
(35) and in 75% of endometrial intraepithelial neoplasias (15).
Thus, in at least half of sporadic MSI+ ECs, somatic PTEN
mutation may precede alteration in MMR. In 20% (Table 2) of
MSI+ sporadic tumors, MMR deficiency may precede somatic
PTEN mutation, although the frameshift mutations in the 6(A)
tracts may be coincidental since approximately the same
frequency of frameshift mutations in one of these two tracts
also occurs in MMR- sporadic ECs.

In the 10 HNPCC ECs with no PTEN expression, only one
had two structural mutations, the remaining nine of which had
monoallelic PTEN mutations. This suggests that even in the
MMR-deficient setting, the complete silencing of PTEN is
accomplished by a combination of structural alteration and
epigenetic silencing. This is similar to the sporadic setting,
where only approximately one-quarter of the tumors with no
PTEN expression were shown to have two genetic hits (15).

In summary, we demonstrate that PTEN inactivation plays
important pathogenic roles in both HNPCC-related ECs and
sporadic tumors. Importantly, somatic PTEN mutational
spectra and frequency of HNPCC-related ECs differ from
those of sporadic ECs with MSI+ phenotype. These findings
suggest that somatic PTEN mutation, especially frameshift, is
a consequence of profound MMR deficiency in HNPCC-related
ECs. In contrast, in sporadic ECs, somatic PTEN mutation or
epigeneric inactivation can be viewed as one of the earliest, if
not the earliest, events that may precede MMR deficiency.

MATERIALS AND METHODS

Tissue samples

Forty-one ECs were collected from a series of well-characterized
HNPCC families carrying germline mutations in either MLH1
or MSH2 (33). The mutations and their present designations
are as described (33) (Table 1). Paraffin-embedded tissue
blocks were cut to 4 µm sections and mounted on Superfrost
Plus slides (Fisher Scientific, Pittsburgh, PA) for immuno-
histochemical studies. Genomic DNA from the paraffin-embedded
tissue blocks or blood was extracted as described previously
(43) and subjected to mutation analysis as described below.

Immunohistochemistry

Immunohistochemical staining for PTEN using the specific
monoclonal antibody 6H2.1 was carried out as described
previously (15,36). Briefly, the sections were deparaffinized
and hydrated by passing through xylene and a graded series of
ethanol to water. Antigen retrieval was performed for 20 min at
98°C in 0.01 M sodium citrate buffer pH 6.4 in a microwave
oven and incubating the sections in 0.3% hydrogen peroxide.
After blocking for 30 min in 0.75% normal horse serum, the
sections were incubated with 6H2.1 (dilution 1:100) overnight
(or 16 h) at 4°C. The sections were washed in PBS pH 7.3 and
then incubated with biotinylated horse anti-mouse IgG
followed by avidin peroxidase using the Vectastain ABC elite
kit (Vector Laboratories, Burlingame, CA). The chromogenic
reaction was carried out with 3’-3’ dianinobenzidine, which
gives a brown reaction product. The immunostaining patterns
and intensities were scored in methyl green-counterstained
slides by two independent observers (X.-P.Z. and C.E.) using
endometrial stroma and/or normal endometrial epithelium as
an internal positive control. Cytoplasmic immunostaining
intensities equal to endometrial stroma and/or normal endometrial
epithelium in a particular sample were scored as ++; weak or
decreased staining intensity as +; and no immunostaining as –.

PTEN mutational analysis of HNPCC-related ECs

DNA from 20 HNPCC-related ECs with absent or weak PTEN
expression were subjected to PTEN mutation analysis of all
nine coding exons, exon–intron junctions and flanking intronic
sequences using PCR-based DGGE and semi-automated
sequencing as described previously (15). Extensive analysis in
the past has demonstrated that PTEN-expressing ECs never
harbor PTEN mutations (15,35).

PTEN mutation data for sporadic ECs

All somatic PTEN mutations found in sporadic ECs with
known MSI status have been selected from our own work
(G.Mutter and C.Eng, unpublished data) as well as from the
existing literature (12,13,26,27,38).

Statistical analysis

Fisher’s exact test was used for statistical analysis. Differences
were considered significant if the two-tailed P-value was <0.05.

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