Colorectal Carcinomas and PTEN/MMAC1 Gene Mutations

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ABSTRACT

Purpose: PTEN/MMAC1/TEP1 is a tumor suppressor gene encoding a dual-specificity protein phosphatase with homology to the cytoskeleton proteins, chicken tensin and bovine auxilin. PTEN mutations have been described in several types of human cancer. Recently, mutations at an (A)6 repeat of PTEN exons 7 and 8 in colorectal cancer (CRC) patients with microsatellite instability have been detected. Moreover, an involvement of the transforming growth factor (TGF)-β pathway in hereditary colorectal syndromes has been proposed.

Experimental Design: In this study, we analyzed the frequency of PTEN gene mutations in 36 CRC patients and 5 colon cancer cell lines. Furthermore, in 16 of 36 patients, microsatellite instability and TGF-β receptor II analysis was possible. The study was performed by PCR and automated sequencing of the entire coding region of the PTEN gene.

Results: About 17% of colon cancer patients and one of five (HSR 320) colon cancer cell lines had mutations. Mutations were detected only among patients with locally advanced or metastatic CRC. PTEN mutations were detected in three of five (60%) patients showing both microsatellite instability and TGF-β receptor II mutations. These patients presented with advanced or metastatic CRC.

Conclusions: Overall, these results show that PTEN alteration together with TGF-β pathway inactivation could contribute to tumorigenesis and metastatic spread of sporadic and microsatellite unstable CRC.

INTRODUCTION

Recently, a new tumor suppressor gene, PTEN/MMAC1/TEP1, has been identified on chromosome 10q23 (1–3). The PTEN gene contains nine exons and encodes a 403-amino-acid protein with the NH2-terminal region characterized by a typical motif HCXXGXXR found in tyrosine phosphatase and dual-specificity protein phosphatases (4). The NH2-terminal region of the PTEN/MMAC1 protein has high sequence homology to the cytoskeleton proteins chicken tensin and bovine auxilin (1, 2).

As a phosphatase, PTEN/MMAC1 can dephosphorylate serine-threonine and tyrosine phosphorylated proteins and PI(3,4,5)P3 (5).

PI(3,4,5)P3 is an important substrate because it can activate the Akt/PKB kinase, inducing antiapoptotic effects in cells. Recently, the role of PTEN/MMAC1 in apoptotic stimuli induction has been reported. This regulation depends on the Akt antiapoptotic protein down-regulation. As a phosphatase, PTEN removes a phosphate from PI(3,4,5)P3 so that Akt is down-regulated. Cells lacking the PTEN gene present high levels of PI(3,4,5)P3 that induce Akt activation (4, 6–8).

Recently, the COOH-terminal region of the PTEN protein has been better described, and its importance in PTEN tumor suppressor function has been established (Ref. 8; Fig. 1). The COOH-terminal region also contains three potential tyrosine phosphorylation sites localized at residues 240, 315, and 336; at residue 338, a serine that functions as a potential Ca2+/calmodulin-dependent protein kinase II site is found. Furthermore, the serine localized at residue 355 could act as a potential casein kinase II site. In addition the last four amino acids of the PTEN/MMAC1 protein (ITKV) represent a PDZ binding domain that could interact with proteins containing PDZ domains (Refs. 1, 2, 9; Fig. 1).

All mutations detected at the COOH-terminal region of PTEN protein are localized to exons 7, 8, and 9 and are determined by early truncation of PTEN mRNA translation. Furthermore, all COOH-terminal region mutants are subject to rapid degradation by which the tumor-suppressor activity of PTEN was inhibited; this happens to another suppressor protein, p53 (9).

Mutations of the PTEN/MMAC1 gene have been described in several types of tumors at different frequencies; germ-line mutations have been associated with Cowden disease (10, 11), as well as the controversial Bannayan-Zonana syndrome (12), and possibly Juvenile polyposis (13). Somatic mutations have been found in endometrial, breast, prostate, and brain cancers and melanoma (1, 2, 14–20).

PTEN mutations in CRC seems to be a rare event (21–23).

Recently, mutation at the (A)6 repeat of PTEN exon 7 and 8 in...
PTEN/MMAC1 Gene Frequency of Mutations in Colorectal Cancer

PTEN/MMAC1 GENE

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
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<tr>
<td>Homology to tensin, auxilin and phosphatase</td>
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</tr>
</tbody>
</table>

Fig. 1 PTEN/MMAC1 gene with homology to tensin, auxilin, and phosphatase.

\(~19\%\) of patients with colorectal tumors showing microsatellite instability was found (24). TGF-\(\beta\) is a cytokine shown to be involved in regulating cell adhesion and cell motility and, therefore, implicated in tumor progression acting on cell-matrix interaction (25). Inactivation of the TGF-\(\beta\) pathway was found in colon cancer cells with microsatellite instability, thus suggesting a possible involvement of TGF-\(\beta\) in CRCs (26). Moreover, it has been demonstrated that PTEN gene transcription is down-regulated by TGF-\(\beta\) (27).

Drawing from this background, in this study we analyzed colon cancer biopsies and cell lines for the sequences of the nine exons of PTEN to evaluate the incidence of PTEN mutations in CRC. Recently, PTEN gene mutations have been related to microsatellite instability and TGF-\(\beta\) RII mutations (28). To evaluate this possibility in 16 of 36 patients studied, microsatellite instability and TGF-\(\beta\) RII analysis was performed.

MATERIALS AND METHODS

Colon Cancer Cell Lines. Five colon cancer cell lines were used in this study: CaCo-2, Colo 205, HSR 320, HT 29, and SW 48. They were obtained from the ATCC (American Type Culture Collection). CaCo-2 was cultivated in Eagle’s MEM supplemented with 1% nonessential amino acids and 10% fetal bovine serum; Colo 205 and HSR 320 were cultivated in RPMI 1640 supplemented with 10% fetal bovine serum; HT 29 was cultivated in McCoy’s 5A Medium with Glutamax-1; SW 48 was cultivated in L-15 (Leibovitz) with Glutamax-1 supplemented with 10% fetal bovine serum. Incubation conditions were at 37°C in the presence of 5% CO2.

Tumor Samples. Tumor specimens were obtained from 36 patients with sporadic CRC; biopsies were collected by endoscopy and frozen immediately at −80°C. Samples were obtained from the University Clinic (Navarra, Spain). The mean age of patients was 60 years, and informed consent was obtained from each patient.

DNA Extraction. Tumor specimens and cells were incubated in the lysis buffer [50 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 2% SDS, and protease from Streptomyces griseus 10 mg/ml] for 12 h at 37°C. The lysis was followed by RNase (20 mg/ml) at 37°C for 30 min and by two phenol-chloroform extractions. DNA was precipitated with 0.2 m NaCl and 100% ethanol and resuspended in TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)] to obtain the final DNA concentration of 1 \(\mu\)g/\(\mu\)l.

PCR and Sequencing. PCR amplification of human-specific PTEN/MMAC1 exons was performed using nine pairs of primers (Table 1). Primers were chosen in intronic regions of the PTEN/MMAC1 gene. For PCR of human-specific TGF-\(\beta\) RII, the following primer pair was used: TA10-F1 5’-TTAT-TCTGGAAGATGCTGC-3’ and TA10-R1 5’-GAAGAAAGTCTCACCCAGG-3’ (25). These primers allowed the amplification of a portion of the exon 3 containing the (A)10 repeat.

PCR was performed in a total volume of 50 \(\mu\)l on a GenAmp PCR System 9700 (Perkin-Elmer, Foster City, CA). After denaturing for 4 min at 94°C, 38 rounds of amplification were performed as follows: 94°C for 30 s; specific annealing temperature (Table 1) for 30 s and 72°C for 1 min; followed by a final extension at 72°C for 7 min. β-Globin was evaluated as a control of DNA purity and amplification. Amplification products were electrophoresed on 2% agarose gel and purified by Centricon columns (Amicon, Beverly, MA). After purification, a sequencing reaction was applied (Prism DyeDeoxy Terminator Cycle Sequencing FS; Perkin-Elmer) according to the manufacturer’s instructions. For sequencing, samples were purified by Centrisep columns (Princeton Separations, Adelphia, New Jersey) and run on a 4.25% denaturing polyacrylamide gel. Both strands of the PTEN gene 9 exons were sequenced. Sequencing was performed on an automated DNA sequencer ABI-377 (Perkin-Elmer), and sequences were evaluated using BLAST software.

Analysis of MSI. We examined DNA for MSI using the following loci (Table 2): DSS254, NM23, D18S35, TP53-Di, DSS346, TP53 Penta, D2S123, D1S2883, D3S1611, and D7S501. PCR was performed by using Microsatellite RER/LOH kit (Perkin-Elmer) according to the manufacturer’s instructions. Fluorescently labeled primers of four different colors were used to amplify the polymorphic markers. The use of the automatic gel electrophoresis apparatus designed for DNA sequencing achieved high resolution. For MSI evaluation, both normal and tumor tissues were studied; electrophoresis profiles were analyzed using Genescan analysis software.

When RER was found in one or more loci in a tumor, that tumor was considered to have a positive RER(+) phenotype.

RESULTS

Colon cancer cell line (\(n = 5\)) sequencing identified 1-bp insertion at exon 7 in 1 of 5 (20%) cell lines screened (HSR 320). This insertion produced protein truncation eight residues downstream, at residue 241.

Tumor staging of patients is summarized in Table 3. All of the data about PTEN gene mutations and their correlations with tumor localization and stage are summarized in Table 4. Mutations of the PTEN gene were detected in 6 of 36 (~17%) tumor samples. We identified point mutations in 2 of 6 (~34%) and frameshifts in 4 of 6 (~67%) samples screened. Cancer localization was to the rectum in 4 of 6 (67%), the sigma colon in 1 of 6 (~17%) and the transverse colon in 1 of 6 (~17%). Tumors were classified according to the Dukes’ System as stage B in 1 of 6 (~17%), C in 1 of 6 (~17%), and D in 4 of 6 (~67%).
patients with metastatic CRC (Dukes' stage D). PTEN gene mutations were found in primary tumor samples; metastasis samples were not available and consequently not included in the present study.

PTEN mutations were found at exons 2, 5, 6, 7, 8, and 9. Mutations detected were 2 of 6 (~34%) deletions, 2 of 6 (~34%) insertions, and 2 of 6 (~34%) base changes. The effect of these mutations was the insertion of early stop codon in 3 of 6 (50%). Among these, one insertion occurred at exon 5 at residue 132 in the core motif (122–134) of the phosphatase domain, one at exon 6 at residue 198, and one at exon 7 at residue 254. Furthermore, in the last one, the frameshifts derived from a 2-bp insertion caused the change of residue 240 from Phe to Leu. This residue is a potential tyrosine phosphorylation site.

In 1 of 6 (~17%) specimens, the detected point mutation resulted in no amino acid change. The point mutation occurred in a codon related to the potential tyrosine phosphorylation residue 366. The insertion of an additional lysine was observed in 1 of 6 (~17%) tumor specimens screened. This was caused by a 3-bp insertion (AAA) at exon 2 in a region characterized by sequence similarity to the tensin protein and with high homology to auxilin. Amino acid change at residue 298 (Glu→Asp) was detected in 1 of 6 mutations examined as result of a point mutation at exon 8. All mutations found were homozygous.

Distant metastasis were detectable in 4 of 6 (~67%) positive patients, whereas 1 of 6 (~17%) presented with advanced local nodal involvement only and 1 of 6 was affected by early CRC (Dukes' stage B).

No PTEN mutations were found in 30 of 36 (~84%) patients studied. For these patients, cancer localization was in the rectum in 17 of 30 (~57%), in the sigma colon in 6 of 30 (20%), in the ascendant colon 2 of 30 (6.66%), in the transverse colon in 4 of 30 (~13%), and in the cecum in 1 of 30 (~3%). Tumors were classified as Dukes' A and B in all negative patients except 7 (~23%) that resulted as Dukes' C.

In 16 of 36 (~44%) patients, MSI analysis was possible. By the comparison of normal and tumor sample profiles, alterations were found in 5 of 16 colorectal tumors screened. All MSI+ tumors had also mutations in the poly(A) region of TGF-β RII (Table 5).
**Table 4** PTEN gene mutations and their correlation with tumor localization and stage

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Localization of tumor</th>
<th>Stage of tumor</th>
<th>Mutation</th>
<th>PTEN exon</th>
<th>Predicted effect</th>
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</thead>
<tbody>
<tr>
<td>82</td>
<td>R</td>
<td>B</td>
<td>2-bp del</td>
<td>6</td>
<td>Termination at AA 198</td>
</tr>
<tr>
<td>104</td>
<td>R</td>
<td>C</td>
<td>2-bp ins</td>
<td>7</td>
<td>Termination at AA 254</td>
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<tr>
<td>139</td>
<td>T</td>
<td>D</td>
<td>pm</td>
<td>9</td>
<td>Silent</td>
</tr>
<tr>
<td>193</td>
<td>R</td>
<td>D</td>
<td>3-bp ins</td>
<td>2</td>
<td>Additional Lys</td>
</tr>
<tr>
<td>218</td>
<td>S</td>
<td>D</td>
<td>4-bp del</td>
<td>5</td>
<td>Termination at AA 118</td>
</tr>
<tr>
<td>522</td>
<td>R</td>
<td>D</td>
<td>pm</td>
<td>8</td>
<td>Glu→Val</td>
</tr>
</tbody>
</table>

* R, rectum; S, sigma; T, transverse colon; del, deletion; ins, insertion; pm, point mutation.

**Table 5** Incidence of PTEN gene mutations in MI+ tumors with TGF RII receptor alterations

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Dukes’ stage</th>
<th>PTEN mutations</th>
<th>TGF RII mutations</th>
<th>MI</th>
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<tbody>
<tr>
<td>522</td>
<td>D</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>218</td>
<td>D</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
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<tr>
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<td>A</td>
<td>–</td>
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<td>+</td>
</tr>
<tr>
<td>452</td>
<td>A</td>
<td>–</td>
<td>+</td>
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</tbody>
</table>

PTEN mutations were detected in 3 of 5 MI+ tumors with TGF-β RII mutations. These patients were affected by advanced and metastatic CRC (Dukes’ stage D), and PTEN gene mutations were localized at exons 2, 5, and 8 (Table 5). Two of 5 MI+ tumors with TGF-β RII mutations did not present PTEN alterations; these patients were affected by early CRC, classified as Dukes’ stage A.

**DISCUSSION**

**PTEN** mutations have been described in a wide range of human cancers (1). Different types of mutations have been found such as frameshifts, missense mutations, nonsense mutations, and splicing variants. Most of the missense mutations are localized at the NH2-terminal portion within the phosphatase domain, whereas protein truncations occur throughout the entire gene. It has been proposed that the COOH-terminal region plays an important role in active protein expression and that both gene mutations. In the other biopsies, PTEN/MMAC1 nonsense mutations were detected in only 1 patient with locally advanced CRC of stage C and 1 patient affected by early CRC of stage B. Therefore, mutations were detectable in only 1 patient with absence of nodal and/or distant metastases; other positive samples were from patients with locally advanced and metastatic CRC.

From these data, it could be hypothesized that there is a correlation between **PTEN/MMAC1** mutations and the tendency...
to produce nodal or distant metastases, and there appears to be a high correlation between PTEN/MMAC1 mutations and the presence of both MI and TGFβ-RII mutations. This observation is potentially very important, taking into account also the recent interest in the TGF-β pathway in CRC (25).

If this could be confirmed, PTEN mutation, linked to TGF-β inactivation, could be one of the molecular events contributing to the neoplastic progression of CRC and, therefore, could be used as an additional prognostic factor for CRC.

REFERENCES


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