American Journal of Pathology, Vol. 161, No. 2, August 2002

Mutational Spectra, Expression Levels, and Subcellular Localization in Microsatellite Stable and Unstable Colorectal Cancers

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PTEN **on 10q23.3 encodes a dual-specificity phosphatase that negatively regulates the phosphoinositol-3-kinase/Akt pathway and mediates cell-cycle arrest and apoptosis. Germline *PTEN* mutations cause Cowden syndrome and a range of several different hamartoma-tumor syndromes. Hereditary nonpolyposis colon cancer (HNPCC) syndrome is characterized by germline mutations in the mismatch repair (*MMR*) genes and by microsatellite instability (MSI) in component tumors. Although both colorectal carcinoma and endometrial carcinoma are the most frequent component cancers in HNPCC, only endometrial cancer has been shown to be a minor component of Cowden syndrome. We have demonstrated that somatic inactivation of PTEN is involved in both sporadic endometrial cancers and HNPCC-related endometrial cancers but with different mutational spectra and different relationships to MSI. In the current study, we sought to determine the relationship of *PTEN* mutation, 10q23 loss of heterozygosity, PTEN expression, and MSI status in colorectal cancers (CRCs). Among 11 HNPCC CRCs, 32 MSI+ sporadic cancers, and 39 MSI− tumors, loss of heterozygosity at 10q23.3 was found in 0%, 8%, and 19%, respectively. Somatic mutations were found in 18% (2 of 11) of the HNPCC CRCs and 13% (4 of 32) of the MSI+ sporadic tumors, but not in MSI− cancers ($P = 0.015$). All somatic mutations occurred in the two 6(A) coding mononucleotide tracts in *PTEN*, suggestive of the etiological role of the deficient MMR. Immunohistochemical analysis revealed 31% (14 of 45) of the HNPCC CRCs and 41% (9 of 22) of the MSI+ sporadic tumors with absent or depressed PTEN expression. Approximately 17% (4 of 23) of the MSI− CRCs had decreased PTEN expression, and no MSI− tumor had complete loss of PTEN expression. Among the five HNPCC or MSI+ sporadic CRCs carrying frameshift somatic mutations with immunohistochemistry data, three had lost all PTEN expression, one showed weak PTEN expression levels, and one had mixed tumor cell populations with weak and moderate expression levels. These results suggest that *PTEN* frameshift mutations in HNPCC and sporadic MSI+ tumors are a consequence of mismatch repair deficiency. Further, hemizygous deletions in MSI− CRCs lead to loss or reduction of PTEN protein levels and contribute to tumor progression. Finally, our data also suggest that epigenetic inactivation of PTEN, including differential subcellular compartmentalization, occurs in CRCs. *(Am J Pathol 2002, 161:439–447)***

Germline mutations of , /, AC1/, 1, a tumor suppressor gene on 10q23.3, are associated with 80% of Cowden syndrome as well as seemingly unrelated developmental disorders Bannayan-Riley-Ruvalcaba syndrome, syndrome, and -like syndromes.[1–5] PTEN is a phosphatase that negatively regulates the phosphoinositol-3-kinase/Akt pathway and mediates cell-cycle arrest and apoptosis.[6–14]

Somatic intragenic mutations or deletions of , have been found, to a greater or lesser extent, in a wide variety of sporadic tumors, especially glioblastoma multiforme, and endometrial and advanced prostate cancers.[15–18] Somatic , mutations were found in both sporadic microsatellite unstable (MSI+) endometrial cancers and MSI− tumors, without significant differences in mutational frequency and spectra.[19] We have recently

Partially funded by the American Cancer Society (RPG98-211-01 to C. E.), the United States Department of Defense (DAMD-00-1-0390 to C. E.), the National Cancer Institute, Bethesda, MD (R01CA82282 to P. P., R01CA67941 to A. D. L. C, and P30CA16058 to The Ohio State University Comprehensive Cancer Center), the Academy of Finland (to P. P.), the Finnish Cancer Foundation (to P. P.), the Sigrid Juselius Foundation (to M. N.-L. and P. P.), the European Commission (QLG1-CT-2000-01230 to M. N.-L.), and a generous gift from the Brown family (to C. E.) in memory of Welton D. Brown.

Accepted for publication April 25, 2002.

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demonstrated, moreover, that a high frequency of somatic mutations in , found in endometrial carcinomas arising in individuals with hereditary nonpolyposis colon cancer syndrome (HNPCC), in which germline deficiency of mismatch repair results in the MSI phenotype, to be exclusively frameshift. Further, >50% of these frameshift mutations were found to occur in the two (A)6 mononucleotide repeats in the , -coding sequence, suggesting that , mutations in HNPCC endometrial cancers result from profound DNA mismatch repair deficiency.[19] Although both colorectal carcinoma and endometrial carcinoma are the most frequent component cancers in HNPCC, only endometrial cancer has been shown to be a minor component of Cowden syndrome.[20] Approximately 15% of sporadic colorectal cancers (CRCs) exhibit the MSI phenotype.[21–23] Existing data to date suggest that the immediate downstream pathways of HNPCC-related component tumors and those of their sporadic counterparts are quite different, although the final common pathway might be similar.[24]

Among MSI+ sporadic CRCs, ~19% were found to have somatic frameshift mutations almost exclusively in one of two (A)6 tracts in exons 7 and 8 in , .[25,26] In contrast, in MSI unknown or MSI− sporadic colorectal tumors, ≪5% have been shown to have somatic , mutations, and none have occurred in any mononucleotide tracts (PLM Dahia and C Eng, unpublished).[27,28] Further, ~10 to 30% of MSI− and MSI unknown sporadic CRCs have loss of heterozygosity (LOH) of markers at or close to , (PLM Dahia and C Eng, unpublished).[29] However, whether structural alterations lead to loss of activity of the PTEN tumor suppressor contributing to the pathogenesis of CRCs remains to be elucidated. In this study, we sought to determine the relationship of , mutation, LOH at 10q23, PTEN expression, and MSI status in CRCs. Further, we sought to determine whether structural alterations in , lead to loss of function of the gene by investigating the expressional levels of the gene product in HNPCC CRCs, sporadic MSI+, and MSI− tumors. We also investigated if there are any correlations between PTEN inactivation and other genetic alterations established to be associated with the MSI phenotype in CRCs.

Materials and Methods

C C n

Forty-six CRCs from individuals with HNPCC classified according to the consensus Amsterdam criteria were obtained for this study. Among these 46, 42 occurred in 29 HNPCC families carrying germline mutations in either *1* or *2*, whereas the remaining four had family histories of CRCs but no mutation in genes were detected.[30,31] Forty-five had pathological slides available for immunohistochemical analysis, 11 had paired normal and tumor DNA available for , mutational and LOH analyses.

Thirty-two sporadic MSI+ CRCs and 62 MSI− tumors were investigated. MSI status was determined by analyzing BAT-26 and , β mononucleotide (polyA) markers by fluorescence-based polymerase chain reaction (PCR), as previously described.[32] None of the 32 individuals with sporadic MSI+ tumors were found to carry germline mutations.[30] Twenty-two of the 32 MSI+ and 23 of the 62 MSI− CRCs had paraffin-embedded tissue blocks available for immunohistochemistry analysis.

Paired normal and tumor DNA were isolated from blood, fresh-frozen tissue, or paraffin-embedded pathological blocks using techniques described previously.[33] Pathological blocks were cut to 4-μm sections and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) for immunohistochemistry studies.

A n

Amplicons that harbor the 8(G) mononucleotide repeat tracts in and *BAT*, and the 6(A) tract in , *53* were generated in the following manner. The corresponding PCR primers and conditions for and *BAT* have been described previously.[34,35] The primers used to amplify the mononucleotide repeat within exon 11 of , *53* were P53–6AF 5′-TGTCATCTCTCCTCCCTGCT-3′, and P53–6AR 5′-TCAAAGACCCAAAACCCAAA-3′. PCR reactions were performed in a 25-μl reaction volume containing 50 ng of genomic DNA, 1× PCR buffer (Qiagen, Valencia, CA), 200 μmol/L of each dNTP (Life Technologies, Inc., Rockville, MD), 400 μmol/L of each primer, and 1.0 U of HotStart- polymerase (Qiagen). The concentration of $MgCl_2$ was 1.5 mmol/L. The following PCR cycles were used for amplification for the pertinent , *53* amplicon: 95°C for 15 minutes, 35 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute. Final extension was 72°C for 10 minutes. All PCR products were gel and column purified and subjected to semiautomated sequencing as previously described.[36]

, A

, mutation analysis of all nine coding exons, exon-intron junctions, and flanking intronic sequences was performed using PCR-based denaturing gradient gel electrophoresis and semiautomated sequencing as previously described.[18,37]

, A

The specificity of PTEN monoclonal antibody 6H2.1 has been proven previously.[38–40] This antibody, raised against the last 100 C-terminal amino acids of human PTEN, was used essentially as previously described[38] with minor modifications. In brief, 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 and incubating the sections in 0.3% hydrogen peroxide. After blocking for 30 minutes in 0.75% normal horse serum, the

sections were incubated with 6H2.1 (dilution 1:100) overnight (or 16 hours) at 4°C. The sections were washed in phosphate-buffered saline, 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 performed with 3′,3′ diaminobenzidine (Sigma Chemical Co., St. Louis, MO) which gives a brown product. After counterstaining with methyl green and mounting, the slides were evaluated under a light microscope. The immunostaining patterns and intensities were determined by two independent observers (XPZ and CE) who each examined and independently scored the slides on two separate occasions. As previously described,[38–40] the vascular endothelium serves as an internal positive control and the immunostaining of the endothelium is scored as ++. Levels of immunostaining in vascular endothelium are remarkably constant among various tissues, including breast,[38] thyroid,[39] pancreas,[41] and colon (XP Zhou and C Eng, this report and unpublished). Immunostaining intensities equal to that of vascular endothelium in a particular sample were scored as ++; weak or decreased staining intensity as +; and no immunostaining as −. An immunostaining intensity greater than that of vascular endothelium are operationally graded as +++.

10 ,23.3 , A ·

LOH analysis at the ´ locus was performed using dinucleotide markers D10S1765 and D10S541, which are within 300 kb and 600 kb, respectively, of ´ . In addition, two intragenic intronic polymorphic markers, IVS4 + 109 ins/del TCTTA and IVS8 + 32t/g, within ´ were also used for LOH analysis. Assessment of the status at IVS8 + 32t/g was performed by differential digestion with the restriction endonuclease II as described.[36] The status at IVS4 + 109 ins/delTCTTA was screened by PCR-based differential digestion with restriction endonuclease *A* II following the manufacturer's instructions (New England Bio-

MSI− tumors (Table 2, Figure 1). All CRC sections had accompanying vascular endothelial cells present, which showed strong PTEN immunostaining (scored ++) in the cytoplasm, and served as internal positive controls as previously described.[38–40] The strong immunoreactivity in the endothelial cells showed a nuclear predominance (+++). In all samples in which normal colonic epithelium was visible (= 56), the cytoplasm of the normal epithelial cells all expressed PTEN (++ immunoreactivity), with a moderate to slightly weaker nuclear immunoreactivity (+/++, = 47; +, = 9). In contrast, among all CRCs examined for PTEN protein expression with adjacent normal colonic epithelium present on the same slide, the neoplastic nuclei had moderate (+/++, = 12), weak (+, = 29) or no (−, = 15) PTEN immunostaining. Thus, in 44 of 56 (79%) of the samples, there was a clear decrease in nuclear expression of PTEN in cancers compared to their corresponding normal epithelium.

Twenty-three of 67 (34%) MSI+ CRCs had weak (+) or no (−) PTEN expression in the cytoplasm (Table 1). Of these 23, 8 had no PTEN expression and the remaining 15, weak expression. Of the 44 tumors without decreased or no expression, 35 had ++ immunoreactivity, and 9 MSI+ tumors had nonuniform staining with moderate, weak, and absent staining in different cell populations throughout the section.

PTEN immunostaining was performed in 23 MSI− CRCs with paraffin-embedded tissue available. Four (17%) CRCs showed weak (+) PTEN cytoplasmic immunostaining, and no tumor lacked PTEN expression. The remaining 19 tumors (83%) had ++ cytoplasmic immunostaining, 3 of which showed nonuniform staining patterns within a sample (Table 2).

The frequency of samples with either no or depressed PTEN expression, detected by immunohistochemistry, was not statistically different when pairwise comparisons were made between HNPCC CRCs, sporadic MSI+, and MSI− tumors (= 0.43 for HNPCC sporadic MSI+, 0.10 for sporadic MSI+ sporadic MSI−, 0.26 for HNPCC sporadic MSI−). However, there seemed to be an associative trend for complete loss of PTEN expression and MSI+ status (chi-square = 3.21; Mantel-Haenzel, = 0.07), whether in the hereditary or sporadic setting.

C n A

Among all MSI+ tumors, 13 had data from genetic and immunohistochemical analyses and were informa for both (Table 2). None of these se seinforma08.8(expression.)-228.8(Ofi

Figure 1. PTEN protein expression (brown chromogenic reaction) in colorectal carcinomas (CRCs). **a** and **b:** Adjacent normal colonic epithelium showing strong cytoplasmic (++) and nuclear PTEN expression (+/++). **c** and **d:** CRC showing cytoplasmic PTEN expression (++). **e** and **f:** CRC with weak cytoplasmic PTEN immunoreactivity (+). Of note, the PTEN nuclear staining in colon carcinoma cells is remarkably weaker or absent compared to that of adjacent normal colonic epithelium. **g** and **h:** CRC exhibiting no PTEN expression (−) in all tumor cells. Original magnifications: ×4 (**a**, **c**, **e**, **g**); ×20 (**b**, **d**, **f**, **h**).

In the 23 MSI− CRCs with immunohistochemical data, none had complete loss of PTEN immunoreactivity. Only one of the four tumors with uniform + immunoreactivity had LOH whereas the remaining three had no structural alteration. Three of the 23 tumors had mixed populations with varying PTEN expression. Of these three, two had LOH (Table 2). LOH analysis was not performed for MSI− tumors without aberrations in PTEN immunoreactivity because extensive data to date demonstrate that no structural alterations (mutations, deletions) have been found in tissue with PTEN immunoreactivity graded ++ or +++.[38–45]

As a general trend, therefore, PTEN expression levels as detected by immunohistochemistry were correlated with the structural status of the gene although it should be noted that no tumor, whatever the expressional levels, carried two structural hits (Table 2).

To help dissect out the significance of the coding region frameshift mutations in one of the two (A)6 repeat tracts in , we then analyzed our MSI+ tumors for the frequency of frameshift mutations in the (A)10 mononucleotide repeat tract within and the (G)8 tracts within and *BAX*. The frameshift frequencies in each of these three mononucleotide tracts were not different between the hereditary and sporadic MSI+ CRCs. Somatic frameshift mutations in the , , , and *BAX* genes were detected in 28 of 38 (74%), 8 of 38 (21%), and 21 of 38 (55%) of MSI+ CRCs, respectively (Table 1). Of note, no correlation could be found between the presence or absence of frameshift mutations in these three mononucleotide repeat tracts and aberrations (Table 1). Because the two pertinent mononucleotide repeat tracts each comprise a run of six As, a search for (A)6 mononucleotide tracts in coding regions yielded one in the *53* gene. In contrast to the relatively frequent frameshift mutations in the (A)6 tracts of in MSI+ CRCs, no mutations were found in the *53* (A)6 tract in these MSI+ tumors.

Discussion

Although we have shown that 15 to 20% of MSI+ CRCs, whether sporadic or HNPCC-related, have a structural alteration, 35% have no or decreased PTEN protein in the cytoplasm of the neoplastic cells. Further, in the four MSI− tumors with decreased PTEN expression, one had LOH at 10q23.3. These observations suggest that mechanisms of silencing other than structural pertain in CRCs. We will refer to all such nonstructural alterations (methylation, transcript stability, protein stability, and so forth) as epigenetic. Interestingly, our observation of differential nuclear-cytoplasmic PTEN expression between normal colonic epithelial cells and adenocarcinomas might suggest inappropriate subcellular compartmentalization as another epigenetic mechanism of PTEN inactivation. It has become clear that different mechanisms of PTEN inactivation can occur in various solid tumors. More than one mechanism can co-exist within a single tumor type, although a particular tumor type might have a predominant mechanism of inactivation. For example, up to 93% of all sporadic endometrial carcinomas have absent or markedly diminished PTEN protein.[18] Of these, 25% have two structural hits, usually one intragenic mutation accompanied by allele loss; in the remainder of these tumors, therefore, must undergo epigenetic inactivation.[18,42,46] In glioblastoma multiforme and primary cervical carcinomas, the predominant mechanism of inactivation is biallelic structural alteration, typically intragenic mutation and deletion of the remaining wild-type allele.[43,44] Biallelic epigenetic silencing predominates in metastatic malignant melanoma but occurs in rare subsets of epithelial ovarian carcinomas.[40,45] The mechanism of loss of PTEN expression in CRCs seems to be similar to that in the majority of primary epithelial ovarian cancers and primary breast cancers, in which a mixed genetic/epigenetic (intragenic mutation/epigenetic or LOH/epigenetic) mechanism predominates.[38,45] In nonmedullary thyroid tumors, islet cell tumors, and perhaps primary, but not metastatic, cutaneous melanomas, differential subcellular compartmentalization might mediate PTEN inactivation.[39,41,47] In all these tissues, strong nuclear PTEN expression in normal cells is lost with transformation to neoplasia. If this mechanism is also germane in CRCs, then such a mechanism appears to be pertinent in many CRCs. Nonetheless, the precise mechanism of how inappropriate subcellular compartmentalization can mediate PTEN dysfunction is unknown and has yet to be elucidated.

When decreased or no PTEN expression is because of structural alteration in MSI+ CRC, we noted that this is because of somatic frameshift mutations in one of two poly-A tracts in the 3′-coding region of in direct contrast to MSI− tumors, where no somatic mutations occur. However, when deletion or insertion of a nucleotide occurs in mononucleotide tracts in a gene(s) in MSI+ tumors, it is difficult to determine initially whether these somatic frameshift changes in a particular gene, in this case , contribute to pathogenesis or whether they merely reflect chance occurrence. Our current observations provide some evidence that suggests that inactivation, by whatever mechanism, can contribute to the pathogenesis of CRC. First, a frameshift mutation in the exon 7 or 8 (A)6 repeat tract is predicted to result in truncated protein lacking several predicted tyrosine phosphorylation sites and the important C-terminal C2 domain that is important for phospholipid membrane binding.[48] Second, we demonstrate for the first time that both the hemizygous deletion, manifested by LOH of markers within and flanking , and the frameshift mutations in one or the other (A)6 tract does result in decreased or absent protein. Third, although we demonstrate the well-documented intragenic, likely pathogenic, frameshift mutations in the (A)10 tract of , and the (G)8 tracts in and *BAX* genes in both hereditary and sporadic MSI+ CRCs,[34,35,49–52] these are unrelated to mutation status in the current series of tumors.

Fourth, although the , (A)6 repeat tract is shorter than the extensively studied (N)8-10 repeats, such as those in , β , , and *BA* genes, the relatively frequent, ie, 15 to 20%, frameshift mutations in the two (A)6 tracts in MSI+ CRCs suggest that , should belong to the category of real target genes with a cutoff mutation frequency of 12% described by a recent systematic study on instability at coding and noncoding repeat sequences in human MSI+ colon cancers.[53] Corroborating this, a coding mononucleotide tract of equal length to those in , , (A)6, in , *53* was not found to be a target for MMR deficiency in our MSI+ tumors, suggesting again that , is likely a functional downstream target of MMR deficiency.

Together with previous observations in sporadic MSI+ CRCs,[25,26] our observation that every somatic intragenic , mutation in MSI+ tumors, whether HNPCC-related or sporadic, has occurred in a mononucleotide repeat suggests that MMR deficiency precedes , mutation in MSI+ CRCs. That somatic , mutation as a consequence of MMR deficiency applies equally to sporadic MSI+ and HNPCC MSI+ CRCs is worthy of note. The observation that one of four CRCs originating from germline , *1*, , *2* mutation-negative classic HNPCC individuals carried the somatic intragenic delA is consistent with the former statement. This is in contrast to the timing and extent of , alterations in endometrial carcinomas. In HNPCC-related endometrial carcinomas, we recently demonstrated that MMR deficiency results in a high frequency of somatic intragenic , mutations affecting the coding mononucleotide repeat tracts.[19] In MSI+ sporadic endometrial carcinomas, however, we demonstrate that somatic , mutations can precede mismatch repair deficiency.[19] Thus, it would seem that , is a structural target of MMR deficiency in ~15 to 20% of MSI+ CRCs, perhaps arguing that , alteration occurs as one of the later steps in tumorigenesis.

The results of PTEN immunohistochemistry demonstrating mixed cellular populations and the corresponding LOH data from three MSI tumors (Table 2; tumors 362, 158, and 146) appears discordant and merits explanation. For tumor 362, LOH analysis shows LOH at 10q22-q24 markers and a tumor population with no PTEN expression and one with full (++) immunostaining. It is possible that the LOH result originated from template sampled from the cells that were not expressing PTEN. If so, then the second silencing hit must still be postulated to be other than structural. It is almost certain that the cellular populations with ++ expression did not serve as template for the LOH analysis. Similarly, for tumor 146 in which there is LOH and mixed populations either with decreased PTEN expression or full (++) expression, it is more likely than not that the LOH results were from template obtained from the cellular population with decreased expression. In tumor 158, no LOH was noted but there were two tumor populations, one expressing PTEN (++) and the other without any expression. These observations may be consistent with either the DNA showing no LOH being sampled from the tumor cells expressing PTEN or that despite the intact alleles, PTEN was completely silenced by mechanisms other than genetic thus resulting in lack of PTEN protein expression.

In summary, we have demonstrated that loss of PTEN function by loss or reduction of protein expression contributes to the development or progression of CRC. PTEN is a selected target in CRCs with deficient mismatch repair; somatic mutations in one of two coding mononucleotide tracts in , result in loss of or diminished protein expression. In MSI− CRCs on the other hand, allele loss of , leading to partial loss of protein expression may represent haploinsufficiency contributing to tumor progression. However, it is always difficult to exclude whether LOH, in the absence of intragenic mutations, could represent non- , -specific (other genes) loss in the 10q23 region. Further, epigenetic silencing and perhaps inappropriate subcellular compartmentalization might be two other important mechanisms of PTEN inactivation in both MSI+ and MSI− CRCs.

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