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Short Communication

Genetic Pathways of Colorectal Carcinogenesis Rarely Involve the *PTEN* and *LKB1* Genes Outside the Inherited Hamartoma Syndromes

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Germline mutations of the *PTEN/MMAC1/TEP* and *LKB1* genes cause hamartomas to develop in the gastrointestinal tracts of patients with Cowden syndrome and Peutz-Jeghers syndrome, respectively. *PTEN* mutations may also be responsible for some cases of juvenile polyposis. Histologically, hamartomas appear benign, but there is good evidence that in these syndromes, the hamartomas can progress to colorectal carcinoma. It remains unknown whether or not cancers that develop from hamartomas acquire a spectrum of mutations similar to those in sporadic colon cancers. *PTEN* and *LKB1* are candidate genes for mutations in sporadic colon cancers, either as initiating events in tumorigenesis or providing a selective advantage during tumor growth. Using single-strand conformational polymorphism analysis, we have screened a set of sporadic colon cancers for somatic mutations in *PTEN* and *LKB1*. No variants predicted to alter protein function were detected in *LKB1*, but 1 of 72 cancers showed a somatic mutation in *PTEN*, together with allele loss. This cancer did not have a detectable *APC* mutation or allele loss at *APC*. It remains possible that *PTEN* and *LKB1* are inactivated in other sporadic colon cancers by means such as deletion or promoter methylation. Like *BRCA1* and *BRCA2*, however, it appears that *PTEN* and *LKB1* mutations can cause cancers when present in the germline, but occur rarely in the soma. *(Am J Pathol 1998, 153:363–366)*

Mendelian diseases that predispose to colorectal cancer include familial adenomatous polyposis (FAP; MIM175100); hereditary nonpolyposis colon cancer (MIM120435/6); and the hamartoma syndromes Peutz-Jeghers syndrome (PJS, MIM175200), juvenile polyposis syndrome (MIM174900), and Cowden syndrome (CS, MIM158350). The genes responsible for FAP and hereditary nonpolyposis colon cancer have been shown to play important roles in the pathogenesis of sporadic cancers of the colon and of other sites. The *APC* gene (which is mutated in the germline of FAP patients) is involved in up to 80% of all sporadic colon cancers,[1] and the mismatch repair loci (which are responsible for hereditary nonpolyposis colon cancer) are mutated or silenced in up to 10% of cancers of the colorectum and endometrium.[2]

CS is known as the multiple hamartoma syndrome, and individuals with this condition develop characteristic features such as cobblestone papules of the mouth and juvenile polyps of the gastrointestinal tract. CS predisposes to cancers of the thyroid, breast, and gastrointestinal tract, including the colorectum in some reported cases.[3] The CS gene has been shown to be *PTEN/MMAC1/TEP* ($10q^{22}$ to q^{23}),[4] a dual-specificity phosphatase that acts as a tumor suppressor and is mutated in several tumor types, including glioblastomas, prostate carcinoma, and a small proportion of breast cancers.[5–13] Inherited *PTEN* mutations are also responsible for Bannayan-Zonnona syndrome (MIM153480)[8,14]; its features include macrocephaly, lipomas, hemangiomas, and juvenile polyps. There is conflicting evidence concerning the suggestion that germline *PTEN* mutations can also cause juvenile polyposis of the gastrointestinal tract in the

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Table 1. Oligonucleotides Used for SSCP Analysis of LKB1

Exon	Oligonucleotide	Sequence	Ta* (°C)	Size (bp)
1F	GB1727	AGG GCT GGC GGC GGG ACT CC	61	363
1R	GB2090	AGG CCC CGC GGT CCC AAC AC		
2F	GC1289	CTG ATA CAC CCC TGT CCT CTC TGT C	54	120
2R	GC1409	AGG CCC CGC GGT CCC AAC AC		
3F	GD5531	CTC CAG AGC CCC TTT TCT G	59	255
3R	GD5786	TCA ATG ACT ATC AGG CCA CG		
4/5F	GA826	GGC CCC AGG ACG GGT GTG TG	61	397
4/5R	GA1223	AGT GTG CGT GTG GTG AGT GC		
6F	GA1659	TGA CTG ACC ACG CCT TTC TT	57	218
6R	GA1877	CCC CCA ACC CTA CAT TTC TG		
7F	GA2412	CTC CTC GCC GGC TTC TCC TC	62	155
7R	GA2567	CCC CAC CAC GCC CTG CTC TA		
8F	GA3439	GAC AGG CGC CAC TGC TTC TG	60	251
8R	GA3690	GGA CAT CCT GGC CGA GTC AG		
9F	GE001	GTA AGT GCG TCC CCG TGG TG	59	337
9R	GE338	GTG GCA TCC AGG CGT TGT CC		

*Ta, annealing temperature used in PCR.

absence of the other features of CS or Bannayan-Zonnona syndrome.[15,16]

PJS is another syndrome of multiple gastrointestinal hamartomas (of a histological type different from juvenile polyps), which are usually associated with characteristic freckling of the lips and buccal mucosa. PJS predisposes to cancers of multiple sites, especially the colon, breast, testis, and ovary.[17] The PJS gene is *LKB1 (STK11)* (19p13.3), a serine/threonine kinase and a tumor suppressor.[18,19]

There is good evidence that the hamartomas in PJS, juvenile polyposis syndrome, and (to a lesser extent) CS can progress to colorectal carcinoma. Allele loss occurs in sporadic colon cancers close to *PTEN* at a frequency of about 30%[20] and close to *LKB1* at a frequency of about 20% (I. Tomlinson, unpublished data). Both *PTEN* and *LKB1* are therefore good candidates for involvement in the pathogenesis of sporadic tumors of the large bowel. *PTEN* and/or *LKB1* mutations might be selected at any stage of colorectal tumorigenesis, the most intriguing possibility being that they can initiate tumorigenesis. We have screened 72 unselected sporadic cancers of the colorectum for mutations in the *PTEN* and *LKB1* genes to test these candidate loci for a role in colorectal tumorigenesis.

Materials and Methods

Using standard methods, DNA was extracted from samples of fresh-frozen sporadic colon cancer and matched normal tissue or blood. These cases had no known family history suggestive of FAP, hereditary nonpolyposis colon cancer, or any of the hamartoma syndromes. Other standard clinicopathological data (age, grade, stage, and tumor site) were obtained from hospital records. Single-strand conformational polymorphism (SSCP) analysis was performed on the cancer samples. Published oligonucleotides and reaction conditions[4–6,15] were used to amplify specifically each exon of *PTEN*; for some longer exons, the products of the polymerase chain reaction were then subjected to restriction endonuclease digestion to render them optimally short for mutation detection by SSCP (*Alu*I for exons 1, 5, 6, 7, and 9 and *Mbo*I for exon 8). New oligonucleotides were designed for exon-by-exon amplification of *LKB1* (Table 1) using a protocol of 94°C for 3 minutes (one time), 35 cycles of 94°C for 1 minute/annealing temperature °C for 1 minute/72°C for 1 minute, and 72°C for 5 minutes (one time). Polymerase chain reaction products were heated to 90°C for 5 minutes and subjected to electrophoresis on a 10% acrylamide gel (30:0.8 acrylamide:bisacrylamide, 10% glycerol) under nondenaturing conditions at 20 mA for about 16 hours. DNA was detected by silver staining of gels using standard methods. For all tumors with possible mutations according to SSCP analysis, that exon was reamplified in duplicate from genomic DNA in the polymerase chain reaction, and these purified polymerase chain reaction products were sequenced in forward and reverse orientations using the Applied Biosystems, Inc. (Foster City, CA) Ready Reaction Dye Terminator Cycle Sequencing kit and the 377 Prism sequencer. All sequencing reactions were performed alongside samples with wild-type genotypes and with known mutations.

Results

For *LKB1*, no variant band was detected in any tumor sample on SSCP analysis. Control samples from three PJS patients with known mutations showed bandshifts. For *PTEN*, however, a small number of bandshifts was observed in the tumor samples using SSCP analysis. Sequencing confirmed a mutation in one tumor (1.4%), resulting from a complex change at the exon 2/intron 2 boundary. This mutation altered the "wild-type" sequence GTA AGG *TAAGAAT* to GTA AGA *GTAATGC* (where exonic sequences are in regular type and intronic sequences are in italics). This results in 1) a silent AGG→AGA Arg→Arg change in codon 53, 2) substitution of the more typical donor splice site consensus sequence *GTAA* for the atypical wild-type sequence *TAAG*, and 3) a 2-bp deletion in intron 2. It is quite possible that this change affects mRNA splicing, although no source of

mRNA was available to prove this contention. Exons 2 and 3 do not constitute a mode 3 number of nucleotides, and aberrant splicing would therefore be expected to lead to a truncated protein. The exon 2/intron 2 change was not present in the germline, and the patient, a 75-year-old male with Dukes' C colorectal carcinoma, had no features of CS, Bannayan-Zonnona syndrome, or juvenile polyposis syndrome. The mutation at codon 53 of *PTEN* has not been reported previously in the germline or soma. Previous studies demonstrated that this tumor showed allele loss close to *PTEN*[20] and did not show a truncating mutation in exon 15 or allele loss at *APC*.[21]

Discussion

The initiating events in the pathogenesis of cancers in the hamartoma syndromes are almost certainly germline and somatic mutations at *LKB1*, *PTEN*, or related, uncharacterized genes. We have found that *PTEN* and *LKB1* mutations in sporadic colon cancers occur at a low frequency or are absent altogether. Thus, the colon cancers in PJS and CS (and possibly some cases of juvenile polyposis syndrome) follow genetic pathways that are distinct from the majority of colorectal tumors (at least regarding their initiating events). Other workers have found *APC* mutations in juvenile polyps with dysplasia,[22] showing that there is partial overlap between the genetic pathways of tumorigenesis in hamartoma syndromes, FAP, and sporadic colon cancers.

Given the reported moderate sensitivity of SSCP of about 80%,[23–25] especially for detecting point mutations, we cannot exclude the possibility that mutations at *PTEN* or *LKB1* occur in a somewhat higher proportion of colon cancers than reported here. It is noteworthy, however, that the spectra of germline and somatic mutations in *PTEN* and of germline mutations in PJS include small deletions that would be easily detected using SSCP analysis [4,5,8,12,19,26]; in addition, SSCP analysis in our study detected positive control samples in both genes that resulted from point mutations. The previously unreported *PTEN* mutation that we detected was accompanied by allele loss and, although its effect at the protein level cannot be proven, we suspect that this mutation was selected for a role in tumorigenesis. Colon cancer may thus resemble carcinoma of the breast, in which somatic *PTEN* mutations occur in a small but important subset of tumors.

Further possibilities for the involvement of *PTEN* or *LKB1* that we have not excluded are gene silencing by promoter methylation or hemi- or homozygous deletion of either locus (whether the entire gene or whole exons). The latter possibility would be consistent with the observed allele loss close to *PTEN* and *LKB1* in colon cancers, and homozygous deletions have been observed at *PTEN* in a variety of tumors.[5] It remains entirely possible, however, that the allele loss close to *PTEN* and/or *LKB1* in colon cancers targets different loci in both cases.

There is, in general, a far from perfect association between the spectrum of tumors in Mendelian cancer syndromes and the range of sporadic tumors in which the same gene is mutated. The familial breast/ovarian cancer genes, *BRCA1* and *BRCA2*, for example, cause cancer when mutant in the germline, but are hardly ever mutated in sporadic cancers. There is, however, evidence that *BRCA1* is inactivated by promoter methylation in some sporadic breast cancers,[27] thus suggesting that defective *BRCA1* can provide a selective advantage to breast tumors whether derived from the germline or soma, albeit through different mechanisms. There will be great potential interest in studying the expression of *PTEN* and *LKB1* mRNA in colorectal tumors and in screening for mutations of *LKB1* in sporadic tumors of other sites.

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References

1. Beroud C, Soussi T. APC gene: database of germline and somatic mutations in human tumors and cell lines. Nucleic Acids Res 1996, 24:121–124
2. Kane MF, Loda M, Gaida GM, Lipman J, Mishra R, Goldman H, Jessup JM, Kolodner R: Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. Cancer Res 1997, 57:808–811
3. Lyons CJ, Wilson CB, Horton JC: Association between meningioma and Cowden's disease. Neurology 1993, 43:1436–1437
4. Liaw D, Marsh DJ, Li J, Dahia PLM, Wang SI, Zheng ZM, Bose S, Call KM, Tsou HC, Peacocke M, Eng C, Parsons R: Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat Genet 1997, 16:64–67
5. Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittmann M, Tycko B, Hibshoosh H, Wigler MH, Parsons R: PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 1997, 275:1943–1947
6. Steck PA, Pershouse MA, Jasser SA, Yung WKA, Lin H, Ligon AH, Langford LA, Baumgard ML, Hattier T, Davis T, Frye C, Hu R, Swedlund B, Teng DHF, Tavtigian SV: Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat Genet 1997, 15:356–362
7. Rhei E, Kang L, Bogomolniy F, Federici MG, Borgen PI, Boyd J: Mutation analysis of the putative tumor suppressor gene *PTEN/MMAC1* in primary breast carcinomas. Cancer Res 1997, 57:3657–3659
8. Guldberg P, thor Straten P, Birck A, Ahrenkiel V, Kirkin AF, Zeuthen J: Disruption of the *MMAC1/PTEN* gene by deletion or mutation is a frequent event in malignant melanoma. Cancer Res 1997, 57:3660–3663
9. Tashiro H, Blazes MS, Wu R, Cho KR, Bose S, Wang SI, Li J, Parsons R, Ellenson LH: Mutations in PTEN are frequent in endometrial carcinoma but rare in other common gynecological malignancies. Cancer Res 1997, 57:3935–3940
10. Wang SI, Puc J, Li J, Bruce JN, Cairns P, Sidransky D, Parsons R: Somatic mutations of PTEN in glioblastoma multiforme. Cancer Res 1997, 57:4183–4186
11. Rasheed BK, Stenzel TT, McLendon RE, Parsons R, Friedman AH, Friedman HS, Bigner DD, Bigner SH: *PTEN* gene mutations are seen in high-grade but not in low-grade gliomas. Cancer Res 1997, 57:4187–4190

12. Dahia PL, Marsh DJ, Zheng Z, Zedenius J, Komminoth P, Frisk T, Wallin G, Parsons R, Longy M, Larsson C, Eng C: Somatic deletions and mutations in the Cowden disease gene, *PTEN*, in sporadic thyroid tumors. Cancer Res 1997, 57:4710–4713
13. Risinger JI, Hayes AK, Berchuck A, Barrett JC. PTEN/MMAC1 mutations in endometrial cancers. Cancer Res 1997, 57:4736–4738
14. Marsh DJ, Dahia PL, Zheng Z, Liaw D, Parsons R, Gorlin RJ, Eng C: Germline mutations in PTEN are present in Bannayan-Zonnona syndrome. Nat Genet 1997, 16:333–334
15. Marsh DJ, Roth S, Lunetta KL, Hemminki A, Dahia PLM, Sistonen P, Zheng ZM, Caron S, vanrsouw NJ, Bodmer WF, Cottrell SE, Dunlop MG, Eccles D, Hodgson SV, Jarvinen H, Kellokumpu I, Markie D, Neale K, Phillips R, Rozen P, Syngal S, Vijg J, Tomlinson IPM, Aaltonen LA, Eng C: Exclusion of PTEN/MMAC1/TEP1 and 10q22–24 as the susceptibility locus for juvenile polyposis syndrome (JPS). Cancer Res 1997, 57:5017–5020
16. Olschwang S, Serova-Sinilnikova OM, Lenoir GM, Thomas G. PTEN germ-line mutations in juvenile polyposis coli. Nat Genet 1998, 18: 12–14
17. Tomlinson I, Houlston R: Peutz-Jeghers syndrome. J Med Genet 1997, 34:1007–1011
18. Hemminki A, Tomlinson I, Markie D, Jarvinen H, Sistonen P, Bjorkqvist AM, Knuutila S, Salovaara R, Bodmer W, Shibata D, delaChapelle A, Aaltonen LA: Localization of a susceptibility locus for Peutz-Jeghers syndrome to 19p using comparative genomic hybridization and targeted linkage analysis. Nat Genet 1997, 15:87–90
19. Hemminki A, Markie D, Tomlinson IPM, Avizienyte E, Roth S, Loukola A, Bignell G, Warren W, Aminoff M, Hoglund P, Jarvinen H, Kristo P, Pelin K, Ridanpaa M, Salovaara R, Toro T, Bodmer W, Olschwang S, Olsen AS, Stratton MR, delaChapelle A, Aaltonen LA: A serine/threonine kinase gene defective in Peutz-Jeghers syndrome. Nature 1998; 391:184–187
20. Frayling IM, Bodmer WF, Tomlinson IP: Allele loss in colorectal cancer at the Cowden disease/juvenile polyposis locus on 10q. Cancer Genet Cytogenet 1997, 97:64–69
21. Homfray TFR, Cottrell SE, Ilyas M, Rowan A, Talbot IC, Bodmer WF, Tomlinson IPM: Defects in mismatch repair occur after APC mutations in the pathogenesis of sporadic colorectal tumours. Hum Mutat 1998, 11:114–120
22. Wu TT, Rezai B, Rashid A, Luce MC, Cayouette MC, Kim C, Sani N, Mishra L, Moskaluk CA, Yardley JH, Hamilton SR: Genetic alterations and epithelial dysplasia in juvenile polyposis syndromes and sporadic juvenile polyps. Am J Pathol 1997, 150:939–947
23. Sheffield VC, Beck JS, Kwitek AE, Sandstrom DW, Stone EM: The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitutions. Genomics 1993, 16:325–332
24. Vidal PA, Moller DE: Comparative sensitivity of alternative single-strand conformation polymorphism (SSCP) methods. Biotechniques 1994, 17:490–492
25. Ravnik GM, Glavac D, Dean M: Sensitivity of single-strand conformation polymorphism and heteroduplex method for mutation detection in the cystic fibrosis gene. Hum Mol Genet 1994, 3:801–807
26. Arch EM, Goodman BK, Van Wesep RA, Liaw D, Clarke K, Parsons R, McKusick VA, Geraghty MT: Deletion of PTEN in a patient with Bannayan-Riley-Ruvalcaba syndrome suggests allelism with Cowden disease. Am J Med Genet 1997, 71:489–493
27. Dobrovic A, Simpfendorfer D: Methylation of the *BRCA1* gene in sporadic breast cancer. Cancer Res 1997, 57:3347–3350